CHEMICAL BIOLOGY APPROACHES FOR TRACKING AND MANIPULATION OF MACROPHAGE PHENOTYPES

A Dissertation Presented

by

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DEDICATION

To my patient and loving wife, Gabriela; my parents, Aníbal and Rosalind; and my brothers, Jorge Iván, Iván Alexis, and Aníbal Xavier. Thank you all from the bottom of my heart. This would not have been possible without you. ¡Los amo eternamente!
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ABSTRACT

CHEMICAL BIOLOGY APPROACHES FOR TRACKING AND MANIPULATION OF MACROPHAGE PHENOTYPES

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Macrophages are white blood cells of the innate immune system that have the ability to change phenotypically depending on the stimuli present in their surroundings through a process commonly referred to as polarization. Macrophage phenotypes broadly range from pro-inflammatory, anti-tumor (M1) to immune-suppressing (M2). Of particular interest to this work, breast cancer progression and metastasis rely on the presence of M2-like tumor-associated macrophages (TAMs). While many studies have shown the involvement of macrophages in tumor progression and metastasis, there remains a need to further explore these interactions and the polarization process, including tracking of macrophage subtypes. Toward this end, I have sought to develop more reliable and efficient polarization-probing tools for the study of macrophage phenotypes, including in complex environments.

In this thesis, I used novel chemical biology methods to track and manipulate macrophage phenotypes, including in the interactions between macrophages and breast cancer. First, I used an innovative sensor array that tracks macrophage polarization phenotypes in a time-efficient, high-throughput manner. I also studied the effects of phenotypic changes and cancerous environments on cellular circadian rhythms via
luminometry. I found that different macrophage polarization states displayed differences in their circadian rhythms and that conditioned media derived from aggressive breast cancers affected macrophages’ circadian rhythms to a greater extent.

The potential of re-programming macrophages as treatment strategies for diseases has gained a lot of attention in the immunology field. In cancers, the use of small molecules to activate macrophages to inflammatory states can induce specific immune responses. At the same time, there are other diseases where the reduction of inflammation is sought. Toward these ends, I evaluated the abilities of small molecules to modulate macrophage phenotypes. Specifically, I investigated the potential of curcumin-derivatives to reduce inflammation in macrophages and a TLR-4 agonist to induce immune-stimulating, anti-cancer responses.

To overcome some of the limitations in studying macrophage phenotypes in complex, multi-cellular environments, I generated a promoter-based reporter cell line that is indicative of macrophage polarization state through fluorescence. This model allows for tracking of macrophage phenotypes in multi-cellular experimental formats and, therefore, facilitates the study of macrophage responses in the context of diseases, like cancer. In summary, this study provides new ways to probe macrophage phenotypes in real time and denotes the necessity of using more representative models of the tumor microenvironment (TME), when studying the interactions between cancer and the immune system.
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CHAPTER 1

INTRODUCTION

1.1 Innate Immunity and Macrophages

Our bodies protect themselves against pathogens and other aberrant entities via two major processes -- innate and adaptive immunity,\(^1\) each of which has a different function and mechanism. Innate, or natural, immunity refers to the defense system with which humans were born. Associated responses are not specific to individual entities, but depend on proteins and phagocytic cells that recognize conserved features of pathogens and other foreign elements, and become quickly activated to help destroy them.\(^2\) While the innate immune system attacks only based on identification of general threats, adaptive (or acquired) immunity is activated when the host is exposed to an entity and uses immunological memory to learn about the threat and enhance the immune response accordingly for the long term.\(^2\) Multiple studies have shown that there is crosstalk between the innate and adaptive immune responses, which includes the involvement of natural killer (NK) cells, dendritic cells and macrophages (innate immunity), and B cells and T cells (adaptive immunity).\(^3\)

Macrophages are of particular interest due to their implication in many diseases including atherosclerosis, asthma, inflammatory bowel disease, rheumatoid arthritis, fibrosis, and many cancer types.\(^4,5\) These immune cells are present in every tissue type and necessary for homeostasis. They possess the ability to sense and respond to pathogens and other environmental challenges and also participate in tissue repair after injury. Their plasticity (ability to change) allows them to be epigenetically programmed in response to signals originating from within the tissue environment.\(^5,6\) Additionally, macrophages can
interact directly with surface receptors on other tissue-resident cell populations, immune cells recruited during injury, and extracellular proteins. As a result, macrophages play diverse roles in development, the acute response to infection and tissue injury, and tissue repair. These cells also have the ability to phagocytose (engulf and degrade) bacteria, dead cells, cell debris, tumor cells and foreign materials, and secrete both pro-inflammatory and antimicrobial agents.5,8

1.2 Macrophage Subtypes, Phenotype-Specific Characteristics, and Functions

Macrophages are considered to be highly dynamic cells due to their ability to change their constitution and carry out varying roles in response to different stimuli. This ability to change, also known as plasticity, allows macrophages to adopt different phenotypes or behaviors through a process called macrophage polarization.9,10 These phenotypic changes range from a pro-inflammatory (M1-like) to an anti-inflammatory (M2-like) polarization state and vice versa (Figure 1.1).11,12

**Figure 1.1: Macrophage polarization and phenotype-associated markers.** Schematic of the macrophage polarization process. Shown are non-differentiated (M0) and differentiated macrophages of two subtypes, M1 (pro-inflammatory, anti-tumor) and M2 (anti-inflammatory, pro-tumor). The orange and blue arrows depict the up-regulation of M1-associated markers (iNOS-TNF-α, TLR-2 and ICAM1) and M2-associated makers (EGR2, CD206, NRP1 and CD36), respectively.
M1-like, or “classically activated” macrophage phenotypes, can be induced by some pro-inflammatory agents that are secreted by immune cells, such as tumor necrosis factor-alpha (TNF-α), interferon gamma (IFN-γ) or by foreign entities, like bacterial lipopolysaccharide (LPS). This subtype, commonly induced by exposure to LPS and/or IFN-γ, is characterized by higher phagocytic activity and production of pro-inflammatory entities such as TNF-α, and nitric oxide (NO), as well as reactive oxygen species (ROS). M1-like macrophages have the ability to protect against bacterial and viral infections, and target, engulf, and eliminate aberrant cells from the body.

At the other end of the spectrum, macrophages can assume roles associated with immune suppression and wound-healing responses. Due to their differing properties, polarizing stimuli, and characteristic markers, M2-like macrophages are further categorized into M2a, M2b, M2c, and M2d subtypes. M2a macrophages are activated by interleukin (IL)-4 or IL-13, and possess increased expression of IL-10, Tumor Growth Factor-Beta (TGF-β), chemokine ligand (CCL)-17, CCL-18, and CCL-22. Cells of this subtype possess higher endocytic activity and promote cell growth and tissue repair. M2b macrophages are activated by immune complexes, toll-like receptor (TLR) ligands, and IL-1β, and release both pro- and anti-inflammatory cytokines, such as TNF-α, IL-1β, IL-6, and IL-10. M2b macrophages regulate the extent of immune responses and inflammatory reactions. M2c macrophages are induced by glucocorticoids, IL-10, and TGF-β, and are considered to be “inactivated macrophages.” These cells have been shown to secrete IL-10, TGF-β, CCL-16, and CCL-18, and play key roles in the phagocytosis of apoptotic cells. M2d macrophages, activated by toll-like receptor antagonists and exposure to IL-
6, lead to the release of IL-10 and vascular endothelial growth factors (VEGF) and promote angiogenesis and tumor progression in multiple cancer types.\textsuperscript{16,19,20}

1.3 Macrophage Plasticity and Involvement in Diseases

Macrophages’ ability to change their phenotypes in the presence of different stimuli is essential for homeostasis and proper functioning of bodily tissues. However, some major diseases, such as sepsis, infection, chronic inflammatory diseases, neurodegenerative diseases, and cancers,\textsuperscript{5,9,15} can progress more rapidly depending on the polarization state of the macrophages present at the disease site. The involvement of macrophages in these disorders makes these cells an attractive therapeutic target.\textsuperscript{21}

1.3.1 Inflammatory and Auto-Immune Diseases

M1 macrophages secrete pro-inflammatory cytokines to fight infections and kill aberrant cells, which is favorable when properly orchestrated.\textsuperscript{4,5,8} However, diseases like systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), type 1 diabetes (T1D), inflammatory bowel disease (IBD), and atherosclerosis promote excessive inflammation by polarizing macrophages toward the pro-inflammatory M1 subtype, which fosters the right conditions for the progression of the disease.\textsuperscript{22,23}

1.3.2 Resolution of Inflammation and Immunosuppressive Diseases

On the other hand, M2 macrophages have wound-healing or tissue-repairing capabilities, and can aid in the resolution of inflammation.\textsuperscript{7} While these processes are naturally occurring, they may also lead to immunosuppression and unfavorable outcomes in some conditions. In fact, some diseases, like \textit{Leishmania}-caused infections, induce polarization of macrophages toward an anti-inflammatory M2 phenotype, which results in an
immunosuppressive environment that leaves the host unable to fight the disease.\textsuperscript{24} This constant state also provides the right environment for some cancer types to grow and metastasize (described further in \textit{Section 1.4}).\textsuperscript{25} Additionally, M2 macrophages are known to be important regulators of fibrogenesis, which facilitates the progression of pulmonary fibrosis.\textsuperscript{26}

### 1.3.3 Macrophages and Cancer

Our bodies use the immune system to efficiently eliminate aberrant cells and maintain a homeostatic environment. However, in some instances, abnormal cells find ways to evade cell integrity checkpoints and the body’s immune response, and thus keep thriving, leading to cancer.\textsuperscript{27} For many years, cancer has been characterized by the uncontrolled growth and spread of abnormal cells, which can result in metastasis and ultimately death if not treated.\textsuperscript{28} The disease is a leading cause of death worldwide, accounting for nearly 10 million deaths in 2020 (or nearly one in six).\textsuperscript{29} Interestingly, macrophages have been found to be relevant to many cancer types, including breast,\textsuperscript{30} bladder,\textsuperscript{31} head and neck,\textsuperscript{32} glioma,\textsuperscript{33} melanoma,\textsuperscript{34} and prostate cancer,\textsuperscript{35} comprising up to 50\% of the tumor mass in some cases.\textsuperscript{36-38} These tumor-associated macrophages, or TAMs, are one of the main tumor-infiltrating immune cell types present in the tumor microenvironment (TME; \textbf{Figure 1.2}). While often associated with being M2-like, TAMs can be categorized as either M1- or M2-like.
Figure 1.2: Macrophage roles in cancer and metastasis. Macrophages contribute to the epithelial mesenchymal transition (EMT), invasion, and metastasis. In the primary tumor environment, macrophages enable angiogenesis, assist in microenvironment remodeling, assist with intra- and extra-vasation of mesenchymal tumor cells, and are required for the establishment and maintenance of metastases. They are recruited to cancer sites by chemoattractants produced by the tumors. Figure adapted from ref. 39.

1.3.4 Anti-tumor, M1-like TAMs

In the context of cancer, M1-like TAMs exert anti-tumor functions, including regulation of cytotoxicity and/or antibody-dependent cell-mediated cytotoxicity (ADCC) to kill tumor cells.\textsuperscript{40} For the cytokine-mediated killing of tumor cells, which is a slow process that generally takes one to three days and involves multiple mechanisms, macrophages secrete toxic entities such as reactive oxygen species (ROS) and nitric oxide (NO).\textsuperscript{41} On the other hand, ADCC requires less time to kill tumor cells, but requires participation of anti-tumor antibodies. On the other hand, ADCC requires less time to kill tumor cells, but requires participation of anti-tumor antibodies.\textsuperscript{42}

1.3.5 Pro-tumor, M2-like TAMs

M2-like macrophages promote the presence and spread of tumor cells, inhibit T cell-mediated anti-tumor immune responses, facilitate tumor angiogenesis, and lead to tumor progression.\textsuperscript{40,43} However, the presence of these cells does not necessarily correlate with
negative outcomes in all cancer types. Previous studies have shown that high densities of TAMs correlate with better overall survival in patients with colorectal cancer, non-small cell lung cancer (NSCLC), and ovarian cancer. Nevertheless, a worse overall survival was observed in patients with glioma, gastric cancer, urogenital cancer, head and neck cancer, and breast cancer.

The polarization of TAMs is influenced by multiple microenvironmental cytokines, chemokines, growth factors, and other signals derived from the TME. Initially, these tumors secrete cytokines like CCL2 and colony stimulating factor (CSF)-1 to lure macrophages to the tumor site. Once at the tumor site, macrophages sense the presence of other cytokines and chemokines that are highly expressed by tumors, including IL-4, IL-6, IL-13, CCL7, CCL8, CCL9, CCL18, and CXCL12, and are then polarized to the tumor-promoting, M2 phenotype. Additionally, the presence of growth factors, such as vascular endothelial growth factor A (VEGF-A), epithelial growth factor (EGF), platelet-derived growth factor (PDGF), TGF-β1, hepatocyte growth factor (HGF), and basic fibroblast growth factor (BFGF), and over-activation of epidermal growth factor receptor (EGFR) signaling also promote TAM infiltration and M2-like polarization. M2-like macrophages can destroy the matrix membrane of endothelial cells by secreting matrix metalloproteinases (MMPs), serine proteases, and cathepsins, and decompose various collagen and other components of extracellular matrix. This crosstalk between TAMs and tumor cells also leads to epithelial-mesenchymal transition (EMT) and allows tumor cells to migrate and gain stem cell-associated properties resulting in tumor invasion and metastasis.
Tumor-induced hypoxia arises from uncontrolled cell proliferation that restricts blood vessel growth, oxygen supply, and nutrition. The hypoxic response induces angiogenesis, metabolic reprogramming, proliferation, self-renewal, and autophagy in attempt to restore oxygen levels.\textsuperscript{55,56} Macrophages are enriched in hypoxic areas with insufficient blood supply, and have been found to react to these environments.\textsuperscript{57} Hypoxia in tumors causes release of prostaglandin E2 and IL-10 by TAMs that create an immune-suppressive environment and stops the immune response through several mechanisms.\textsuperscript{55} This in turn helps to facilitate tumor progression and metastasis.\textsuperscript{58} Tumor cells are also known to secrete a wide range of proangiogenic cytokines, including BFGF, VEGF, IL-1, IL-8, TNF-\(\alpha\), MMP-9, and MMP-2.\textsuperscript{40} The expression of these molecules results in proliferation of endothelial cells, matrix remodeling, and vascularization.

Since macrophages can assist or destroy tumor cells depending on the phenotype they adopt, they have risen as a promising alternative target in the treatment of cancer.\textsuperscript{59} While it is important to address macrophage contributions to all cancer types, this dissertation is largely focused on tracking macrophage phenotypes in the context of breast cancer.

\textbf{1.4 Breast Cancer and Classifications}

Of particular interest and among the more common forms of cancer, breast cancer accounts for up to 50\% of new cancer cases among women.\textsuperscript{60} Understanding how mammary carcinomas operate, and their genetic and molecular backgrounds are crucial to the generation of therapies targeting breast cancer. Fortunately, multiple studies have shown there are fundamental differences among breast cancer types, which can serve as a metric for tumor aggressiveness and behavior. One categorization that is applicable to many
different cancer types is tumor grade. Also referred to as histologic grade, this is a description of a tumor based on how morphologically aberrant the cancer cells and tissue appear, and how quickly they grow and spread. Low-grade cancer cells resemble normal cells and tend to grow and spread more slowly than high-grade cancer cells.\textsuperscript{61}

Breast cancer tumors are also described based on their molecular profiles. The luminal A subtype is classified as the most common type of breast cancer, representing up to 50-60\% of all breast cancers.\textsuperscript{62} This tumor type, which has high expression of hormone receptors (estrogen (ER) and progesterone receptor (PR)) and low human epidermal growth factor receptor 2 (HER-2), tends to be slow-growing and less aggressive than other subtypes. Tumors with a luminal A background are associated with the most favorable prognosis, and they respond well to available therapies.\textsuperscript{63,64}

Luminal B tumors comprise 15-20\% of breast cancers and are known to have a higher cell division rate.\textsuperscript{65} These cells are ER-positive, HER2-positive and may be PR-positive or -negative. Due to how quickly these tumor cells divide, these tend to be higher grade than luminal A and thus are associated with poorer outcomes.\textsuperscript{63,64} HER2-enriched tumors are ER- and PR-negative and HER2-positive. HER2-enriched cancers tend to grow faster than luminal cancers and can have a worse prognosis. Advances in medicines including targeted therapies aimed at the HER2 protein have made treatments of this disease more successful.\textsuperscript{66,67}

Triple-negative or basal-like breast cancers are ER-, PR-, and HER2-negative. This category is considered to be the most aggressive form of breast cancer due to its enhanced growth rates and metastatic potential. Patients with triple-negative breast cancers (TNBC) have poorer prognoses than other subtypes. This cancer often does not respond well to
existing therapies and new ones are difficult to design in part because of the lack of molecular targets, such as hormone receptors or HER-2, that could be used for cancer therapy.\textsuperscript{66,68}

1.5 Commonly Used Models and Techniques to Study Macrophage Phenotypes

Today, there are multiple macrophage models to study the polarization process and its implication in different diseases. For example, primary macrophages are naturally occurring macrophages that stand as the most accurate representation of how a macrophage should behave. In the work presented herein, the primary cells used were obtained from either wild-type (WT) C57BL/6 or WT BALB/c mouse strains.\textsuperscript{69} These are commonly used mouse models for the study of immune responses, as they possess intact immune systems. Two common sources of primary macrophages from mice are the peritoneal cavity and the bone marrow.\textsuperscript{70}

While these cells are accurate \textit{in vitro} representations of macrophages, their isolation is time consuming and the cells are unable to divide once differentiated. Immortalized cells are derived from primary cells that have undergone manipulations so that they are able to divide indefinitely.\textsuperscript{71} The resulting cells are fairly robust, but the changes incurred may result in altered morphological, functional, and genetic characteristics compared to the parent cells. Hence, it is important to ensure that the immortalized model to be utilized behaves similarly to the primary cells with regard to the element(s) to be studied.

Two frequently utilized immortalized macrophage models are RAW264.7 and J774 cells.\textsuperscript{72,73} The RAW264.7 is a murine macrophage cell line that originated from an \textit{Abelson leukemia} virus-transformed cell lineage derived from BALB/c mice. This cell line is well-characterized with regard to macrophage-mediated immune, metabolic, and phagocytic
functions. J774 murine macrophages were isolated from the ascites of an adult, female BALB/c mouse with reticulum cell sarcoma.

Studying macrophage behavior does not only require appropriate macrophage models, but also a suitable method to keep track of their phenotypic changes. To date, the scientific community has used various optical imaging techniques for macrophage tracking in vivo and in vitro using different cancer models. Some of the commonly used optical techniques for macrophage imaging in animal models are bioluminescence imaging (BLI) and intravital microscopy, which require luminescent or fluorescent probes for macrophage labeling (e.g., intrinsic fluorophores, genetic probes, and commercial chemical probes).

However, these agents do not report on changes in macrophage biology. As such, there is still a need for tools that can be used to visualize changes in macrophage phenotypes in real time, including within multi-cellular systems, e.g., interactions with cancer cells in the tumor microenvironment (TME), both in vivo and in vitro.

At the same time, there are several assays that are used to monitor macrophage polarization, but these can only be used with fixed cells or lysates, and are not typically amenable for use with multiple cell-types or three-dimensional models. Real Time Polymerase Chain Reaction (RT-PCR) and ELISA, for instance, have been commonly used to identify macrophage phenotypes in vitro, but are limited because they require isolation of a single cell type and only provide average expression data for mRNA (for RT-PCR) and protein (for ELISA) levels for a given population. Immuno-staining and flow cytometry are two other approaches that can be used, and can provide data for individual
cells. But they are also limited to evaluations at single time points, and both require the use of macrophage-identifying probes when used in concert with multicellular models.

1.6 Commonly Used Platforms for Macrophage Studies
To better understand the effects of cell types on macrophages, studies have used different platforms that range from simply exposing macrophages to cell-derived conditioned media to co-culture with actual cells or even more complex experimental designs. The use of these models, however, depends on the applications and limitations of each model.

First, conditioned media or the cell secretome refers to the collection of proteins that contain a signal peptide and are processed via the endoplasmic reticulum and Golgi apparatus through the classical secretion pathway. More generally, conditioned media also contains proteins that are shed from the cell surface and intracellular proteins released through non-classical secretion pathway or exosomes. These secreted proteins include numerous enzymes, growth factors, cytokines and hormones or other soluble mediators that potentially influence the behavior of other cell types. For instance, conditioned media derived from cancer cells is commonly used to study the effects of cancer-derived secretomes on other cell types, such as macrophages and other immune cells. This model is fairly simple to generate and use, and is amenable with all of the methods for assessing macrophage behavior described in Section 1. However, it excludes other factors like cell-to-cell interactions, which may be important to account for in evaluating macrophage responses.

To overcome the lack of cell-to-cell interactions when using conditioned media, many studies use a two-dimensional or 2D co-culture setting. In adherent 2D co-cultures,
cells grow in a monolayer in a culture flask or plate, attached to a plastic surface. This method is especially helpful when it comes to studying cell-to-cell interactions, due to simple and low-cost maintenance of the cell culture and the feasibility of performing functional tests such as macrophage phagocytosis of cancer cells. Unfortunately, 2D cultures also have some limitations that include the disturbance of interactions between the cellular and extracellular environments, and changes in cell morphology, polarity, and method of division. These drawbacks led to the generation of experimental formats that more accurately mimic conditions found in vivo.

Three-dimensional or 3D co-cultures are in vitro generated environments in which cells are permitted to grow and interact with their surroundings in all three dimensions, similarly to how they would in vivo. 3D co-cultures better represent cell-to-cell and cell-to-extracellular environment interactions, morphology and cell division preservation, and access to oxygen, nutrients, metabolites, and signaling molecules or cytokines. Furthermore, the characteristics of cells and their responses, including to drugs, may differ based on whether cells are cultured in two versus three dimensions. However, 3D cancer models are hard to generate, culture and maintain, and include many other variables, such as tumor size and shape, that need to be considered for each individual experiment.

1.7 Macrophage Reprogramming Strategy for Treatment of Immune-Associated Diseases

The plasticity of macrophages, including their ability to be re-polarized, has drawn the attention of the scientific and medical fields as a therapeutic target for the treatment of many immune-associated diseases, including auto-immune diseases and cancers. For this reason, pharmaceutical companies have attempted to develop drugs targeting signaling
pathways or surface receptors, such as the TLR series or other cytokine-related receptors, that could be used to modulate macrophage phenotypes in the context of the disease.\textsuperscript{86} In auto-immune diseases like rheumatoid arthritis (RA), for instance, the presence of pro-inflammatory M1 macrophages worsens the symptoms of the disease, therefore, an anti-inflammatory, M2 phenotype is desirable.\textsuperscript{87} While RA treatments using anti-TNF-\(\alpha\) antibodies have shown promising results, their off-target effects leave the patient exposed to severe infection, which represents a significant drawback.\textsuperscript{88}

The development of a class of small molecule inhibitors referred to as targeted synthetic disease-modifying antirheumatic drugs (tsDMARDs), represents a breakthrough in rheumatoid arthritis (RA) treatment. tsDMARDs target intracellular JAKs/MAPKs/NF-\(\kappa\)B/SYK-BTK signaling pathways, which inhibit pro-inflammatory responses in macrophages, thus ameliorating the symptoms associated with the disease. Unfortunately, there are risks and adverse effects associated with tsDMARDs administration that include infections and thromboembolism.\textsuperscript{89} Therefore, there is a need to find more suitable small molecule inhibitors for the treatment of diseases like RA.

Of particular interest, curcumin, a constituent of turmeric, has emerged as a potential anti-inflammatory small molecule for the treatment of chronic inflammation and auto-immune diseases.\textsuperscript{90} However, this anti-inflammatory agent displays some limitations in terms of bioavailability and biodistribution due to its hydrophobicity. Therefore, some studies (including this thesis) are focusing their efforts on generating alternate forms of curcumin as a promising avenue for disease treatment.\textsuperscript{91}

In the context of cancer, small molecules are desired that induce a pro-inflammatory phenotype in macrophages or inhibit signals coming from tumor cells, thus preventing
macrophage polarization to a tumor-helping subtype.\textsuperscript{86,92} Macrophage surface receptors make ideal targets for small molecule drugs due to their accessibility. Imiquimod, for instance, an FDA-approved TLR-7 agonist for topical treatment of genital warts and basal carcinoma, is a successful example of a small molecule drug targeting macrophages.\textsuperscript{93} Unfortunately, previous studies developing drugs targeting macrophages have encountered limitations, including but not limited to, solubility.\textsuperscript{94} This is of particular relevance because many of the antigens that bind macrophage-specific receptors are highly hydrophobic, which translates to the drugs designed to bind those receptors also being hydrophobic. This results in solubility issues that ultimately represent a major limitation \textit{in vivo}. For instance, its low solubility in circulation is a major reason why imiquimod is only approved for topical skin use.\textsuperscript{95}

Most small molecule drugs encounter issues with regard to target specificity. While the therapeutics are designed to be specific for a particular target, they are not selective for a given tissue or cell type. This can result in a lack of accumulation at the intended site and/or off-target effects, which can cause reduced therapeutic activity or toxicity. Allowing small molecule immune-modulating drugs to freely interact with other components of the immune system also poses a major risk, since unintentional exacerbated activation of the immune system can lead to conditions such as cytokine storms and autoimmune disorders.\textsuperscript{96} These downsides inopportunistely limit the use of conventional small molecule drugs for macrophage polarization and reprogramming \textit{in vivo}. Therefore, it is of utmost importance to generate more effective and less toxic small molecules that can activate or suppress macrophage inflammatory activities to treat different diseases.
1.8 Dissertation Overview

Macrophages are cells of the innate immune system that change their phenotype depending on the stimuli present. Macrophage phenotypes and functions range from pro-inflammatory (M1) to anti-inflammatory (M2). Multiple studies have demonstrated the relevance of macrophage phenotypes, both M1 and M2, in cancer progression and metastasis. For this reason, understanding the interactions between macrophages and tumors/tumor cells, has never been more important. Unfortunately, current macrophage and tumor models, as well as the techniques used to study macrophage phenotypes in the context of cancer, have limitations that prevent a more accurate understanding of these interactions. In this thesis, I generate tools to understand and track macrophage phenotypes, including in the context of cancer, and develop the means to alter them via pro- or anti-inflammatory small molecule treatments.

In chapter two, we demonstrated the ability of a sensor array that provides an information-rich 5-channel output to successfully determine macrophage responses in a matter of minutes, and can be used in a high-throughput manner. The simple and robust sensor generates a high dimensional data array that enables accurate macrophage evaluations and discrimination in standard cell lines and primary cells. This platform was evaluated using cytokines as stimuli, as well as exposure to a model disease environment in the form of cancer conditioned media from different cell lines. In all cases, the sensor was able to discern the different treatments from one another.

In chapter three, we hypothesized that macrophage circadian rhythms are differentially affected based on the stimuli to which they are exposed. To test this hypothesis, we used luciferase reporters for circadian genes in macrophage cells,
RAW264.7-\textit{Bmal1}:luc and \textit{-Per2}:luc. We tracked the circadian rhythms in cytokine polarized RAW264.7 cells and in cells exposed to conditioned media from less and more aggressive breast cancer cells. Following M1 and M2 polarization, results showed differently altered circadian rhythmicities, periods, and amplitudes for each subtype. For the conditioned media treatment, we found that the conditioned media from the more aggressive breast cancer cells affected oscillations of the macrophages to a greater extent than did that from the less aggressive type.\textsuperscript{98}

In chapter four, towards development of small molecules for polarizing macrophages, we investigated the anti-inflammatory effects of a set of curcumin-derivatives, including in murine RAW264.7 macrophages. As described in \textit{Section 1.}, curcumin has beneficial properties including reduction of inflammation, but its lack of aqueous solubility and poor biodistribution have limited its use as a treatment for diseases. To try to overcome these issues while retaining curcumin’s biological activity, a series of derivatives were synthesized. These compounds showed enhanced solubility and similar anti-inflammatory effects compared to curcumin.

In chapter five, we explored the ability of a small molecule TLR-4 agonist to induce anti-cancer activity in macrophages. This compound, referred to as PBI1, demonstrated macrophage activation through activation of TLR4. Macrophage treatment with the compound also enhanced macrophage phagocytic efficiency versus non-treated macrophages. Additive effects were observed via use of a complementary strategy (anti-CD47 antibody), suggesting that this small molecule approach could be used in conjunction with other therapeutics.\textsuperscript{99}
In chapter six, we used biological and chemical biology-based approaches to study macrophages, including their associations with breast cancer and re-conversion via chemical entities. In initial work, we studied the regulation of eight polarization-associated markers (*Appendix A*). Then, we generated reporter cell lines based on inducible nitric oxide synthase (*iNos*) expression and epidermal growth receptor (*Egr*)2 expression in RAW264.7 cells. The Egr2 reporter did not function as desired (*Appendix B*). Using RAW264.7:*iNos*-eGFP cells, we showed that conditioned media derived from and co-culture with 4T1 or EMT6 cells resulted in reduced fluorescence. However, the extents and trends observed between the two cancer models were not similar.

Additionally, we showed that cell debris-containing (non-filtered) conditioned media had a greater capacity of inducing anti-inflammatory phenotypes compared to filtered versions lacking debris, however, was not cell-line dependent (*Appendix C*). Ultimately, co-culture of the RAW:*iNos*-eGFP cells with *in vitro* three-dimensional models of 4T1 or EMT6 cells also induced decreases in fluorescence, with the results were similar to those of the 2D co-culture model. Using PBI1, we then demonstrated that macrophages can be re-polarized into a pro-inflammatory (M1) phenotype and tracked, even in the presence of cancer stimuli. This reporter-based approach overcomes the limitations encountered when using other techniques and provides a way to study macrophage interactions with cancer in more realistic and complex environments.100

Overall, in this thesis I intended to aid the immuno-oncology field by generating tools to further understand and affect macrophage phenotype dynamics, including their interactions with breast cancer tumors and cells.
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CHAPTER 2

HIGH-CONTENT AND HIGH-THROUGHPUT IDENTIFICATION OF MACROPHAGE POLARIZATION PHENOTYPES


2.1 Introduction

Macrophages are plastic leukocytes that perform a vast range of immune- and homeostasis-related functions, with their function and behavior dictated by environmental stimuli. Macrophages can be characterized as being activated into two major phenotypes, M1 and M2.\(^1\) M1 macrophages are associated with inflammation, including secretion of pro-inflammatory cytokines, engulfment of foreign entities, generation of reactive oxygen and nitrogen species, and assistance in T-helper type1 (Th1) cell responses to fight infection. Conversely, M2 macrophages perform anti-inflammatory and wound repair functions.\(^2,3\) Disturbance of the mechanisms that govern the balance of M1 and M2 states can result in a number of health problems, including infections, cancer, pregnancy complications, and inflammatory and autoimmune diseases.\(^4,5\) Given the significance and complexity of the roles macrophages play in biology and disease, knowledge of their activation and polarization state can provide critical information regarding the disease microenvironment, and be useful in selecting therapeutic approaches. For example, manipulation of tumor-associated macrophages provides a potential means to combat cancer. The tumor microenvironment releases factors that drive macrophages toward an M2-like phenotype,\(^6\)
resulting in secretion of anti-inflammatory cytokines, promotion of tumor growth and invasion, and facilitation of metastases. Therapies are being developed to “re-educate” these tumor-associated macrophages (TAMs) from this immune-suppressing state to an antitumor M1 phenotype as a more effective, less toxic cancer treatment.\textsuperscript{7,8} The development of such entities would be facilitated by a means to evaluate macrophage characteristics in a straightforward and high-throughput manner.

Efforts to generate therapies based on macrophage phenotypic conversion (to stimulate immune activation or suppression) and evaluate macrophage immune responses to other agents in drug discovery and toxicology are challenging due to the complexity of the polarization process. An increasing body of research reveals that macrophage polarization is more intricate than a two-state, M1/M2 conversion; rather, a spectrum of states exists.\textsuperscript{9-11} M2 macrophages can be further subclassified into M2a, M2b, M2c, among others, depending on the activating stimulus and resulting surface markers displayed.\textsuperscript{12} In addition, the macrophage polarization/sub-polarization process is dynamic and can evolve based on changes in the microenvironment.\textsuperscript{13-15} Complicating the matter further, macrophages can have mixed or overlapping M1- and M2-associated indicators. For instance, macrophages isolated from patients with advanced gastric and pancreatic cancers show high levels of both pro-inflammatory and anti-inflammatory cytokines. Both sets expressed IL-10 (M2-associated), while the former also had high levels of IL-12, and the latter IL-1β and TNF-α (M1 associated).\textsuperscript{16,17} These factors make it challenging to identify macrophage polarization states for diagnostic applications and fundamentally, to understand or identify phenotypes that are relevant to disease states.
Currently, the presence or levels of cellular and/or secreted biomarkers is most commonly used to detect and characterize macrophage polarization. While providing useful information, this approach is reliant on the specificity of the markers and requires multiple assays to obtain sufficient information for cellular evaluation. Additional limitations include: expression overlap between different polarization states (as mentioned above), poor phenotypic resolution of similar stimuli, non-translatable markers between mice and humans, and the fact that mRNA levels do not necessarily signal a robust difference in protein expression/at the functional level. In addition, the techniques used to identify the presence of biomarkers, such as RT-PCR, Western blot, and flow cytometry, are expensive and not amenable to multiplexing or high-throughput applications. Thus, there is a strong need for a general high-throughput method that can be used to evaluate these cells and their characteristics to facilitate therapeutic design and understand phenotypic responses of macrophages to stimuli.

As an alternative to marker-specific approaches, chemical nose or array-based sensing employs and discerns selective interactions between analytes and sensor elements to generate unique patterns for each analyte. The resulting pattern can be further analyzed for quantitative classification. This approach has been successfully employed in a wide range of systems including mammalian cells, bacteria, and proteins in biofluids. The strategy is ideal for cell phenotyping because changes in cellular responses yield variations in surface composition (e.g., protein, lipids, glycans, etc.) that result in different fingerprints, providing high-content information for each cellular state. Because macrophage polarization is accompanied by changes in cellular metabolism and surface protein expression, we hypothesized that an array-based sensing strategy would
provide a general platform for discriminating macrophage phenotypic and sub-phenotypic states. Incorporation of this strategy into a multi-channel format would enable multidimensional, high-content output from a single microwell, rendering this method readily applicable to high-throughput screening.33

In this chapter, we describe the development and application of a polymer-protein supramolecular assembly as a sensor array to gather high-throughput, high-content information on macrophage polarization state. The sensor is composed of only two elements: a guanidine-functionalized cationic poly(oxanorborneneimide) (PONI) polymer, and an anionic green fluorescent protein (GFP). The two entities form a complex through electrostatic interactions, resulting in a Förster resonance energy transfer (FRET) pair. When this sensor is applied to macrophages in different polarization/sub-polarization states, it yields fluorescent signals in five channels. The multidimensional output is then quantitatively analyzed using linear discriminant analysis (LDA) to reproducibly classify different macrophage activation states (Figure 2.1). To the best of our knowledge, the 5-channel output sensor has not been reported previously and the high level of information density enables us to accurately profile a spectrum of activation state of macrophages. We validated the sensor with model macrophage RAW264.7 cells and primary bone marrow-derived macrophages (BMDMs) stimulated with known M1 and M2 polarizing cytokines. The successful discrimination of M1 and M2 macrophages among the five subtypes demonstrates the ability of the sensor to accurately differentiate subtle phenotypic changes. We further evaluated the efficacy of the sensor system in a model disease environment, where macrophages were cultured in cancer cell-conditioned media, generating distinct patterns for macrophages exposed to different cancer types. Taken together, the sensor
platform can classify macrophage phenotypes in a matter of minutes. Furthermore, this platform can read out the effects of subtle environmental changes on macrophages, providing a new tool for diagnostics and for fundamental studies of macrophage behavior. The information generated can provide valuable insights on macrophages in diseases, potentially improving efficiency of existing therapies and facilitating the development of new treatments.

Figure 2.1: Schematic illustration of phenotyping macrophage activation states using array-based sensor. (A) FRET-based sensor assembly was formed between PONI-C3-Guanidine-Pyrene and GFP. Selective interactions of sensor elements at cell surface membrane resulted in fluorescence changes in all five channels, generating a distinct fingerprint for each cell activation state. (B) Chemical structure of PONI-C3-Guanidine-Pyrene and the resulting five fluorescence channels in the FRET complex.
2.2 Materials and Methods

2.2.1 Materials

All reagents were purchased from Thermo-Fisher Scientific except where otherwise noted. All DMSO utilized was cell culture grade (Sigma). RT-PCR data was generated using a CFX Connect Real-Time PCR Detection System (Biorad). For assays requiring absorbance and fluorescence measurements, a SpectraMax M2 plate reader was used (Molecular Devices).

2.2.2 PONI-C3-Guanidine Polymer Synthesis

Monomers and polymers were synthesized according to previous reports.\textsuperscript{34}

2.2.3 Green Fluorescent Protein Expression

EGFP was constructed and characterized according to reported protocols.\textsuperscript{35} In short, \textit{Escherichia coli} strain BL21 was transformed with plasmids containing EGFP recombinant protein. After transformation and induction with IPTG, cells were lysed and purified by Co2+ nitrilotriacetate columns. Fluorescent proteins were further characterized by SDS-PAGE gel, scanning absorbance, and emission spectrum. The results are consistent with previously reported work.\textsuperscript{30}

2.2.4 Transmission Electron Microscopy

TEM samples were prepared with either 0.5 μM of C3-Gu-Py only or by mixing 0.5 μM of C3-Gu-Py with 50 nM of EGFP in 10 mM HEPES buffer for 30 min in the dark at room temperature. 5 μL of the solutions were then placed on 300 mesh Copper grids (with formvar films) obtained from Electron Microscopy Sciences (EMS FF300-Cu) and allowed
to dry overnight. The samples were analyzed using a TEM JOEL 2000FX at an acceleration voltage of 200kV.

2.2.5 FluorescenceTitration

0.5 μM C$_3$-Gu-Py polymer was titrated with EGFP at a concentration range from 0 to 200 μM in a black 96 well-microplate. The solution was mixed in 10 mM HEPES buffer. After 30 min incubation at room temperature in the dark, the fluorescence spectrum was measured at an excitation wavelength of 344 nm.

2.2.6 Binding Affinity Calculation

Fluorescence titration was utilized to calculate the binding affinity of the C$_3$-Gu-Py polymer with EGFP. The fluorescence decay of the C$_3$-Gu-Py excimer as a function of EGFP concentration was fitted to a one-site binding equation,$^{36}$ which is:

\[
I = I_0 + \left( I_{\text{lim}} - I_0 \right) \times \frac{C_0 + C + \frac{1}{K}}{2C_0} \times \left[ \sqrt{(C_0 + C + \frac{1}{K})^2 - 4CC_0} - (C_0 + C + \frac{1}{K}) \right]
\]

where I is the fluorescence intensity of C$_3$-Gu-Py excimer at a given concentration of EGFP, $I_0$ is the fluorescence intensity of C$_3$-Gu-Py in the absence of EGFP, $I_{\text{lim}}$ is the fluorescence intensity when the quenching reaches a plateau, $C_0$ refers to the concentration of C$_3$-Gu-Py, and C is the concentration of EGFP. Based on the equation, microscopic binding constant K was determined by using the non-linear least-squares curve fitting analysis in OriginPro (OriginLab Co.).
2.2.7 Linear Discriminant Analysis

Linear discriminant analysis (LDA) was applied on normalized fluorescence data to statistically classify each group, using SYSTAT software (version 11.0, SystatSoftware). All variables were used in the complete mode and the tolerance was set as 0.001. Input data was transformed to canonical scores to best separate each group where the between-class variance was maximized while the within-class variance was minimized.

2.2.8 Unknown Identification

The identity of unknown samples was predicted by computing the Mahalanobis distance of the unknown data to the training groups using LDA. First, the normalized fluorescence responses of the unknown samples were converted to canonical scores in LDA, using the discriminant functions established from the reference set. Next, Mahalanobis distance of that case to the centroid of each training cluster in the LDA space was computed. The unknown sample was predicted to belong to the closest group, defined by the shortest Mahalanobis distance.

2.2.9 Cell Culture

RAW264.7 cells, HeLa, and MCF7 cell lines were purchased from American Type Culture Collection (ATCC). Primary bone marrow derived macrophages (BMDMs) were isolated from freshly euthanized C57/B6 mice, donated generously by Dr. Jessie Mager, Department of Veterinary and Animal Science, University of Massachusetts Amherst. The BMDMs were isolated, differentiated and cultured according to previously reported methods. All cells were cultured at 37 °C under a humidified atmosphere containing 5% CO₂. Standard growth media consisted of high glucose Dulbecco's Modified Eagle
Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (100 μg/ml penicillin and 100 μg/ml streptomycin). Under the above culture conditions, the cells were sub-cultured approximately once every two to five days.

2.2.10 Macrophage Polarization via Activation Agents

Both RAW264.7 cells and BMDMs were treated with the following polarization stimuli for 48 h to induce the desired polarization state: LPS group, 50 ng/mL; IFN-γ group, 50 ng/mL; combo group, 50 ng/mL LPS and IFN-γ; IL-4 group, 30 ng/mL; and IL-10 group, 30 ng/mL. After 2-day polarization, cells were washed with PBS, trypsinized, and plated as 10,000 cells per well on a 96-well plate overnight before proceeding to sensing studies.

2.2.11 Macrophage Polarization via Cancer Cell Conditioned Media

HeLa and MCF7 cell lines were cultured using DMEM medium supplemented with 10% FBS and 1% antibiotics for 2 days to reach above 90% confluence. At this time, the media was replaced with fresh standard, growth media and cells were incubated for an additional 5-7 days. The supernatant from each cell line was then collected, centrifuged for 5 min, and filtered using a 0.45 μm syringe filter. Subsequently, growth and conditioned media at a 1:1 ratio were transferred into a T25 culture flask containing RAW cells. After 48 h of culture, RAW264.7 cells were washed with PBS, trypsinized and plated as 10,000 cells/well on a 96-well plate for overnight attachment.

2.2.12 Sensing Studies

The sensor was prepared by mixing 0.5 μM of C₃-Gu-Py with 50 nM of EGFP in 10 mM HEPES buffer for 30 minutes in dark at room temperature. Subsequently, 150 μL of sensor solution was incubated with and without the cell populations (washed once with PBS) in
96-well microplates. The change in fluorescence intensity for each channel was recorded every 15 minutes at its respective wavelength (pyrene monomer: 344/390 nm and 344/420 nm, pyrene excimer: 344/470 nm, EGFP: 475/510 nm, FRET: 344/510 nm) on a Molecular Devices SpectraMax M2 microplate reader using appropriate filters.

2.2.13 RT-PCR Preparation

Cells were plated in 24-well plates at a density of 50,000 cells/well. Cells were treated with the appropriate polarization stimulus for 48 h. Following treatments, RNA was extracted following the procedure below.

2.2.14 RNA Extraction and cDNA Conversion

Approximately 1.5 μg RNA was harvested using the PureLink RNA Mini Kit (Ambion) following the manufacturer's instructions. SuperScript IV Reverse Transcriptase was used for the conversion of approximately 150 ng of RNA to cDNA, along with RNaseOut, 10 mM dNTPs, and 50 μM Random Hexamers (ThermoFisher), also following the manufacturer's instructions.

2.2.15 Quantitative RT-PCR

RT-PCR was performed on cDNA as prepared above using a CFX Connect real-time system with iTaq Universal SYBR Green Supermix (Biorad). All DNA primers were purchased from Integrated DNA Technologies. The following primer sequences were used:

- **β-actin** (forward) 5’-GATCAGCAAGCAGGAGTACGA-3’,
- **β-actin** (reverse) 5’-AAAACGCAGCGCAGTAACAGT-3’;
- **iNos** (forward) 5’-GTTCTCAGCCCCAACAATACAAGA-3’
- **iNos** (reverse) 5’-GTGGACGGGTCGATGTCAC-3’;
- **Tnf-α** (forward) 5’-CCTGTAGCCCCACGTCGTA-3’,
- **Tnf-α** (reverse) 5’-GGGAGTCAAGGTACAACCC-3’;
- **Mannose receptor (MR)** (forward) 5’-GGATGTTGATGCTACTGGA-3’,
Mannose receptor (MR) (reverse) 5'-AGTAGCAGGGATTTCTGCTG-3';
Egr2 (forward) 5'-TGA GAGAGCAGCGATTGATT-3',
Egr2 (reverse) 5'-ATAACAGTCAGTGTGTC CCC-3',
Tlr-2 (forward) 5'-GCATCCGAATTGCATCA CCG-3'
Tlr-2 (reverse) 5'-ACAGCGTTTGCTGAAGAGGA-3',
cd-36 (forward) 5'-TTGAAGAAGGAACC ACTGCT-3',
cd-36 (reverse) 5'-AACAGACAGTGAAGGCTCAA-3';
Icam1 (forward) 5'-GGACTTTCCATCTTCCAGC-3',
Icam1 (reverse) 5'-CCAGGTATATCCGAGCTTCA-3';
Nrp1 (forward) 5'-GACCATA CAGGAGATGGCAA-3',
Nrp1 (reverse) 5'-GTAGCGT A GTGGACCCTCA-3'.
Tgf-β (forward) 5'-GCGGA CTACTATGTAA AAGA-3',
Tgf-β (reverse) 5'-TTCTCATAGATGGCGTTGTT-3'.

Analyses were performed as follows: the samples were first activated at 50 °C for 2 min, then 95 °C for 2 min. Then denaturing occurred at 95 °C for 30 s followed by annealing at 57 °C; the denature/anneal process was repeated over 40 cycles. Relative gene expression was determined by comparing the Ct value of the gene of interest to that of the β-actin housekeeping gene, by the $2^{\Delta\Delta Ct}$ method. Three biological replicates were performed for each control group and three technical replicates were used for each biological replicate.

2.3 Results and Discussion

2.3.1 Supramolecular Assembly of Sensor

The sensor is designed to provide an information-rich, five-channel output with only two sensor elements. The first element of the sensor is a cationic poly(oxanorbornene) (PONI) random copolymer scaffold that incorporates a guanidine group and a pyrene dye molecule ($C_3$-Gu-Py). The positively charged guanidine group ensures that selective interactions occur only when the complex is close to negatively charged cell surface functionalities. The solvatochromic pyrene molecule will alter its spectral properties when local environmental factors, such as polarity and hydrophobicity, change. In this way, both
selectivity and sensitivity of the sensor are ensured. Through electrostatic interactions, cationic C₃-Gu-Py forms a polymeric complex with an anionic green fluorescent protein (EGFP). In practice, the pyrene unit provides three signals, two corresponding to the free pyrene and one to the excimer form. The EGFP then adds two channels: free GFP fluorescence and FRET with the two pyrene channels (Figure 2.1B).

Initial studies focused on the optical characterization of the C₃-Gu-Py/EGFP supramolecular assembly. Polymer C₃-Gu-Py was titrated with increasing concentrations of EGFP. After 30 min of incubation, a simultaneous decrease in pyrene emission at 470 nm and increase of GFP emission at 510 nm was observed upon irradiation with 344 nm light (Figures 2.2 and 2.3A). Efficient fluorescence quenching of C₃-Gu-Py was observed (Figure 2.3B) at higher concentrations of assembly. The association constant Ka of $7.17 \times 10^5$ M⁻¹ was derived by fitting the fluorescent titration curve.
Figure 2.3: Fluorescence titrations (a) and quenching (b). 0.5 μM C₃-Gu-Py was titrated with varying concentrations of EGFP in 10 mM HEPES buffer. Fluorescence spectrum was recorded at pyrene excitation of 344 nm. A decrease in pyrene emission at 470 nm and increase of EGFP emission at 510 nm was observed. Each value is the average of three independent measurements.

The overall spectrum featured five output peaks that can be recorded from the sensor: pyrene monomers at 344/390 and 344/420, pyrene excimer at 344/470, EGFP at 475/510, and FRET signal at 344/510. Based on the spectral flexibility, a concentration of 0.5 μM C₃-Gu-Py and 50 nM EGFP was selected for experiments. Dynamic light scattering (DLS) data revealed the polymer assembly was ~230 nm in diameter and the size slightly increased to ~237 nm when EGFP was added (Figure 2.4). Transmission electron microscopy (TEM) images confirmed these results (Figure 2.5), indicating that a supramolecular assembly was formed between C₃-Gu-Py and EGFP.
Figure 2.4: Hydrodynamic size of PONI-C3-Gu-Py polymer (a) and polymer-GFP assembly (b) in 10 mM HEPEs buffer. C3-Gu-Py polymer formed a complex with an average diameter of 230 ± 84.4 nm. With the addition of EGFP, the size of polymer-EGFP assembly is approximately 237 ± 97.7 nm in diameter.

Figure 2.5: TEM images of PONI-C3-Gu-Py polymer (a) and polymer-EGFP assembly (b) in 10 mM HEPEs buffer. C3-Gu-Py polymer formed a complex of size ~25 nm. The difference in sizes measured by DLS and TEM can be attributed to the drying process during TEM sample preparation as well as the high vacuum conditions in the TEM chamber. Upon the addition of EGFP, larger complexes were observed.
2.3.2 Discrimination of M1 and M2 Subtypes Using RAW264.7 Cells

We first tested the ability of the sensor system to distinguish among macrophage phenotypes using the RAW264.7 macrophage cell line. Established cytokines were used, each activating macrophages through a different mechanism (Table 2.1), generating a distinct phenotypic state. In general, RT-PCR results assessing standard M1 and M2 markers confirmed that after 48 h activation, cells were polarized into corresponding states (Figure 2.6). LPS and IFN-γ treated cells (M1 stimulation) showed significant increases in Tnf-α and iNos mRNA expression whereas the IL-4 (M2a stimulation) group had an increase in Egr2 and mannose receptor (MR) expression. Although the IL-10 group (M2c stimulation) was tested against multiple M2 markers, including Egr2, MR, and Tgf-β, as well as the reduction of M1 marker iNos, no significant changes in the levels of expression of any associated genes were observed (Figure 2.7).

Table 2.1: Mechanisms and effects of in vitro macrophage polarization via different cytokines.

<table>
<thead>
<tr>
<th>Polarization</th>
<th>Mechanism</th>
<th>Surface marker change</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipopolysaccharide</td>
<td>Binds TLR4, induces pro-inflammatory cytokines&lt;sup&gt;42&lt;/sup&gt;</td>
<td>Increased expression levels of MHC-II, CD80, CD86; decreased levels of MRC1 or Fc-γ RII&lt;sup&gt;43&lt;/sup&gt;</td>
<td>M1</td>
</tr>
<tr>
<td>LPS</td>
<td>Interferon-γ (IFN-γ)</td>
<td>Binds IFN-γ receptor&lt;sup&gt;44&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Combo (LPS + IFN-γ)</td>
<td>Synergizes LPS and IFN-γ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interleukin 4 (IL-4)</td>
<td>Binds IL-4Rα and IL-2R, down-regulates proinflammatory mediators&lt;sup&gt;1,32&lt;/sup&gt;</td>
<td>Decreased expression of CD14 and CCR5&lt;sup&gt;45&lt;/sup&gt;; regulation of MHC-II, β2 integrins, chemokine CCL22/MDC&lt;sup&gt;46&lt;/sup&gt;</td>
<td>M2a</td>
</tr>
<tr>
<td>Interleukin 10 (IL-10)</td>
<td>Inhibits production of pro-inflammatory cytokines</td>
<td>Down-regulation of MHC II and co-stimulatory molecules</td>
<td>M2c</td>
</tr>
</tbody>
</table>
Figure 2.6: Macrophage activation confirmed by RT-PCR. (A) mRNA quantification of M1-associated genes, Tnf-α and iNos. (B) mRNA quantification of M2-associated genes, Egr2 and MR, according to treatment group. Control = non-treated cells, combo = LPS + IFN-γ treated. Fold changes in mRNA level were normalized to β-actin. Statistical significance was determined by two-tailed student t-test. *= p < 0.1, **= p < 0.05, ***= p < 0.005, n = 3 biological replicates (3 technical replicates were used each). n.s. = not significant.

Figure 2.7: RT-PCR quantification of M2 state in IL-10 activated RAW264.7 macrophages. Tgf-β is M2-associated gene. Reduction of M1-associated gene iNos is used to evaluate M2 state. Control = non-treated cells. Fold changes in mRNA level were normalized to β-actin. Statistical significance was determined by two-tailed student t-test. *= p < 0.1, **= p < 0.05, ***= p < 0.005, n = 3 biological replicates (3 technical replicates were used each). n.s. = not significant.
Having confirmed that polarization had occurred, cells from each treatment group were plated on a 96-well microplate for overnight attachment. Equivalent cell numbers (10,000 per sample) were used to ensure that changes in sensor response were due to alterations in cell surface functionalities, not density. For the sensing process, C₃-Gu-Py and EGFP were premixed for 30 min to allow formation of stable FRET complexes. Subsequently, cells were washed once with phosphate buffered saline (PBS) and incubated with the sensor complex in the dark. Fluorescence signals were recorded every 15 min until equilibrium was reached. The 5-channel readout generated a distinct fluorescence pattern for each treatment group (Figure 2.8A). We further utilized linear discriminate analysis (LDA) to test whether the six cell phenotypes could be robustly discriminated based upon their fluorescent signatures. As shown in Figure 2.8B, the LDA plot revealed six distinct clusters for M1 and M2 subtypes with a correct classification of 100%, demonstrating that each activation pathway resulted in a distinct cellular response.

We further validated the reliability of the sensor by performing unknown sample identification. Among the 45 tested unknowns, 41 samples were binned correctly into their corresponding group, giving a high percentage of correct unknown identification at 91%. Next, we investigated the necessity of having 5 channels of information from the sensor by comparing the performance of classification and unknown identification using either an individual sensor element or different combinations. The highest percentage of accuracy was achieved when all 5 channels were used, demonstrating the importance of multidimensional data in discriminating complex cell phenotypes. (Figure 2.8C-D).
Figure 2.8: Discrimination of RAW264.7 macrophages activated by M1 or M2 subtype stimuli using sensor complexes. (A) Fluorescence intensities of each treatment group were obtained at 30 min and normalized against sensor only. n = 8 biological replicates. (B) The fluorescence patterns were analyzed through linear discriminant analysis (LDA) and the first two canonical scores were plotted with 95% confidence ellipse (n=8). Correct classification percentage (C) and unknown identification (D) of M1 and M2 subtypes using different combinations of sensor channels.

Accurate phenotyping was obtained in less than an hour, which is highly desirable for high-throughput applications. By generating a distinct fluorescence pattern corresponding to each activation stimulus, this method can discriminate among major cell polarization states (M1 and M2) in a simple, robust, and rapid manner. In addition, this method can further distinguish different subtypes of major macrophage phenotypes. Since this system does not rely on specific markers, the global information gathered from the 5-channel sensor can discern less-characterized sub-phenotypes, such as IL-10 stimulated macrophages, which are challenging to identify using traditional methods like RT-PCR.
2.3.3 Discrimination of M1 and M2 Subtypes with Primary Macrophages

Immortalized macrophage cell lines provide a useful tool for assessing sensor response, however these models differ in multiple aspects from their primary cell analogs. We next tested the sensor using physiologically relevant primary bone marrow-derived macrophages (BMDM). Progenitor cells were isolated from C57/B6 mice and induced to differentiate into macrophages using previously reported procedures. Once macrophage cells were obtained, we exposed them to M1 and M2 subtype polarization stimuli for 48 h as used above for RAW264.7 cells. RT-PCR results confirmed appropriate activations in each case, with increases in Tnf-α and iNos mRNA expression for M1 related stimuli (LPS and/or IFN-γ) and Egr2 and MR mRNA levels for IL-4 stimulated M2 cells. Although IL-10 activation did not show substantial enhancement in Egr2 levels, a nearly 6-fold increase in MR expression was observed (Figures 2.9 and 2.10). Following macrophage polarization, the sensor complex was added to equal numbers of cells under each treatment group and fluorescence results were obtained after 15 min. Complete discrimination among the five assessed groups of M1 and M2 phenotypes was achieved with 96% correct classification (Figure 2.11). 92% of correct unknown identification confirmed the high reliability of our sensor.
Figure 2.9: RT-PCR quantification of M1-associated genes in primary bone marrow-derived macrophages, according to treatment group. Control = non-treated cells; combo = cells treated with both IFN-γ and LPS. Fold changes in mRNA level were normalized to β-actin. *= p < 0.1, **= p < 0.05, ***= p < 0.005, n = 3 biological replicates (3 technical replicates were used each). n.s. = not significant.

Figure 2.10: RT-PCR quantification of key M2 genes in primary bone marrow-derived macrophages, according to treatment group. Control = non-treated cells. Fold changes in mRNA level were normalized to β-actin. *= p < 0.1, **= p < 0.05, ***= p < 0.005, n = 3 biological replicates (3 technical replicates were used each). n.s. = not significant.
When the sensor complex interacts with cells, in most cases, all monitored fluorescence channels show an increase in signal intensity. This suggests that upon interacting with macrophages, the sensor complex disaggregates, exposing its individual components to interact with the cell surface. Depending upon the local environment, the fluorescence intensities for individual molecules (pyrene, EGFP, and FRET) also change. Since distinct fluorescence patterns were consistently observed for each stimulus, we believe this disruption process is modulated by cell surface functionalities and composition. Our previous studies have indicated that the sensor complex is highly sensitive to glycosylation patterns on cell surfaces. However, more mechanistic studies are needed in order to elucidate what other cell components are also interacting with sensor elements.
2.3.4 Discrimination of Macrophages Exposed to Conditioned Media from Different Cancer Cells

The above studies demonstrate that our sensor array was able to discriminate macrophages polarized with specific cytokines. However, biological microenvironments are often far more complex and have multiple stimuli. Hence, we assessed whether the sensor could discern macrophage phenotype in a model disease environment to address this issue. First, conditioned media was generated by culturing different types of cancer cells (HeLa, cervical carcinoma, and MCF7, mammary carcinoma) until ~80% confluence was reached. Then, the culture media was extracted and used to stimulate macrophages for 48 h. RT-PCR results revealed different activation patterns for macrophages activated with media conditioned from different cells (Figure 2.12).

![Figure 2.12: RT-PCR quantification macrophages exposed to conditioned media from different cancer cells.](image)

(a) mRNA quantification of M1-associated genes, Tnf-α and iNos. (b) mRNA quantification of M2-associated genes, Egr2 and MR. Control = non-treated cells. MCF7-M and HeLa-M = conditioned media from MCF7 and HeLa cells, respectively. Fold changes in mRNA level were normalized to β-actin. *= p < 0.1, **= p < 0.05, ***= p < 0.005, n = 3 biological replicates (3 technical replicates were used each). n.s. = not significant.
C3-Gu-Py and EGFP complexes were added to cells and the 5-channel fluorescence readouts were collected. Distinct fluorescence signals were obtained for macrophages subjected to each of the conditioned media types. An LDA plot showed three well-separated clusters with 100% classification accuracy (Figure 2.13). When macrophages were exposed to cultured media conditioned by cervical cancer versus breast cancer cells, the sensing readout was dramatically different, indicating that a unique state of activation was present following each type of stimulation. A high percentage (96%) of correct unknown identification was also achieved. These results are exciting because they demonstrate that this method not only functions following single cytokine stimulation, but also in more complex environments. This is promising evidence that with careful evaluation, this sensing method could be applied to profile macrophages from individual patients, offering insights for precision medicine.

Figure 2.13: Discrimination of macrophage cells cultured under exposure to conditioned media from different cancer cell types for 48 h. The LDA plot of the first two canonical scores was obtained and plotted with 95% confidence ellipses (n=8). CM is cancer cell conditioned medium, with the cell line type preceding it, control represent macrophages cultured using standard growth media.
2.4 Conclusion

Macrophage polarization is a complex and dynamic process. With its roles in homeostasis and disease, it is important to be able to discern macrophages characteristics in a rapid and straight-forward manner. In this chapter, we report a simple and robust sensing method that can quickly capture the overall responses of activated macrophages under different environmental stimuli.

Compared with current methods of characterizing macrophage polarization, the sensor reported in this study has advantages of generating a multidimensional, high-content chemical readout regarding the cell surface, in a high-throughput matter. Standard methods such as RT-PCR and ELISA, can only capture a limited number of well-established markers for each cell activation state, and are independent (not multiplexed) assays, requiring a separate analysis for each. Considering the heterogeneity of macrophage polarization and the overlapping nature of M1 and M2 markers,\textsuperscript{11,12} it is also difficult to elucidate and differentiate activation states with standard methods. For instance, the multiple IL-10 markers used in our RT-PCR studies did not reveal significant changes. The ambiguity of a less-well characterized sub-phenotype could be because the end-point evaluation missed the dynamic changes in macrophage marker expression during the 48 h activation.

In contrast, the array-based sensor utilizes selective interactions between sensor elements and the entire analyte surface to generate high-content fingerprints for each activation state. Once trained, the sensor can rapidly identify target analytes through pattern recognition. Although the C\textsubscript{3}-Gu-Py moiety has been utilized for bacterial sensing,\textsuperscript{48} its
capability in mammalian cell sensing has not been investigated. By coupling the polymer with GFP through supramolecular interactions, we observed that the IL-10 treated group has a distinct set of characteristics in comparison with the other M2 subgroups in both tested cell models (Figures 2.8 and 2.11). The 5-channel, high-content information gathered from the sensor is crucial in achieving a high level of classification accuracy and allows us to address challenging biological questions from a chemical perspective. In addition, running assays like RT-PCR and ELISA can be time-consuming and error prone, with relatively high costs for thorough characterizations consisting of multiple markers. In contrast, the sensor material used here is synthetically easy to generate, and all components can be mixed in one microplate well, which not only reduces sensor material but is also compatible for high-throughput screening applications.

Due to the robust and facile nature of the system, there are many potential applications for the array-based sensing strategy. Altered immune states are a major factor in diseases including cancer, atherosclerosis, and auto-immune disorders. Macrophage polarization states are key in driving forward disease progression. Rapid assessment of their activation states can provide valuable information in selecting appropriate therapeutic strategies. Notably, the high-throughput nature of the method would facilitate the rapid screening of immune states for individual patients, enabling personalized medicine approaches in tackling these immune-driven diseases. Furthermore, this strategy could be applied to other plastic immune cells, such as dendritic cells and T cells. By extending this sensor to other cell types, the status of major components of the immune system could be rapidly determined. This strategy can also greatly improve the drug discovery process, by allowing for rapid identification of altered cell states, and/or evaluation of...
immunogenicity following agent treatment. Potential immune adjuvants or anti-inflammatory entities could be screened together by using the sensor on immune cells in a multi-well plate format. With these capabilities, the sensor system not only has utility as a fundamental research tool, but as a high-throughput, high-content means for therapeutic screening against other plastic cell types.

2.5 References


CHAPTER 3

MACROPHAGE CIRCADIAN RHYTHMS ARE DIFFERENTIALLY AFFECTED BASED ON STIMULI


3.1 Introduction

The immune system is a network of cells, organs, and biomolecular entities that protects organisms against foreign pathogens. Multiple immune-related activities are regulated in a circadian manner, meaning that they occur with an approximately 24 hour cycle. These include natural killer (NK) cell cytokine production and immunity against pathogens, numbers of particular white blood cell types (e.g., hematopoietic stem cells, B cells, and T cells) in the blood, and/or lymph nodes, migration of hematopoietic cells, neutrophils, and monocytes to tissues, phagocytic activity of neutrophils, and expression of cytolytic factors by NK cells. As a result, circadian time can determine immune responses to stimuli. For example, sensitivity to lipopolysaccharide (LPS, an endotoxin found in outer membranes of gram negative bacteria) and inflammatory responses against Salmonella enterica serovar Typhimurium have been found to be circadian, with increased responses at the start of the active phase and early rest period of mice, respectively. Mice infected with Leishmania or Trichuris muris pathogens in the morning showed lower parasite loads relative to those infected in the evening. Mouse models of peritoneal inflammation show more recruitment of inflammatory monocytes to the peritoneal cavity, spleen, and
liver in the afternoon versus the morning,\textsuperscript{10} while in mouse models of myocardial infarction, neutrophil recruitment to cardiac tissue peaks in the evening.\textsuperscript{11} In humans, which are diurnal (as opposed to mice, which are nocturnal), immune responses can also vary in a circadian manner. Allergic symptoms are increased between midnight and the early morning,\textsuperscript{11,12} influenza vaccinations in the morning result in greater antibody concentrations compared to those administered in the afternoon,\textsuperscript{13} and asthma worsened lung function and resulted in increased sputnum leukocytes and eosinophils in the early morning, compared to in the late afternoon.\textsuperscript{14}

Macrophages are phagocytic white blood cells involved not only in the innate immune response, but also in organismal development, homeostasis, and tissue repair.\textsuperscript{15} Macrophages shift their functional state to respond to physiological challenges or environmental factors. Specific characteristics and roles are associated with their polarization to subtypes broadly classified as classically (M1) or alternatively (M2) activated, which are inflammatory or immune-suppressing, respectively.\textsuperscript{15,16} M2 macrophages may be further categorized into M2a, M2b, M2c, and M2d subtypes, which arise from activation by different cytokines and have different responses. M2a macrophages are activated by IL-4 or IL-13 and lead to increased expression of IL-10, TGF-\(\beta\), CCL17, CCL18, and CCL22; they possess endocytic activity and promote cell growth and tissue repair.\textsuperscript{17} Because we are using IL-4 to polarize macrophages, exclusively resulting in the M2a subtype, we refer to these cells as being M2.

Macrophages have been shown to possess their own intrinsic clocks that operate autonomously,\textsuperscript{18} with a functional core molecular circadian clock (comprised of a negative feedback loop with BMAL1 and CLOCK proteins as activators, and PER and CRY
proteins as repressors).\textsuperscript{19} Approximately 8-15\% of the macrophage transcriptome has circadian oscillations, including genes required for pathogen recognition and responses.\textsuperscript{18,20} Furthermore, multiple macrophage functions are circadian-dependent, such as recruitment to infected tissue,\textsuperscript{8} generation of chemokines and cytokines, and phagocytosis.\textsuperscript{21,22} Considering that macrophages have highly divergent functions, from tissue remodeling\textsuperscript{23} and angiogenesis\textsuperscript{24} to generation of reactive oxygen species (ROS)\textsuperscript{25} and phagocytosis,\textsuperscript{21,22} it is important to address how circadian oscillations might change concomitantly with phenotype.

Previous studies have indicated that there is a link between macrophage activation and circadian rhythms and/or molecular clock elements. These evaluated the impacts of LPS (which is pro-inflammatory) and other cytokines on circadian clocks in primary, peritoneal macrophages\textsuperscript{26} and bone marrow-derived macrophages (BMDMs)\textsuperscript{27} derived from mPER2:luc mice, finding that immune-activating treatments affected circadian period, phase, and amplitude. Curtis et al. also observed LPS-mediated effects, and assessed additional characteristics in BMDMs and peritoneal macrophages, and isolated human macrophages, at two individual time points. In this instance, \textit{Per2} and \textit{Bmal1} were evaluated: while \textit{Per2} mRNA levels and protein expression were significantly increased, those of \textit{Bmal1} decreased.\textsuperscript{28} In separate work, downregulation of \textit{Bmal1} in mouse BMDMs increased the expression of proinflammatory cytokines, further suggesting that \textit{Bmal1} may act as an anti-inflammatory molecule in macrophages.\textsuperscript{29}

Associated with \textit{Bmal1}, Curtis et al. also found that LPS significantly decreased levels of its transcriptional repressor, \textit{REV-ERBa}, but did not affect the presence of its transcriptional activator, \textit{RORa}.\textsuperscript{28} However, another study showed that stimulation with a
combination of LPS and IFN-γ, which polarizes macrophages to the pro-inflammatory M1 subtype, increased REV-ERBα mRNA and protein levels in differentiated THP-1 macrophages. Concurrently, treatment with IL-4, which polarizes macrophages to the anti-inflammatory M2 subtype, resulted in REV-ERBα reduction. These studies suggest that pro- and anti-inflammatory polarizations can differently affect circadian clock gene expression. However, the implications of macrophage stimulation on circadian oscillations of the positive and negative arms of the core clock are largely unknown and have not been assessed with significant detail. Because the circadian clock is a dynamic system, it requires continuous monitoring to obtain sufficiently high-resolution data for the accurate characterization of its patterns. For this reason, we have applied the use of luciferase reporters for real-time tracking of circadian oscillations in macrophages and thorough analyses of these data to study the relationship between macrophage activation and the biological clock.

On account of their distinct and opposing functions, and the results of prior studies, we hypothesized that polarization of macrophages to pro- and anti-inflammatory subtypes would affect their circadian rhythms differently. To this end, we generated stable reporter cell lines assessing positive (Bmal1) and negative (Per2) components of the core circadian clock, using RAW264.7 macrophages. We tracked their oscillations via real-time luminometry following polarization to M1 and M2 subtypes using standard cytokine treatments (M1 via LPS or a combination of LPS/IFN-γ, M2 via IL-4). Polarization states of macrophages were verified by evaluating levels of M1- and M2-associated markers via RT-PCR. M1 polarization resulted in decreased amplitudes and rhythmicities of both Bmal1:luc and Per2:luc reporters, but did not significantly affect periods. On the other
hand, M2 polarization resulted in increased periods, but not significant alterations to amplitudes or rhythmicity. It is also important to note that responses of Bmal1:luc and Per2:luc were not always similar. While the mechanisms for this outcome are unknown, it has been previously observed in macrophages and other cellular models.28,31–34

Because macrophages play major roles in cancers, which can also affect their phenotypes, we also assessed the effects of cancer cell-conditioned media on macrophage oscillations. Exposure to media from 4T1 cells, generally considered highly aggressive, resulted in significant loss of rhythmicity, while media from EMT6 (less aggressive) cells yielded no detectable changes. Finally, considering the impacts of macrophages on cancers, including in terms of aggression, we sought to determine whether macrophages could affect circadian rhythms of cancer cells. Following treatment with RAW264.7-conditioned media, human osteosarcoma cells (U2OS-Per2:luc) showed reduced circadian rhythmicity and period, and enhanced circadian amplitudes. Taken together, our results suggest that macrophage polarization, and therefore function, is linked to circadian oscillations and changes therein, and put forth that it is important to assess these oscillations with sufficient attention to detail to understand the changes occurring.

3.2 Materials and Methods

3.2.1 Cell Culture

RAW264.7 murine macrophage, and 4T1 and EMT6 murine mammary carcinoma cells were obtained from the ATCC; 293T human embryonic kidney cells were obtained from the Jerry Laboratory (Dept. of Veterinary and Animal Sciences, UMass Amherst); U2OS human osteosarcoma cells were obtained from the Wadsworth laboratory (Dept. of
Biology, UMass Amherst). RAW264.7, EMT6, and 4T1 cells were maintained in high glucose DMEM (Gibco), supplemented with 10% Fetal Bovine Serum (FBS; Corning), 1% penicillin-streptomycin (Gibco), and 1% L-glutamine (Gibco). 293T cells were maintained in DMEM/F12 media (Gibco), supplemented with 10% FBS, 1% penicillin-streptomycin, and 0.015 mg/mL gentamicin (Gibco). U2OS cells were maintained in DMEM, supplemented with 10% FBS, 1% penicillin-streptomycin, 1% L-Glutamine, 1% non-essential amino acids (NEAA; HyClone), and 1% sodium pyruvate (Gibco). Experiment-specific media preparations are indicated below. All cells were incubated at 37 °C under 5% CO₂ atmosphere, unless otherwise noted.

3.2.2 Lentiviral Transfections

The generation of Bmal1:luc and Per2:luc plasmids have been described previously, as has their transfection into U2OS cells. Subsequent stable transfections of these reporters into RAW264.7 cells were performed similarly to as in other studies. Briefly, 3 x 10⁶ 293T cells were seeded in 60 mm culture dishes and transiently transfected with 3 µg psPAX2 packaging plasmid, 2 µg pMD2.G envelope plasmid, and 5 µg Bmal1:luc or Per2:luc plasmid constructs using Lipofectamine3000 (Thermo Fisher Scientific). After 48 h incubation, lentiviral particles were harvested and filtered through a 0.45 µm membrane (Thermo Fisher Scientific). 9 mL lentivirus-containing supernatant was combined with 9 mL RAW264.7 growth media containing 10 µg/mL polybrene (Sigma). Cells were seeded in T25 culture flasks at 2 x 10⁵ cells/mL and incubated under standard conditions until 70-80% confluence was reached. At this stage, culture media was removed and 6 mL lentivirus-containing media was added. After 2 days of infection, media was replaced with selection media (DMEM with all growth supplements plus 4 µg/mL
puromycin (Gibco)). The selection media was changed once every 3 days for 4 weeks.

### 3.2.3 Luciferase Assay

Cells were seeded in 24-well plates with 500 µL cells at a density of 2 x 10^5 cells/mL. Once the cells reached confluence (approximately 48 h), they were lysed and assessed using the Luciferase Assay System (Promega) according to the manufacturer’s instructions. Luminescence from the RAW264.7-Bmal1:luc and -Per2:luc cells was measured via a SpectraMax M5 multi-mode microplate reader.

### 3.2.4 Cell Synchronization

For RAW264.7 cell synchronization, cells were seeded in 35 mm culture dishes at 2 x 10^5 cells/mL and incubated for approximately 24 h. Media was then aspirated, cells washed with phosphate-buffered saline (PBS; Gibco), and synchronized by subjecting to starvation conditions by adding starvation media (L-15 media (Gibco) with 1% penicillin-streptomycin and 1% L-glutamine) and incubating for 18 h. Starvation media was removed and replaced with L-15 media containing only 100 nM dexamethasone (Sigma-Aldrich) for 2 h. Both starvation and synchronization were carried out at 37 °C under 5% CO₂ atmosphere. For U2OS cell synchronization, cells were seeded in 35 mm culture dishes at 2 x 10^5 cells/mL and incubated for approximately 24 h. Media was then aspirated and replaced with U2OS culture media containing 100 nM dexamethasone, followed by incubation for 2 h at 37 °C under 5% CO₂ atmosphere.

### 3.2.5 Cell Treatments with Cytokines and Conditioned Media

Lipopolysaccharide (LPS; Sigma-Aldrich), IFN-γ (BD Biosciences), and IL-4 (BioLegend) were prepared in PBS at concentrations of 1000 µg/mL, 100 µg/mL or 200
μg/mL, and 100 μg/mL, respectively, and stored at -20 °C as single-use aliquots. For cell treatments, cytokines were serially diluted in PBS to achieve the desired concentration, maintaining a final PBS concentration of 0.2% in cultures. Cytokines in PBS were added to bioluminescence recording media (for luminometry) or the equivalent lacking luciferin (for RT-PCR assays). For LPS-only treatments, concentrations are as indicated; for LPS/IFN-γ, 5 ng/mL of LPS and 12 ng/mL of IFN-γ were used; for IL-4 50 ng/mL was used. The RAW264.7 recording media was prepared by dissolving powdered DMEM (Sigma-Aldrich) in Millipore-purified water (18.2 Ω resistance) to give a final concentration of 11.25 mg/mL, followed by sterile filtration using a 0.2 μm filter (Thermo Fisher Scientific), supplemented with 5% FBS, 1% HEPES (HyClone), 1% penicillin-streptomycin, 1% L-glutamine, 1% sodium pyruvate (Gibco) and 150 μg/mL D-luciferin (Pierce; not added for RT-PCR). The cytokine-containing solutions were mixed well and added to cells as designated following synchronization and removal of synchronization media. Cells were then incubated at 36.5 °C under ambient atmosphere for the duration of the respective experiment.

Conditioned media derived from EMT6 and 4T1 cells was generated as follows. Cells were plated and grown to confluence in T175 flasks using culture conditions described above. Then, growth media was removed, cells were washed with PBS, and media was replaced with conditioning media (powdered DMEM in Millipore-purified water (18.2 Ω resistance) to 13.5 g/mL, sterile filtered using a 0.2 μm filter, supplemented with 1% FBS, 1% HEPES, 1% penicillin-streptomycin, and 1% sodium pyruvate). Cells were incubated at 36.5 °C under ambient atmosphere for 72 h, after which media was removed, filtered via a 0.45 μm filter, and stored at -20 °C in single-use aliquots. Following
synchronization, for experiments involving treatment with 4T1 or EMT6 conditioned media (and controls), media was removed and replaced from all samples, which received 50% RAW264.7 bioluminescence recording media (with or without luciferin, depending on experiment), and 50% of one of the following: for non-treated (NT) control, conditioning media with 5% FBS; for FBS control, conditioning media; for 4T1 or EMT6, conditioned media from the designated breast cancer cell line. Independent of treatment, each sample had a final concentration of 1% L-glutamine and 150 µg/mL D-luciferin, which were supplemented as needed.

Conditioned media from RAW264.7 cells was prepared similarly to above, using RAW264.7 bioluminescence recording media (without luciferin) containing 1% FBS as conditioning media. After synchronization, for experiments involving treatment of U2OS cells with RAW264.7 conditioned media (or controls), U2OS media was removed from all samples and replaced as follows. For NT controls, samples received 100% U2OS bioluminescence recording media (described below). Other treatments used 50% U2OS bioluminescence recording media, and 50% of one of the following: for FBS control, RAW264.7 conditioning media; for conditioned samples, RAW264.7 conditioned media. U2OS bioluminescence recording media was prepared by dissolving powdered DMEM (Sigma-Aldrich) in Millipore-purified water (18.2 Ω resistance) to give a final concentration of 11.25 g/mL, followed by sterile filtration using a 0.2 µm filter, supplemented with 5% FBS, 1% HEPES (HyClone), 0.25% penicillin-streptomycin, 0.35 g/L sodium bicarbonate (Fisher Scientific) and 150 µg/mL D-luciferin. Independent of treatment, each sample had a final concentration of 0.35 g/L sodium bicarbonate and 150 µg/mL D-luciferin, which were supplemented as needed.
3.2.6 RT-PCR Experiments

Each condition used for RT-PCR experiments was performed in biological triplicates. Following treatment of cells with cytokines or conditioned media and incubation for 48 h, RNA was harvested as described previously. Briefly, RNA was harvested from cells using a PureLink RNA Mini Kit (Ambion) according to the manufacturer’s instructions. Each RNA sample was reverse transcribed to cDNA using 50 μM random hexamers (Applied Biosystems), 10 mM dNTPs (Thermo Scientific), 40 U/μL RNaseOut (Invitrogen), 200 U/μL SuperScript IV Reverse Transcriptase (Invitrogen), 100 mM DTT (Invitrogen), and 5x Super Script IV buffer (Invitrogen). RT-PCR was performed in 96-well plates using a CFX Connect Real-Time System (Bio-Rad). Each reaction consisted of 100 ng cDNA, 10 μL iTaq universal SYBR Green Supermix (Bio-Rad), 4 μM each forward and reverse primer (Integrated DNA Technologies), and RNAse-free water (Fisher) to 20 μL. The samples were centrifuged and processed using an initial denaturation at 95 °C for 3 min, followed by 40 cycles of 95 °C denaturation for 10 s, and 58 °C annealing/extension for 30 s. The primers used were β-actin forward (5’- GAT CAG CAA GCA GGA GTA CGA -3’), reverse (5’- AAA ACG CAG CGC AGT AAC AGT -3’); iNos forward (5’- GTT CTC AGC CCA ACA ATA CAA GA -3’); reverse (5’- GTG GAC GGG TCG ATG TCA C -3’); Tnf-α forward (5’- CCT GTA GCC CAC GTC GTA G -3’), reverse (5’- GGG AGT AGA CAA GGT ACA ACC C -3’); CD206 forward (5’- GGA TGT TGA TGG CTG GTA G -3’), reverse (5’- AGT AGC AGG GAT TTC GTC TG -3’). Relative Tnf-α, iNos, and CD206 expression levels were determined by comparing Ct values for these genes to β-actin (control) via $2^{\Delta\Delta Ct}$ method. Three biological replicates with three technical replicates for each were analyzed for each condition.
3.2.7 Bioluminescence Recording and Analysis of Time Series

Following synchronization and addition of cytokines or conditioned media, dishes were sealed with 40 mm sterile cover glass using silicon vacuum grease and subjected to a LumiCycle 32 System (Actimetrics) for monitoring at 36.5 °C for 5-7 days. Bioluminescence signals were measured every 10 min.

Bioluminescence recordings were pre-processed to exclude the initial 24-h transient and spikes. Recordings were considered arrhythmic outliers and excluded from further analysis if their range (maximum minus minimum over time) was less than 1/3rd that of the median range for that reporter and treatment. To assess the strength of rhythmicity for each rhythmic recording, a quadratic trend was removed, and then three measures were computed: the relative power of the band in the power spectral density corresponding to periods of 16h to 32h (RelPow), the rhythmicity index computed as the height of the third peak of the correlogram (RI), and the maximum value of the chi square periodogram (MaxQp). To assess circadian period and amplitude for each rhythmic recording the average of a 24-h moving window was removed, resulting in detrended data with 12 h of data eliminated from the beginning and end of the recordings, and then the average of a 3-h moving window was used to smooth the data. The resulting time series (t=36 h to t=132 h) were fit to a damped cosine curve for one estimate of the period and amplitude. Furthermore, phase markers (peak, trough, mean-crossings) were identified to compute additional measures: the period as estimated by the difference in marker times from cycle to cycle, and the amplitude as measured by the difference in peak and trough heights for cycle 1 or cycle 2.
3.3 Results and Discussion

3.3.1 M1 and M2 Polarization Conditions Differently Alter the Circadian Rhythms of RAW264.7 Macrophages

To evaluate how circadian rhythms change in opposing macrophage subtypes, it is critical to generate data with sufficient resolution to facilitate detailed analyses of oscillations. To track circadian rhythms in a time-resolved manner, with frequent sampling over the course of multiple circadian cycles, we opted to use luciferase reporters for promoter activity. Here, we stably transfected RAW264.7 macrophage cells with a reporter for a positive (Bmal1) and a negative (Per2) component of the core circadian clock, yielding RAW264.7-Bmal1:luc and RAW264.7-Per2:luc cells, respectively (Figure 3.1). LPS is an endotoxin found in outer membranes of gram negative bacteria, and results in M1 responses in RAW264.7 macrophages. IL-4 is secreted by T helper 2 (T\textsubscript{H}2) cells and cancers, yielding M2 responses. To investigate circadian alterations that occur in M1 and M2 subtypes, RAW264.7 reporter cells were polarized under the following conditions: for the M1 subtype, 5 ng/mL, 20 ng/mL, or 50 ng/mL of LPS, and a combination of 5 ng/mL of LPS and 12 ng/mL of IFN-γ (LPS/IFN-γ) were used; for the M2 subtype, 50 ng/mL of IL-4 was used. To confirm cell polarization following treatments, we carried out RT-PCR to assess the presence of M1 and M2 specific markers (Figure 3.2). As in other studies, polarization to the M1 subtype using the aforementioned conditions resulted in enhanced levels of the M1 markers Tnf-α (Figure 3.2A) and iNos (Figure 3.2B), which increased with LPS concentration and LPS/IFN-γ. Under these conditions the M2 specific marker Cd206 was down-regulated. In contrast, when RAW264.7 macrophages were
polarized to the M2 subtype via IL-4, the expression of M1 markers Tnf-α and iNos decreased, as expected\(^{14}\) while M2-associated Cd206 increased (Figure 3.2C).

Figure 3.1: Luciferase assay data obtained following the stable transfections of (A) Bmal1:luc and (B) Per2:luc into RAW264.7 cells. Each condition has three technical replicates (n=3). Paired student T-tests were used to calculate significance between conditions (\(*\*\*p<0.0001\)). The error bars represent standard deviation of the mean. NT = non-transfected, parental RAW264.7 cells.

Figure 3.2: Relative mRNA levels of Tnf-α, iNos, and CD206 following polarization via cytokine treatments in RAW264.7 cells. Levels were quantified using RT-PCR. As expected, LPS and LPS/IFN-γ treatments upregulated M1 markers (A) Tnf-α and (B) iNos, and downregulated the M2 marker (C) Cd206. IL-4 treatment downregulated (A) Tnf-α and (B) iNos, and upregulated (C) Cd206. Insets within B and C show data without L/I and IL4, respectively. Each treatment contained three biological replicates, with three technical replicates each. Each mean is shown as a diamond; error bars represent standard error of the mean (SEM). NT = non-treated; L5 = 5 ng/mL LPS; L50 = 50 ng/mL LPS; L/I = 5 ng/mL LPS and 12 ng/mL IFN-γ; IL4 = 50 ng/mL IL-4.

Circadian oscillations based on Bmal1:luc and Per2:luc signals were tracked via real-time luminometry. Raw and detrended circadian data of M1 polarized cells show that average bioluminescence levels of Bmal1:luc reporters are lower (raw data shown in
Figures 3.3A and 3.4A; detrended data shown in Figures 3.3C and 3.5A) while those of Per2:luc reporters vary in terms of levels (raw data shown in Figures 3.3B and 3.4B; detrended data shown in Figures 3.3D and 3.5B) compared to NT samples in the respective RAW264.7 reporter cell lines. In contrast, raw and detrended circadian data of M2 polarized cells show increased average bioluminescence of both Bmal1:luc (raw data shown in Figures 3.3A and 3.4A; detrended data shown in Figures 3.3C and 3.5A) and Per2:luc reporters (raw data shown in Figures 3.3B and 3.4B; detrended data shown in Figures 3.3D and 3.5B) compared to respective NT samples in the same RAW264.7 reporter cell lines.

Figure 3.3. Bioluminescence rhythms of Bmal1 and Per2 promoter activities following cytokine treatments in RAW 264.7-Bmal1:luc and -Per2:luc cells. Shown are raw (A, B) and de-trended, smoothed (C, D) time series averaged across replicates (N=8) for each treatment. The standard error is shown with a light envelope around the mean; in some instances, this is too small to be visualized. The raw and detrended data with individual replicates can be found in Figures 3.4 and 3.5, respectively.
Figure 3.4. Raw bioluminescence following cytokine treatments. Shown are time series for (A) Bmal1 and (B) Per2 promoter activities. Each time series is color-coded by experiment date (N=4 each). There is one outlier (gray, a Per2:luc recording from LPS 5 ng/mL treatment), that when de-trended has a range smaller than one-third of the median range for the given reporter and treatment.
Figure 3.5: Detrended bioluminescence following cytokine treatments. Shown are time series that have been de-trended by subtracting the mean of a 24-h sliding window and smoothed with the mean a 3-h sliding window for (A) *Bmal1* and (B) *Per2* promoter activities. Each time series is color-coded by experiment date (N=4 each). There is one outlier (gray, a *Per2:luc* recording from 5 ng/mL LPS treatment), that when detrended has a range smaller than one-third of the median range for the given reporter and treatment.
Circadian rhythmicity, period, and amplitude were differently altered in the M1 and M2 polarization states, with dissimilar Bmal1:luc and Per2:luc responses. The strength of rhythmicity for each time-series was assessed by three methods: the relative power in the circadian (16-32 h) band of the power spectral density (RelPow), the rhythmicity index computed as the height of the third peak of the correlogram (RI), and the maximum value of the chi-squared periodogram (MaxQp). M1 polarization with 5 and 20 ng/mL of LPS significantly decreased only Per2:luc rhythmicity, while 50 ng/mL of LPS reduced rhythmicity of both Bmal1:luc and Per2:luc reporters (Figures 3.6A top and 3.7A). Like the lower concentration LPS treatments, the combination of LPS/IFN-γ significantly decreased rhythmicity only in the Per2:luc reporter (Figures 3.6B top and 3.7B), compared to respective NT samples. In contrast, M2 polarization with IL-4 significantly increased the rhythmicity only in the Per2:luc reporter when measured using MaxQp, but not using other methods (RI, RelPow).
Figure 3.6: Circadian parameters for (A) *Bmal1* and (B) *Per2* oscillations following cytokine treatments. Shown are measures of strength of rhythmicity (top), period (middle), and amplitude (bottom). The measure of rhythmicity is the relative power in the 16- to 32-h band of the power spectral density. The period and amplitude are estimated by fitting each time series to a damped cosine curve. Data points are color-coded by dose; within each, data is separated by experiment (those to the left are from the first, and those to the right are from the second). The distribution of measures for each treatment is compared to that of the non-treated samples using a randomization test for difference in means (NS indicates “not significant,” * p<0.05, ** p<0.01, and no indicator above a treatment indicates that there were too few data points for the test to have sufficient power). Time series that did not fit well to a damped cosine (GOF<0.9) were excluded from period and amplitude evaluations. NT = non-treated; L5 = 5 ng/mL LPS; L20 = 20 ng/mL LPS; L50 = 50 ng/mL LPS; IFN = 5 ng/mL LPS and 12 ng/mL IFN-γ.
Figure 3.7: Comparison of results from application of multiple measures of rhythmicity for cytokine-treated cells. Shown are data from three analyses: Maximum Qp value from Chi-Square Periodogram (top), Rhythmicity Index from a correlogram (middle), and the relative power in the 16- to 32-h band of the power spectral density (bottom; also shown in Figure 3.6 and duplicated here for comparison) for (A) Bmal1 and (B) Per2 promoter activities. Data points are color-coded by dose; within each, data is separated by experiment (those to the left are from the first, and those to the right are from the second). The distribution of measures for each treatment is compared to that of the non-treated samples using a randomization test for difference in means (NS indicates “not significant”, * p<0.05, ** p<0.01, and no indicator above a treatment indicates that there were too few data points for the test to have sufficient power). NT= non-treated; L5 = 5 ng/mL LPS; L20 = 20 ng/mL LPS; L50 = 50 ng/mL LPS; IFN = 5 ng/mL LPS and 12 ng/mL IFN-γ.

Circadian periods were also affected by polarization. M1 polarization with LPS altered the period of RAW264.7 cells differently depending on treatment. Decreased periods were observed following LPS treatments with 5 ng/mL (Bmal1:luc, mean-crossing up), 20 ng/mL (Per2:luc, trough to trough, mean CWT period), and 50 ng/mL (Per2:luc, mean CWT period) (Figure 3.8). However, increased periods were observed following 50 ng/mL LPS (Per2:luc, mean-crossing up, peak to peak) (Figures 3.6 middle and 3.8). Furthermore, period values exhibited wider spreads with increasing LPS concentration in both reporters, which may be attributed to lower rhythmicity. In contrast, M2 polarization via IL-4 treatment increased periods in both reporters when measured by mean-crossing up
and down, and trough to trough (Figure 3.8), but this period enhancement was only found
in the Bmal1:luc reporter when determined by DC Fit, peak to peak, and mean CWT
methods (Figures 3.6A middle and 3.8A). No significant period reductions were found in
any of the methods used. For samples with reduced rhythmicity (time series that did not fit
well to a damped cosine (GOF<0.9)), no period was calculated.
Figure 3.8: Comparison of results from application of multiple types of measures for period determination for cytokine-treated cells. Shown are results from six methods for estimating period: damped cosine fit (DC Fit, top; also shown in Figure 3.6 and reproduced here for comparison), average time between mean-crossings as bioluminescence rises (Mean-Cross (Up), second row), average time between mean-crossings as bioluminescence falls (Mean-Cross (Down), third row), average time between peaks (Peak-to-Peak, fourth row), average time between troughs (Trough-to-Trough, fifth row), and the continuous wavelet-estimate averaged across time (Mean CWT Period, bottom) for (A) Bmal1 and (B) Per2 promoter activities. Data points are color-coded by dose; within each, data is separated by experiment (those to the left are from the first, and those to the right are from the second). The distribution of measures for each treatment is compared to that of the vehicle (non-treated samples) using a randomization test for difference in means (NS indicates “not significant”, * p<0.05, ** p<0.01, and no indicator above a treatment indicates that there were too few data points for the test to have sufficient power). Veh=Vehicle; L5 = 5 ng/mL LPS; L20 = 20 ng/mL LPS; L50 = 50 ng/mL LPS; IFN = 5 ng/mL LPS and 12 ng/mL IFN-γ.
Lastly, amplitudes were altered depending on polarization treatment. M1 polarization with 20 ng/mL and 50 ng/mL of LPS, and the combination of LPS/IFN-γ significantly reduced the amplitudes of Bmal1:luc reporters in RAW264.7 cells when analyzed using DC fit amplitude and peak1-trough1 (Figures 3.6A bottom and 3.9A), while the peak2-trough2 method showed significant period reductions for the 20 ng/mL and 50 ng/mL LPS treatments, but not LPS/IFN-γ (Figure 3.9A). Amplitude reductions were also observed for LPS/IFN-γ (DC fit amplitude, peak to trough) and 50 ng/mL LPS (peak2-trough2) conditions in Per2:luc reporter (Figures 3.6B bottom and 3.9B). No significant amplitude increases were observed under any M1 polarization conditions for either Bmal1 or Per2. In contrast, amplitude enhancement was observed in IL-4 treated Per2:luc samples via the DC fit method (Figures 3.6B bottom and 3.9B), and no significant amplitude reductions were observed under M2 polarization conditions. Where samples showed reduced rhythmicity (e.g., LPS-treated RAW264.7-Per2:luc), no amplitudes were calculated.
3.3.2 Exposure to Breast Cancer-Conditioned Media Alters Circadian Rhythms of RAW264.7 Cells

Previous studies have shown that macrophages play critical roles in the dissemination of cancer cells, and that cancers can polarize macrophages toward the M2 subtype. These studies that imply there is a cross-talk between macrophages and the tumor microenvironment. Hence, we hypothesized that cancer cells would also affect the circadian rhythms of macrophages. To test this, first we assessed the effects of conditioned media derived from 4T1 or EMT6 mouse breast cancer cells on M1 and M2 marker levels.
in RAW264.7 cells via RT-PCR. We observed that both 4T1- and EMT6-conditioned media increased the expression of the M1 markers Tnf-α and iNos, and decreased the expression of the M2 marker Cd206 (Figure 3.10).

Figure 3.10: Relative mRNA levels of Tnf-α, iNos, and Cd206 in RAW 264.7 cells following exposure to cancer cell-conditioned media. Levels were quantified using RT-PCR. EMT6 and 4T1 conditioned media treatments resulted in increased levels of (A) Tnf-α and (B) iNos, and decreased (C) Cd206. Each treatment contained three biological replicates, with three technical replicates each (whose means are shown as diamonds). Error bars represent standard error of the mean (SEM). Vehicle = FBS control, EMT6 = EMT6 cell-conditioned media, 4T1 = 4T1 cell-conditioned media.

Then, we tracked circadian rhythms of RAW264.7 Bmal1:luc and Per2:luc reporters exposed to these conditioned media samples. Raw and detrended circadian data of both EMT6- and 4T1-conditioned media treated samples showed that average bioluminescence of Bmal1:luc reporters were lower (raw data shown in Figures 3.11A and 3.12A; detrended data shown in Figures 3.11C and 3.13A) while Per2:luc reporters varied in their in levels (raw data shown in Figures 3.11B and 3.12B; detrended data shown in Figures 3.11C and 3.13B) compared to respective NT samples, in the same RAW264.7 reporter cell lines.
Figure 3.11: Bioluminescence rhythms of Bmal1 and Per2 promoter activities following macrophage exposure to cancer-conditioned media. Shown are raw (A,B) and detrended, smoothed (C,D) time series averaged across replicates (N=12) for each treatment. The standard error is shown with a light envelope around the mean; in some instances, this is too small to be visualized. The raw data and detrended data with individual replicates can be found in Figures 3.12 and 3.13, respectively.

Figure 3.12: Raw bioluminescence following conditioned media treatments. Shown are time series for (A) Bmal1 and (B) Per2 promoter activities. Each time series is color-coded by experiment date (N=4 per experiment). Outliers (gray) are time series that have a range smaller than 1/3rd of the median range for the given reporter and treatment (for Bmal1:luc recordings, N=1 from EMT6 cell-conditioned media; for Per2:luc recordings, N=3 from FBS control and N=2 from EMT6 cell-conditioned media).
Conditioned media derived from the more aggressive 4T1 breast cancer cell line was found to elicit greater circadian effects than that from EMT6 cells, including with regard to reductions in circadian rhythmicity and period distributions (Figures 3.14 and 3.15). Rhythmicity was determined via multiple methods, all of which, except for the rhythmicity index, showed significant decreases for both reporters in 4T1-conditioned media treated cells (Figures 3.14 and 3.15). Concurrently, treatment with EMT6 breast cancer-conditioned media resulted in no significant alterations in rhythmicity by any method used except for the rhythmicity index (Figures 3.14 and 3.15). For additional circadian evaluations (e.g., period and amplitude assessments), samples that had periods...
outside of the 16-32 h circadian period range, or had a damped cosine fit with GOF < 0.9 were considered arrhythmic and excluded.

Figure 3.14: Circadian parameters for (A) Bmal1 and (B) Per2 oscillations following exposure to cancer cell-conditioned media. Shown are measures of strength of rhythmicity (top), period (middle), and amplitude (bottom). The measure of rhythmicity is the relative power in the 16- to 32-h band of the power spectral density. The period and amplitude are estimated by fitting each time series to a damped cosine curve. Data points are color-coded by dose; within each, data is separated by experiment (those to the left are from the first, in the middle are from the second, and to the right are from the third). The distribution of measures for each treatment is compared to that of the vehicle using a randomization test for difference in means (NS indicates “not significant”, * p<0.05, ** p<0.01, and no indicator above a treatment indicates that there were too few data points for the test to have sufficient power). Time series that did not fit well to a damped cosine (GOF<0.9) were excluded from period and amplitude evaluations. (Vehicle = FBS control, EMT6 = EMT6 cell-conditioned media, 4T1 = 4T1 cell-conditioned media).
Figure 3.15: Comparison of results from application of multiple measures of rhythmicity following exposure to conditioned media. Shown are data from three analyses: Maximum Qp value from Chi-Square Periodogram (top), Rhythmicity Index from a correlogram (middle), and the relative power in the 16- to 32-h band of the power spectral density (bottom; also shown in Figure 3.14 and duplicated here for comparison) for (A) Bmal1 and (B) Per2 promoter activities. Data points are color-coded by dose; within each, data is separated by experiment (those to the left are from the first, in the middle are from the second, and to the right are from the third). The distribution of measures for each treatment is compared to that of the vehicle using a randomization test for difference in means (NS indicates “not significant”, * p<0.05, ** p<0.01, and no indicator above a treatment indicates too few data points for the test to have enough power). (Vehicle = FBS control, EMT6 = EMT6 cell-conditioned media, 4T1 = 4T1 cell-conditioned media).

Periods were also affected by conditioned media treatments with Bmal1 and Per2 being dissimilarly affected in some instances (especially in the effects of EMT6 media treatments). Overall, 4T1-conditioned media treated macrophages showed significantly enhanced periods of Bmal1::luc and Per2::luc. Bmal1::luc had longer periods as determined by the DC Fit, mean-crossing up and peak to peak methods, however, it showed a period reduction using the mean-crossing down method (Figure 3.16A). Per2::luc periods were determined to increase via mean-crossing down, peak to peak, and mean CWT period methods; no decreases were found (Figure 3.16B). EMT6-conditioned media treated
samples showed significant period enhancements in the Bmal1:luc reporter using multiple methods (DC fit, mean-crossing up, peak to peak, and trough to trough; Figures 3.14A and 3.16A), while the Per2:luc reporter showed only significant period increases using peak to peak and mean CWT period methods, which were also to a lesser degree (Figures 3.14B and 3.16B). No period reductions for either reporter were observed following EMT-conditioned media treatment, using any tests. It is also noteworthy that the overall distributions of period values for the 4T1-conditioned media treated samples were wider than those of controls and EMT6-conditioned media treated samples.
Figure 3.16: Comparison of results from application of multiple types of measures for period determination following conditioned media treatments. Shown are results from six methods for estimating period: damped cosine fit (DC Fit Mean, top; also shown in Figure 3.14), average time between mean-crossings as bioluminescence rises (Mean-Cross (Up), second row), average time between mean-crossings as bioluminescence falls (Mean-Cross (Down), third row), average time between peaks (Peak-to-Peak, fourth row), average time between troughs (Trough-to-Trough, fifth row), and the continuous wavelet-estimate averaged across time (Mean CWT Period, bottom) for (A) Bmal1 and (B) Per2 promoter activities. Data points are color-coded by dose; within each, data is separated by experiment (those to the left are from the first, in the middle are from the second, and and to the right are from the third). The distribution of measures for each treatment is compared to that of the vehicle using a randomization test for difference in means (NS indicates “not significant”, * p<0.05, ** p<0.01, and no indicator above a treatment indicates that there were too few data points for the test to have sufficient power). (Vehicle = FBS control, EMT6 = EMT6 cell-conditioned media, 4T1 = 4T1 cell-conditioned media).
4T1 cell-conditioned media treated macrophage reporter cells were also affected in terms of amplitude changes, although these were not as substantial as effects on other circadian characteristics. Both *Bmal1:*luc and *Per2:*luc oscillations showed significantly reduced amplitudes following 4T1 conditioned media treatment using the peak2-trough2 method (Figure 3.17). However, the other approaches used did not result in statistically significant changes. No amplitude evaluations showed effects on the *Per2:*luc reporter, or either *Bmal1:*luc or *Per2:*luc signals for cells treated with EMT6-conditioned media. (Figures 3.14 and 3.17).

Figure 3.17: Comparison of results from application of multiple types of measures for amplitude determination following exposure to cancer cell-conditioned media. Shown are results from three methods for estimating amplitude: damped cosine fit (top; also shown in Figure 3.14 and reproduced here for comparison), peak-to-trough amplitude of first cycle (middle), and peak-to-trough amplitude of second cycle (bottom) for (A) *Bmal1* and (B) *Per2* promoter activities. Data points are color-coded by dose; within each, data is separated by experiment (those to the left are from the first, in the middle are from the second, and to the right are from the third). The distribution of measures for each treatment is compared to that of the vehicle using a randomization test for difference in means (NS indicates “not significant”, * p<0.05, ** p<0.01, and no indicator above a treatment indicates that there were too few data points for the test to have sufficient power). (Vehicle = FBS control, EMT6 = EMT6 cell-conditioned media, 4T1 = 4T1 cell-conditioned media).
3.3.3 RAW264.7-Conditioned Media Affects the Circadian Rhythms of U2OS Cells

Macrophages have shown different responses and interactions with cancers. In some cases, macrophages facilitate the metastasis and invasion of breast cancer cells\textsuperscript{48,49} while in others, they reduce cell proliferation.\textsuperscript{50,51} Here, we investigated whether and how conditioned media derived from naïve macrophages affects osteosarcoma cells, which have diminished growth following macrophage exposure, and their circadian outputs. We used the human osteosarcoma cell line U2OS, which is a well-established model for circadian studies\textsuperscript{33,52,53} to evaluate the effects of macrophage-conditioned media on circadian oscillations of cancer cells. While the cell lines are derived from different species, interactions between the two have been previously confirmed.\textsuperscript{54} We used U2OS cells, stably transfected with Bmal1: luc and Per2: luc luciferase reporters,\textsuperscript{31} and treated them with conditioned media harvested from naïve RAW264.7 cells. The circadian oscillations of U2OS-Bmal1: luc and –Per2: luc were subsequently tracked via luminometer for 5-7 days.

Raw and detrended circadian data of RAW264.7-conditioned media treated U2OS cells showed that the average bioluminescence levels of Bmal1: luc reporters were lower (raw data shown in \textbf{Figures 3.18A} and \textbf{3.19A}; detrended data shown in \textbf{Figures 3.18C} and \textbf{3.20A}), while Per2: luc reporter bioluminescence levels were higher (raw data shown in \textbf{Figures 3.18B} and \textbf{3.19B}; detrended data shown in \textbf{Figures 3.18C} and \textbf{3.20B}), compared to respective NT samples, in the same U2OS reporter cell line.
Figure 3.18: Bioluminescence rhythms of Bmal1 and Per2 promoter activities in U2OS cells following exposure to conditioned media from macrophages. Shown are raw (A,B) and de-trended, smoothed (C,D) time series averaged across replicates (N=8) for each treatment. The standard error is shown with a light envelope around the mean; in some instances, this is too small to be visualized. The raw and detrended data with individual replicates can be found in Figures 3.19 and 3.20, respectively.

Figure 3.19: Raw bioluminescence following conditioned media treatments in U2OS cells. Shown are time series for (A) Bmal1 and (B) Per2 promoter activities. Each time series is color-coded by experiment date (N=4 each).
Figure 3.20: De-trended bioluminescence following conditioned media treatments in U2OS cells. Shown are time series that have been de-trended by subtracting the mean of a 24-h sliding window and smoothed with the mean a 3-h sliding window for (A) Bmal1 and (B) Per2 promoter activities. Each time series is color-coded by experiment date (N=4 each).

The Per2:luc reporter showed significantly decreased rhythmicity in the RelPow 16-32 h and rhythmicity index methods while the Bmal1:luc reporter did not show any significant rhythmicity alterations (Figures 3.21 and 3.22). No samples showed increased rhythmicity. Period was also altered due to the conditioned media treatment. The Per2:luc reporter showed significantly decreased periods in the DC fit, mean-crossing up, peak to peak methods while Bmal1:luc reporter did not show any significant period alterations (Figures 3.21 and 3.23). No samples showed increased period. Overall, the conditioned media treated samples showed wider distribution of period values compared to NT samples. Furthermore, the Per2:luc reporter showed significantly increased amplitude in across all methods used (DC fit mean damped amplitude, peak1- trough1, and peak2- trough2), while Bmal1:luc was not significantly affected in terms of amplitude (Figures
3.21 and 3.24). In follow-up experiments, we also evaluated U2OS cell treatments with conditioned media from M1- and M2-polarized macrophages, however there were no changes in results obtained and thus are not presented.

**Figure 3.21. Circadian parameters of (A) Bmal1 and (B) Per2 in U2OS cells following exposure to macrophage conditioned media.** Shown are measures of strength of rhythmicity (top), period (middle), and amplitude (bottom). The measure of rhythmicity is the relative power in the 16- to 32-h band of the power spectral density. The period and amplitude are estimated by fitting each time series to a damped cosine curve. Data points are color-coded by dose; within each, data is separated by experiment (those to the left are from the first, in the middle are from the second, and to the right are from the third). The distribution of measures for each treatment is compared to that of the vehicle using a randomization test for difference in means (NS indicates “not significant”, *** p<0.001, ** p<0.01, and no indicator above a treatment indicates that there were too few data points for the test to have sufficient power). Time series that did not fit well to a damped cosine (GOF<0.9) were excluded from period and amplitude evaluations. (Vehicle = FBS control, RAW264.7 = RAW 264.7 cell-conditioned media).
Figure 3.22. Comparison of results from application of multiple measures of rhythmicity following U2OS cell exposure to macrophage-conditioned media. Shown are data from three analyses: Maximum Qp value from Chi-Square Periodogram (top), Rhythmicity Index from a correlogram (middle), and the relative power in the 16- to 32-h band of the power spectral density (bottom; also shown in Fig. 3.21 and duplicated here for comparison) for (A) Bmal1 and (B) Per2 promoter activities. Data points are color-coded by dose; within each, data is separated by experiment (those to the left are from the first, and to the right are from the second. The distribution of measures for each treatment is compared to that of the vehicle using a randomization test for difference in means (NS indicates “not significant”, *** p<0.001, **p<0.01, and no indicator above a treatment indicates too few data points for the test to have enough power). (Vehicle = FBS control, RAW 264.7 = RAW 264.7 cell-conditioned media).
Figure 3.23. Comparison of results from application of multiple types of measures for period determination following U2OS cell exposure to macrophage-conditioned media. Shown are results from six methods for estimating period: damped cosine fit (DC Fit Mean, top; also shown in Fig. 3.21 and duplicated here for comparison), average time between mean-crossings as bioluminescence rises (Mean-Cross (Up), second row), average time between mean-crossings as bioluminescence falls (Mean-Cross (Down), third row), average time between peaks (Peak-to-Peak, fourth row), average time between troughs (Trough-to-Trough, fifth row), and the continuous wavelet-estimate averaged across time (Mean CWT Period, bottom) for (A) Bmal1 and (B) Per2 promoter activities. Data points are color-coded by dose; within each, data is separated by experiment (those to the left are from the first and to the right are from the second). The distribution of measures for each treatment is compared to that of the vehicle using a randomization test for difference in means (NS indicates “not significant”, *** p<0.001, ** p<0.01, and no indicator above a treatment indicates that there were too few data points for the test to have sufficient power). (Vehicle = FBS control, RAW 264.7 = RAW 264.7 cell-conditioned media).
Figure 3.24. Comparison of results from application of multiple types of measures for amplitude determination following U2OS cell exposure to macrophage-conditioned media. Shown are results from three methods for estimating amplitude: damped cosine fit (top; also shown in Fig. 3.21, and reproduced here for comparison), peak-to-trough amplitude of first cycle (middle), and peak-to-trough amplitude of second cycle (bottom) for (A) Bmal1 and (B) Per2 promoter activities. Data points are color-coded by dose; within each, data is separated by experiment (those to the left are from the first, and to the right are from the third. The distribution of measures for each treatment is compared to that of the vehicle using a randomization test for difference in means (NS indicates “not significant”, * p<0.05, ** p<0.01, and no indicator above a treatment indicates that there were too few data points for the test to have sufficient power). (Vehicle = FBS control, RAW 264.7 = RAW 264.7 cell-conditioned media).

3.4 Conclusion

Macrophages have diverse functions that range from pro-inflammatory (e.g., generation of reactive oxygen and nitrogen species) to immune-suppressing (e.g., tissue remodeling and angiogenesis) activities. To produce the appropriate responses to physiological or environmental challenges, macrophages are plastic -- they can be polarized to broadly M1 (pro-inflammatory) or M2 (anti-inflammatory) subtypes. Previous studies have shown that 8-15% of the macrophage transcriptome, including genes involved in pathogen
recognition and responses, has circadian oscillations.18,20 Multiple functions and characteristics of macrophages have also been shown to occur in a circadian manner, such as recruitment to infected tissue,8 generation of chemokines and cytokines, and phagocytosis.21,22 Based on the distinct, opposing functions of the two general macrophage subtypes, and macrophages’ inherent associations with circadian rhythms, we hypothesized that the oscillations of circadian genes are differently altered depending on stimuli.

Here, we studied the relationship between macrophage polarization and core clock oscillations by tracking the signals of Bmal1:luc and Per2:luc reporters in RAW264.7 cells exposed to various stimuli, using real-time luminometry. We performed two series of experiments in this regard: first, we used cytokines to polarize cells to standard immune-stimulating (M1) and –suppressing (M2) states; then, we exposed macrophages to conditioned media from two different murine breast cancer cell types, 4T1 (more aggressive) and EMT6 (less aggressive). We found that opposing polarizations of macrophages differentially altered their circadian rhythms, and that the circadian alterations observed following conditioned media treatments are similar to those of M1 polarized macrophages.

It has been previously shown that mRNA/protein levels of core circadian clock genes in macrophages are altered under pro-inflammatory conditions.26,28 LPS treatment significantly reduced Bmal1 mRNA levels in peritoneal macrophages,26 mouse BMDM and peritoneal macrophages, and human macrophages and peripheral blood mononuclear cells (PBMC).28 In our own study, we similarly saw that LPS-treated Bmal1:luc reporters have lower bioluminescence levels compared to NT samples in both raw (Figures 3.3A
and 3.4A) as well as detrended (Figures 3.3B and 3.5A) data, suggesting that M1 polarization with LPS weakens Bmal1 circadian rhythms. It has also been shown that treatment with a high concentration of LPS significantly increased Per2 mRNA levels in mouse BMDMs. However, a luminometry study carried out with mPer2:luc mouse-derived peritoneal macrophages showed that 5 ng/mL LPS reduced, but 20 ng/mL and 100 ng/mL LPS increased non-detrended signal intensities for a portion of the experiment (24-54 hours) compared to control, while another study showed that 50 ng/mL LPS reduced non-detrended rhythms in mouse BMDMs over four days.

Likewise, our data showed (over 24-72 hours) that 5 ng/mL reduced raw bioluminescence rhythms of Per2:luc in RAW264.7 cells, but (over 24 hours to 1 week) 20 ng/mL and (over 24-72 hours) 50 ng/mL LPS resulted in increases (Figures 3.3B and 3.4B). However, after ~3 days, we saw increased raw bioluminescence levels for 5 ng/mL LPS and reduced bioluminescence for 50 ng/mL LPS-treated samples compared to NT.

For LPS/IFN-γ-treated samples, reduced bioluminescence was observed, compared to NT, for the entire time-series (Figures 3.3B and 3.4B), as seen previously with mPer2:luc BMDM. Our data showed that circadian amplitudes were reduced when cells were exposed to LPS or LPS/IFN-γ (Figures 3.6 and 3.9), which were also similar to previous studies showing that LPS or LPS/IFN-γ treatments can reduce the amplitudes of peritoneal macrophages and BMDMs in mPer2:luc mice. While LPS or LPS/IFN-γ treatments showed no significant period effects in mPer2:luc BMDMs, and in our study (Figures 3.6 and 3.8), LPS treatment has been shown to induce a subtle lengthening of period in mPer2:luc peritoneal macrophages. We also observed that M1 polarization with 5 and 20 ng/mL of LPS, and a combination of LPS/IFN-γ significantly decreased Per2:luc
rhythmicity, while 50 ng/mL of LPS reduced rhythmicity of both Bmal1: luc and Per2: luc reporters (Figures 3.6 and 3.7). However, other studies have not assessed cellular rhythmicity.

Weakened circadian amplitudes observed in cells polarized to greater extents toward M1 states (e.g., via 50 ng/mL LPS) may be caused by the generation of reactive oxygen species (ROS) in these macrophages, which was also found previously.\textsuperscript{26} M1 polarization increases expression of ROS producing genes (e.g., Tnf-α and iNos). Amplitudes and rhythmicity in M1 cells can also be affected by increased levels of the Bmal1 repressor, REV-ERB, which is present following treatment with LPS and IFN-γ.\textsuperscript{30} Under M2 polarization (described further below), such ROS generating genes are down-regulated (Figure 3.2), and enhanced amplitudes were observed (Figures 3.3 and 3.6). However, another likely reason for decreased Bmal1 oscillations is the production of proinflammatory miRNA-155 in response to LPS treatment. Curtis et al. showed that miR-155 levels are inversely correlated with those of Bmal1, which possesses two miR-155 binding sites in its promoter, and is unaffected following LPS treatment in the presence of a miR-155 antagonir.\textsuperscript{28}

While the circadian effects of M2 polarized macrophages have not been as thoroughly studied, M2 polarization/IL-4 (20 ng/mL) treatment has been shown to decrease mRNA and protein levels of REV-ERBα (a Bmal1 transcriptional repressor) in differentiated THP-1 macrophages.\textsuperscript{30} Such reduction of REV-ERBα should increase levels of Bmal1; we also found increased levels of raw bioluminescence for the Bmal1: luc reporter (Figure 3.6A) upon M2 polarization in our study. The increased Bmal1 should also drive Per2 expression, which we also observed via amplitude enhancement in Per2: luc
(Figures 3.6B and 3.9), and was also found by Chen et al.\textsuperscript{27} However, while that study did not find any effects on period, we observed increased periods in both reporters following M2 polarization (Figures 3.6 and 3.8). Furthermore, we also found that M2 polarization significantly increased rhythmicity of the \textit{Per2:luc} reporter (Figures 3.6 and 3.7).

There is significant evidence to indicate cross-talk between macrophages and tumor microenvironments, but the contributions of macrophages also depends on cancer type.\textsuperscript{46,47,55,56} Similarly, macrophage responses to conditioned media can vary. For example, conditioned media derived from high tumor grade MDA-MB-231 cells (human breast cancer) has been shown to upregulate both M1 and M2 markers of human CD14\textsuperscript{+} macrophages,\textsuperscript{47} while murine BMDMs exposed to 4T1 breast cancer conditioned media, and splenocytes cocultured with 4T1 cells expressed higher levels of M1 marker genes including \textit{Tnf-\alpha}.\textsuperscript{55,56} 4T1 cell-conditioned media has been shown to contain significant amounts of IFN-\gamma,\textsuperscript{56} which can itself polarize macrophages to the M1 subtype.\textsuperscript{57,58} However, the effects of tumor microenvironments on macrophage circadian rhythms are unknown. To evaluate the interactions between the two, we treated RAW264.7-\textit{Bmal1:luc} and -\textit{Per2:luc} macrophages with EMT6 (less aggressive) and 4T1 (highly aggressive) murine mammary carcinoma\textsuperscript{59} derived-conditioned media and analyzed their circadian effects. Our data showed that the more aggressive 4T1 breast cancer-conditioned media reduced macrophage rhythmicity akin to M1 polarization conditions, which was not surprising given the similar marker profile as determined by RT-PCR (Figure 3.10). On the other hand, the less aggressive EMT6-derived conditioned media did not affect rhythmicity, but instead yielded significantly altered periods. Altogether our data suggests
that mouse breast cancer conditioned media polarizes macrophages to an M1 subtype as a defense mechanism to protect the host against the cancer.

As mentioned above, macrophages can result in positive or negative effects following interaction with cancers. Previous studies show that macrophages promote metastasis and invasion of breast cancer cells,\textsuperscript{48,49} while others show that macrophages inhibit osteosarcoma cell growth via inhibition of cell proliferation and increased phagocytosis.\textsuperscript{50,51,54} Considering the previously investigated effects of macrophage-conditioned media on cancer cells, and the interactions between macrophages and osteosarcomas, we wished to evaluate whether the former might also influence circadian rhythms of the latter. We treated human osteosarcoma reporter cell lines, U2OS-\textit{Bmal1}:\textit{luc} and -\textit{Per2}:\textit{luc} with conditioned media harvested from naive RAW264.7 cells. We observed reduction in rhythmicity and amplitude enhancement of the U2OS oscillations. As circadian rhythms are found to be increasingly disrupted with disease severity and oncogenic characteristics\textsuperscript{36} and even subtle renormalization of disrupted rhythms can reduce oncogenic features,\textsuperscript{33} our data highlights another connection between circadian oscillations and cancer, which should be studied further.

Taken together, we show that the circadian rhythms of macrophages are influenced by their polarization states. While M1 polarization was associated with significant loss of rhythmicity, M2 polarization resulted in amplitude and period enhancements. We also found that macrophage circadian effects translate to cultures with cancer cell-conditioned media, where M1-like marker characteristics were accompanied by M1-like circadian oscillations. Finally, we show that conditioned media of macrophages enhances the circadian rhythms of another cancer cell type (U2OS), which macrophages act against in
oncogenic environments. This raises a hypothesis that macrophages can alter the circadian rhythms of tumors and cancer cell types to affect their oncogenic features. In the future, this relationship should be examined with other cancer models, including to determine whether macrophages negatively affect the circadian oscillations of cancer cells/types with which they interact to facilitate disease. In terms of macrophages themselves, it is of interest to study whether the lack of rhythmicity in inflammatory macrophages is due to loss of synchrony by performing single-cell experiments, and to assess whether this circadian divergence confers benefits in terms of immune response and host defense.

3.5 References


CHAPTER 4

DIARYLIDENE-N-METHYL-4-PIPERIDONE AND SPIROBIBENZOPYRAN CURCUMIN DERIVATIVES AS ANTIOXIDANT AND ANTI-INFLAMMATORY PHARMACOPHORES

4.1 Introduction

Inflammation is the first line of defense in the body. It is the immune system's response to harmful stimuli, such as pathogens, damaged cells, or toxic compounds, and is responsible for mitigating threats and initiating the healing process.\textsuperscript{1,2} When immune cells sense damaged tissues or pathogens, they signal and recruit more immune cells, such as macrophages and T-cells, by releasing various cytokines and chemokines.\textsuperscript{3,4} Macrophages are typically activated to a pro-inflammatory (M1) sub-type, which can be replicated \textit{in vitro} by treatment with lipopolysaccharide (LPS) and IFN\textgreek{g}. On the other hand, anti-inflammatory (M2) macrophages aid in the resolution of inflammation, generated \textit{in vitro} by treatment with interleukin 4 (IL-4).\textsuperscript{5,6} Unfortunately, there are congenital conditions and diseases that force these immune cells to generate too much inflammation (auto-immune diseases) or a lack thereof (immuno-compromised conditions).\textsuperscript{7-10}

Prolonged inflammation is a hallmark of chronic diseases including rheumatoid arthritis, atherosclerosis, asthma, cystic fibrosis, and cancer.\textsuperscript{1,11} Pro-inflammatory stimuli (inflammation-causing stimuli) can be generated externally, and include pathogens, allergens, or stress, or internally, such as free radical species, interleukin 1\textbeta (IL-1\textbeta), tumor necrosis factor-alpha (TNF-\textalpha), or arachidonic acid (AA).\textsuperscript{4,7,12,13} Of particular interest, AA is a polyunsaturated fatty acid covalently bound in esterified form to the cell membranes of most cells in the body. During inflammation, AA is released and oxygenated by
enzymes, leading to the formation of an important group of inflammatory mediators, the eicosanoids, which include prostaglandins and leukotrienes. Prostaglandins and other prostanoids are generated from AA via the cyclooxygenase (COX) enzyme, and have potent inflammatory properties. On the other hand, lipoxygenase (LOX) metabolizes arachidonic acid to a group of non-cyclized eicosanoids, the leukotrienes, some of which are also important inflammatory mediators.

When auto-immune diseases like asthma and inflammatory bowel disease occur (leading to the release of AA, COX, LOX, prostaglandins, and leukotrienes), resolution of inflammation, represented by downregulation of TNF-α, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and 5-lipoxygenase (5-LOX), is desirable. Toward this goal, nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly used, with more than 30 million daily consumers. Based on data collected from patients in the Arthritis, Rheumatism, and Aging Medical Information System (ARAMIS), concerns associated with NSAIDs include that they are palliative, and can result in serious side-effects including gastrointestinal and renal complications. Thus, there remains a need for efficacious anti-inflammatory lead compounds with fewer side effects. Among natural products having antioxidant and anti-inflammatory activities, phenolics appear to be promising.

Well-known targets of polyphenols and the sources of their anti-inflammatory activities involve arachidonic acid-dependent pathways, including the inhibition of COX, LOX, and phospholipase A2 (PLA2). Curcuminoids and their structurally-related metabolic degradation products, like ferulic acid, share a basic pharmacophore with phenolic acids by possessing a base phenylpropanoid (C6-C3) structure.
These metabolites act on the metabolism of arachidonic acid by inhibiting LOX and COX enzymes.\textsuperscript{22,25,27,32} It has also been shown that curcumin has the ability to prevent macrophage polarization towards a pro-inflammatory phenotype through toll-like receptor 4 (TLR4)-mediated signaling pathway inhibition.\textsuperscript{15} More specifically, curcumin inhibits nuclear factor-κB (NF-κB), 5-LOX, and COX-2,\textsuperscript{35,36} which results in decreased expression of the inflammation marker iNOS and decreased production of pro-inflammatory cytokines such as nitric oxide (NO), TNF-α, IL-1β, prostaglandin E2 (PGE2), and interleukin 6 (IL-6) in macrophages stimulated with LPS, a known TLR-4 agonist (Figure 4.2).\textsuperscript{37} Curcumin’s antioxidant capabilities and its ability to inhibit LOX and COX, and affect pro-inflammatory phenotypes in macrophages, make it an ideal candidate to treat chronic inflammation and auto-immune diseases.

\textbf{Figure 4.1:} Approach for generation of curcumin-related molecules based on original structure and active species.
Unfortunately, Curcumin’s poor \textit{in vivo} bioavailability and biodistribution in phase I clinical trials has prevented its development as an effective therapeutic.\textsuperscript{38,39} Curcumin’s hydrophobic nature leads to poor absorption in oral administration, and its instability and rapid metabolism result in low serum bio-availability and poor tissue distribution when administered systemically.\textsuperscript{40,41} Attempts to deliver curcumin using drug delivery systems, such as liposomes and nanoparticles, have not been very promising due to issues associated with solubility, bioavailability, and bio-degradability.\textsuperscript{42,43}

Curcuminoid metabolites possess a diarylheptanoid base structure consisting of symmetric or asymmetric combination of C\textsubscript{6}-C\textsubscript{3} phenolic moieties (e.g., ferulic acid and vanillin), which are responsible for free radical scavenging, anti-oxidant, and anti-inflammatory properties.\textsuperscript{29,44} Free radical scavengers, such as antioxidants, protect cells from injury caused by free radicals, which are unstable, reactive entities that can damage biological molecules. Their presence can increase the risk of cancer and other diseases.\textsuperscript{45,46}
The biological activities of curcumin and related structures are associated with the presence of unsaturated α,β-ketones. However, the di-carbonyl moiety is conformationally unstable due to keto-enol tautomerism mediated by pH, heat, and light. To address issues of stability and bio-availability, based on our earlier synthetic work on diarylidene-cyclohexanone derivatives as new antiplasmodial pharmacophores, we now report the synthesis of mono-carbonyl symmetric diarylidene- and dihetarylidene-N-methyl-4-piperidone derivatives (Figures 4.1 and 4.3-4.5).

**Figure 4.3:** The base-catalyzed synthesis scheme for Diarylidene-N-methyl-4-piperidones (DANMPs) (1-16)

**Figure 4.4:** The acid-catalyzed synthesis scheme for Diarylidene-N-methyl-4-piperidones (DANMPs) (17-21).
Figure 4.5: The base-catalyzed synthesis scheme for Diheteroarylidene-N-methyl-4-piperidones (DHANMPs) (22-25).

These symmetric and robust structures facilitate the SAR studies presented here. We have generated molecules bearing electron-donating or withdrawing substituents to assess antioxidant and anti-inflammatory activities. We hypothesize that by having suitable substituents on the two aryl rings, free radical scavenging will be improved, thereby achieving potent anti-inflammatory activity. In addition, we synthesized and evaluated spirobibenzopyrans derived from mono carbonyl analogues of curcumin (i.e., diarylidenealkanones; (Figures 4.1 and 4.6)). These molecules offer structural novelty; to date, there have been no reports investigating anti-inflammatory properties of these compounds, although our group has previously reported spirobibenzopyran molecules with potent anti-cancer properties.\(^{50}\)

Figure 4.6: The general synthesis scheme for Spirodibenzopyrans (SBPs) (26-30).
In this study, thirty curcumin derivatives were synthesized of which five were selected for further assessments based on \textit{in silico} evaluations and DPPH assays. The macrophage model cell line RAW264.7 was chosen to test the anti-inflammatory properties of these compounds (Compounds 1, 3, 13, 26, and 27). Results from RT-PCR and Griess assay studies indicate that the selected curcumin-derived small molecules were able to decrease the levels of inflammatory responses in activated macrophages stimulated with LPS and IFN\textgreek{y}. Based on our findings, further studies of these molecules should be undertaken, including in models of disease.

\section{4.2 Materials and Methods}

\subsection{4.2.1 Chemical Synthesis and Characterization}

\textbf{Materials and Instrumentation}

All chemicals used in syntheses, including aldehydes, N-methyl piperidone, and 3-pentanone were obtained from Merck chemicals, HiMedia, or Sigma-Aldrich. Curcumin was obtained from Sigma-Aldrich. All solvents (ethanol, methanol, chloroform and DMSO) used were spectral grade or distilled before use.

\textsuperscript{1}H-NMR and \textsuperscript{13}C-NMR spectra were recorded with Bruker Ascend 400 MHz and Bruker Ascend 100 MHz instruments, respectively, using Tetramethyl silane as an internal standard. For compound 26, \textsuperscript{1}H-NMR and \textsuperscript{13}C-NMR spectra were recorded with Varian 200 MHz and Varian 50 MHz. Mass spectra were obtained via Agilent 6490 Triple Quad LC/MS. An Agilent Cary 630 spectrophotometer was employed to record FT-IR spectra between the range 400 and 4000cm\textsuperscript{-1} using KBr pellets. UV-Vis spectra were obtained on
a Shimadzu 2450 spectrophotometer between the wavelength range 200-600 nm. Melting points for compounds were recorded in open capillary tubes using SMP30 (120-230 volts). An Agilent 1260 Infinity high performance liquid chromatography system equipped with a quaternary solvent delivery system, inline degasser, autosampler, and photo diode array detector was used for HPLC-based evaluation of purity. For all analyses, a Zorbax Extend-C18 (4.6 mm × 250 mm, 5 µm) column was used. Wavelengths for each compound were set at the respective \( \lambda_{\text{max}} \) (characterization data) for detection. The mobile phases were A: water and B: acetonitrile. The flow rate was maintained at 1 ml/min as linear gradient elution with total elution time of 21 minutes. The temperature of the column was maintained at 25 °C. For each sample, an injection volume of 15-30 µL was used.

**General method for the alkali-catalyzed preparation of DANMPs (1-16) and DHANMPs (22-25).** To a 1:1 mixture of ethanol-water solution placed in an ice bath, NaOH (0.05 mol) was added and the solution was stirred for 10 minutes. To this, half of the already prepared ethanolic mixture of N-methyl piperidone (0.01 mol) and aldehyde (0.02 mol) was added at room temperature and stirred for another 30 minutes. Subsequently the other half of the mixture was added and stirred. The entire process was completed under nitrogen atmosphere. Reaction progress was monitored by TLC and after reaction completion, ice-cold water was added. The product precipitated and the resulting solid was filtered, washed thoroughly with ice-cold water and for removal of alkali. The crude solid was then dried and recrystallized.

**General method for the acid-catalyzed preparation of DANMPs (17-21).** For the generation of compounds 17-21, 10-40 mL acetic acid (CH₃COOH) was saturated with
HCl gas by using Kipp’s apparatus. This acetic acid-hydrochloric acid mixture was cooled to 15 °C and the corresponding aldehyde (0.02 mol) was dissolved in it. To the resulting solution, N-methyl-piperidone (0.01 mol) was added and stirred for 10 minutes, following which the reaction was allowed to warm to room temperature. Nitrogen atmosphere was maintained for the reactions, especially in the cases of nitro and hydroxyl derivatives. Reaction progress was monitored by TLC. After completion of the reaction, 10% aqueous NaOH was added to the reaction mixture to make the pH neutral. The product precipitated and the resulting solid was filtered and washed with ice cold water. The crude solid was purified by recrystallization.

**General procedure for the preparation of Spirodibenzopyrans (SBPs) (26-30).** 3-Pentanone (0.01 mol), substituted salicylaldehyde (indicated below; 0.02 mol), and SiCl$_4$ (silicon tetrachloride, 0.03 mol) were added to absolute ethanol (20 ml) at room temperature and the mixture was stirred under nitrogen atmosphere. Formation of solid in the reaction vessel indicated completion of the reaction. The contents were poured onto ice and stirred for approximately 15 minutes to quench unreacted SiCl$_4$. The solid obtained was filtered and washed with water, then dried and recrystallized from distilled chloroform.

**3,5-Bis((E)dibenzylidene) -N-methyl-4-piperidone [1].** Generated using benzaldehyde (2.12 g, 20 mmol) and N-methyl-4-piperidone (1.162 mL, 10 mmol) in 40 ml of ethanol-water 1:1 mixture. To this, NaOH (2 g, 50 mmol) was added and the mixture was stirred for 24 h at room temperature. Recrystallized from pet ether and chloroform to give lightly yellow colored crystals; yield: 88.9 %. mp: 111.5-113 °C. HPLC purity and retention time: 98.2%, 15.72 min. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.82 (H-7, 2H, s), $\delta$ 7.45-7.37 (Ar-H, m), $\delta$ 3.76 (H-2, H-6, 4H, s), $\delta$ 2.45, (N-CH$_3$, 3H, s). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 186.96
(C-4), δ 136.46 (C-5), δ 135.28 (C-7), δ 133.03 (C-1’), δ 130.43 (C-4’), δ 129.03 (C-2’ and 6’), δ 128.58 (C-3’ and C-5’), δ 57.1 (C-6) and δ 45.89 (N-C); IR (KBr, cm\(^{-1}\)): 3014 (sp\(^2\) C-H stretch), 2880 (sp\(^3\) C-H stretch), 1670 (C=O stretch), 1618 (C=C stretch), 1588 and 1572 (Ar skeletal bands), 764 and 693 (mono-substituted aromatic ring); Mass: (m/z): [M+H]\(^+\) calcd for C\(_{20}\)H\(_{19}\)NO, 289.1467; found 290.2000; UV (MeOH) λ\(_{\text{max}}\) (nm): 328 and 231.

3,5-Bis ((E)-2-Chlorobenzylidene)-N-methyl-4-piperidone [2]. Generated using 2-chlorobenzaldehyde (2.81 g, 20 mmol) and N-methyl-4-piperidone (1.162 mL, 10 mmol) in 40 ml of ethanol-water 1:1 mixture. To this, NaOH (2 g, 50 mmol) was added and the mixture was stirred for 12 h at room temperature. Recrystallized from chloroform-methanol to give lightly yellow colored crystals; yield: 81.4%. mp: 145-147.8 °C. HPLC purity and retention time: 99.1%, 18.01 min. \(^1\)H NMR (400 MHz, CDCl\(_3\)): δ 8.00 (H-7, 2H, s), δ 7.45 (H-2’, 2H, overlapping signals), δ 7.31 (H-6’, 2H, overlapping signals), δ 7.29 (H-5’, 2H, overlapping signals), δ 7.24 (H-4’, 2H, overlapping signals), δ 3.61 (H-2, H-6, 4H, s), δ 2.37, (N-CH\(_3\), 3H, s). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): δ 186.15 (C-4), δ 135.16 (C-2’), δ 134.2 (C-5), δ 134.02 (C-7), δ 133.6 (C-1’), δ 130.33 (C-6’), δ 130.01 (C-4’), δ 129.94 (C-3’), δ 126.41 (C-5’), δ 56.67 (C-6) and δ 45.55 (N-C); IR (KBr disc) (cm\(^{-1}\)): 3067 (sp\(^2\) C-H), 2977 (sp\(^3\) C-H), 1675 (C=O), 1623 (C=C), 1588 and 1572 (aromatic skeletal bands), 1053 (C-Cl); Mass: (m/z): [M+H]\(^+\) calcd for C\(_{20}\)H\(_{19}\)NO, 357.0687; found 358.1000; UV (MeOH) λ\(_{\text{max}}\) (nm): 235 and 314 nm.

3,5-Bis ((E)-3-Chlorobenzylidene)-N-methyl-4-piperidone [3]. Generated using 3-chlorobenzaldehyde (2.81 g, 20 mmol) and N-methyl-4-piperidone (1.162 mL, 10 mmol) in 40 mL of ethanol-water 1:1 mixture. To this, NaOH (2 g, 50 mmol) was added and the
mixture was stirred for 23 h at room temperature. Recrystallized from chloroform-methanol to give yellow colored crystals; yield: 78.6%. mp: 172.2-173.6 °C; HPLC purity and retention time: 99.5%, 18.83 min. \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta 7.72\) (H-7, 2H, s), \(\delta 7.38\) (H-2’, H-4’, 4H, overlapping signals), \(\delta 7.35\) (H-5’, 2H, overlapping signals), \(\delta 7.27\) (H-6’, 2H, overlapping signals), \(\delta 3.73\) (H-2, H-6, 4H, s), \(\delta 2.47\), (N-CH\(_3\), 3H, s). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \(\delta 186.41\) (C-4), \(\delta 136.86\) (C-3’), \(\delta 134.99\) (C-5), \(\delta 134.51\) (C-7), \(\delta 133.99\) (C-1’), \(\delta 129.93\) (C-5’), \(\delta 129.83\) (C-2’), \(\delta 129.04\) (C-4’), \(\delta 128.43\) (C-6’) \(\delta 56.81\) (C-6) and \(\delta 45.85\) (N-C); IR (KBr disc) (cm\(^{-1}\))): 3011 (sp\(^2\) C-H), 2930 (sp\(^3\) C-H), 1669 (C=O), 1613 (C=C), 1588 and 1562 (aromatic skeletal stretch), 1096 (C-Cl), 782 (meta-substitution); Mass: (m/z): [M+H]\(^+\) calcd for C\(_{20}\)H\(_{19}\)NO, 357.0687; found 358.1000; UV (MeOH) \(\lambda_{\text{max}}\) (nm): 233 and 322 nm.

3,5-Bis ((E)-4-Chlorobenzylidene)-N-methyl-4-piperidone [4]. Generated using 4-chlorobenzaldehyde (2.81g, 20 mmol) and N-methyl-4-piperidone (1.162 mL, 10 mmol) in 64 mL of ethanol-water 1:1 mixture. To this, NaOH (2 g, 50 mmol) was added and the mixture was stirred for 45 h at room temperature. First recrystallized using chloroform-methanol, after which a second recrystallization using ethyl acetate was performed to give lightly yellow colored crystals; yield: 83.9%. mp: 112.8-114 °C; HPLC purity and retention time: 97.2%,18.77 min. \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta 7.74\) (H-7, 2H, s), \(\delta 7.39\) (H-2’, 6’, 4H, d, J=7.80 Hz), \(\delta 7.31\) (H-3’, H-5’), \(\delta 3.71\) (H-2, H-6, 4H, s), \(\delta 2.46\), (N-CH\(_3\), 3H, s). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \(\delta 186.51\) (C-4), \(\delta 135.20\) (C-4’), \(\delta 135.1\) (C-5), \(\delta 133.5\) (C-7), \(\delta 133.40\) (C-1’), \(\delta 131.61\) (C-2’ and 6’), \(\delta 128.89\) (C-3’ and C-5’), \(\delta 56.98\) (C-6) and \(\delta 45.91\) (N-C); IR (KBr disc) (cm\(^{-1}\))): 3006 (sp\(^2\) C-H), 2937 (sp\(^3\) C-H), 1673 (C=O), 1615 (C=C), 1587 and 1546 (aromatic skeletal bands), 1097 (C-Cl); Mass: (m/z):
[M+H]$^+$ calcd for C$_{20}$H$_{19}$NO, 357.0687; found 358.1000; UV (MeOH) $\lambda_{\text{max}}$ (nm): 235 and 332 nm.

3,5-Bis ((E)-3-bromobenzylidene)-N-methyl-4-piperidone [5]. Generated using 3-bromobenzaldehyde (3.7 g, 20 mmol) and N-methyl-4-piperidone (1.162 mL, 10 mmol) in 50 mL of ethanol-water 1:1 mixture. To this, NaOH (2 g, 50 mmol) was added and the mixture was stirred for 48 h at room temperature. First recrystallized using chloroform-methanol, after which a second recrystallization using pet ether-acetone was performed to give yellow colored powder; yield: 84.3%. mp: 123-125 °C; HPLC purity and retention time: 99.8%, 19.43 min. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.71 (H-7, 2H, s), $\delta$ 7.51-7.49 (H-2', H-4', 4H, overlapping signals), $\delta$ 7.31-7.29 (H-5', H-6', 4H, overlapping signals), $\delta$ 3.72 (H-2, H-6, 4H, s), $\delta$ 2.47, (N-CH$_3$, 3H, s). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 186.39 (C-4), $\delta$ 137.18 (C-3'), $\delta$ 134.04 (C-2'), $\delta$ 132.89 (C-5), $\delta$ 131.97 (C-7), $\delta$ 130.11 (C-1'), $\delta$ 128.84 (C-4'), $\delta$ 128.83 (C-6'), $\delta$ 122.69 (C-3'), $\delta$ 56.78 (C-6) and $\delta$ 45.83 (N-C); IR (KBr disc) (cm$^{-1}$): 3070 (sp$^2$ C-H), 2945 (sp$^3$ C-H), 1670 (C=O), 1610 (C=C), 1595 and 1480 (aromatic skeletal stretch), 995 and 910 (C-H alkene out of plane bend), 845 and 870 (aromatic C-H out of plane bend); Mass: (m/z):[M+H]$^+$calcd for C$_{20}$H$_{17}$Br$_2$NO, 447.1700; found 448.0000; UV (MeOH) $\lambda_{\text{max}}$ (nm): 322 nm.

3,5-Bis ((E)-4-bromobenzylidene)-N-methyl-4-piperidone [6]. Generated using 3-bromobenzaldehyde (3.7 g, 20 mmol) and N-methyl-4-piperidone (1.162 mL, 10 mmol) in 122 mL of ethanol-water 1:1 mixture. To this, NaOH (2 g, 50 mmol) was added and the mixture was stirred for 46 h at room temperature. Recrystallized from chloroform-ethyl acetate to give yellow colored powder; yield: 98%. mp: 183.7-186.3 °C; HPLC purity and
retention time: 97.0%, 19.51 min. $^1$H NMR (400 MHz, CDCl$_3$): δ 7.72 (H-7, 2H, s), δ 7.56 (H-3’, H-5’, 4H, d, J=8.04 Hz), δ 7.24 (H-2’, H-6’, 4H, d, J=8.04 Hz), δ 3.70 (H-2, H-6, 4H, s), δ 2.46, (N-CH$_3$, 3H, s). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 186.5 (C-4), δ 135.29 (C-5), δ 134.01 (C-7), δ 133.49 (C-1’), δ 131.86 (C-3’ and C-5’), δ 131.81 (C-2’ and C-6’), δ 123.51 (C-4’), δ 56.97 (C-6) and δ 45.9 (N-C); IR (KBr disc) (cm$^{-1}$): 3080 (sp$^2$ C-H), 2950 (sp$^3$ C-H), 1670 (C=O), 1612 (C=C), 1595 and 1500 (aromatic skeletal stretch), 1000 and 915 (C-H alkene out of plane bend), 820 and 780 (aromatic C-H out of plane bend); Mass: (m/z):[M+H]$^+$ calcd for C$_{20}$H$_{17}$Br$_2$NO, 447.1700; found 448.0000; UV (MeOH) $\lambda_{\text{max}}$ (nm): 334 and 236 nm.

3,5-Bis ((E)-4-methoxybenzylidene)-N-methyl-4-piperidone [7]. Generated using 4-methoxybenzaldehyde (2.72 g, 20 mmol) and N-methyl-4-piperidone (1.162 mL, 10 mmol) in 60 mL of ethanol-water 1:1 mixture. To this, NaOH (2 g, 50 mmol) was added, and the mixture was stirred for 48 h at room temperature. Recrystallized from chloroform-methanol to give yellow colored crystals; yield: 77.8%. mp: 201-203.2 °C; HPLC purity and retention time: 96.0%, 13.95 min.$^1$H NMR (400 MHz, CDCl$_3$): δ 7.77 (H-7, 2H, s), δ 7.38 (H-3’, H-5’, 4H, d, J=7.84 Hz), δ 6.94 (H-2’, H-6’, 4H, d, J=7.84 Hz), δ 3.84 (O-CH, 3H, s), δ 3.76 (H-2, H-6, 4H, s), δ 2.48, (N-CH$_3$, 3H, s). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 186.83 (C-4), δ 160.2 (C-4’), δ 135.95 (C-5), δ 132.31 (C-2’), δ 131.30 (C-1’), δ 128.0 (C-6’), δ 114.06 (C-3’), δ 57.20 (O-CH$_3$), δ 55.33 (C-6) and δ 45.94 (N-C); IR (KBr disc) (cm$^{-1}$): 3011 (sp$^2$ C-H), 2929 (sp$^3$ C-H), 1670 (C=O), 1609 (C=C), 1163(C-O), 1153(C-O), 1580 and 1510 (aromatic skeletal bands) 830 (para substitution); Mass: (m/z):[M+H]$^+$ calcd for C$_{22}$H$_{23}$NO$_3$, 349.4300; found 350.2000; UV (MeOH) $\lambda_{\text{max}}$ (nm): 242 and 364 nm.
3,5-Bis ((E)-4-methylbenzylidene)-N-methyl-4-piperidone [8]. Generated using 4-methylbenzaldehyde (2.37 g, 20 mmol) and N-methyl-4-piperidone (1.162 mL, 10 mmol) in 60 mL of ethanol-water 1:1 mixture. To this, NaOH (2g, 50 mmol) was added and the mixture was stirred for 24 h at room temperature. Recrystallized from chloroform-methanol to give yellow colored crystals; yield: 82%. mp: 200-202 °C. HPLC purity and retention time: 98.3%, 17.55 min. $^1$H NMR (400 MHz, CDCl$_3$): δ 7.79 (H-7, 2H, s), δ 7.29 (H-2’, H-6’, 4H, d, J=7.52 Hz), δ 7.23 (H-3’, H-5’, 4H, d, J=7.52 Hz), δ 3.76 (H-2, H-6, 4H, s), δ 2.46 (N-CH$_3$, 3H, s), δ 2.38 (CH$_3$, 3H, s). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 186.96 (C-4), δ 139.30 (C-5), δ 132.49 (C-1’), δ 132.42 (C-4’), δ 129.30 (C-3’ and C-5’), δ 120.50 (C-2’ and C-6’), δ 57.17 (C-6) and δ 45.88 (N-C); IR (KBr disc) (cm$^{-1}$): 3040 (sp$^2$ C-H), 2972 (sp$^3$ C-H), 1670 (C=O), 1603 (C=C), 1580 and 1510 (aromatic skeletal bands), 815 (para substitution); Mass: (m/z):[M+H]$^+$ calcd for C$_{22}$H$_{23}$NO = 317.432; found 317.9000; UV (MeOH) $\lambda_{max}$ (nm): 341 and 336 nm.

3,5-Bis ((E)-4-isopropylbenzylidene)-N-methyl-4-piperidone [9]. Generated using 4-isopropylbenzaldehyde (2.96 g, 20 mmol) and N-methyl-4-piperidone (1.162 mL, 10 mmol) in 60 mL of ethanol-water 1:1 mixture. To this, NaOH (2g, 50 mmol) was added and the mixture was stirred for 24 h at room temperature. First recrystallized using chloroform-methanol, after which second and third recrystallizations using pet ether-chloroform and methanol, respectively, were performed to give yellow colored powder; yield: 74.5%. mp: 105.5-108.5 °C; HPLC purity and retention time: 95.4%, 18.60 min. $^1$H NMR (400 MHz, CDCl$_3$): δ 7.80 (H-7, 2H, s), δ 7.34 (H-2’, H-6’, 4H, d, J=7.52 Hz), δ 7.28 (H-3’, H-5’, 4H, d, J=7.52 Hz), δ 3.78 (H-2, H-6, 4H, s), δ 2.95 (H-7’, 1H, sep, J=13.44 Hz), δ 2.46, (N-CH$_3$, 3H, s), δ 1.28 (H-8’, 3H, d, J=6.56 Hz). $^{13}$C NMR (100 MHz, CDCl$_3$):
δ 186.99 (C-4), δ 150.18 (C-4’), δ 136.4 (C-5), δ 132.89 (C-7), δ 132.4 (C-1’), δ 126.72 (C-3’), δ 57.16 (C-6) and δ 45.80 (N-C), δ 34.05 (C-7’); IR (KBr disc) (cm⁻¹): 3050 (sp² C-H), 2970 (sp³ C-H), 1670 (C=O), 1610 (C=C), 1515 and 1475 (aromatic skeletal stretch), 990 and 920 (C-H alkene out of plane bend), 825 (aromatic C-H out of plane bend); Mass: (m/z):[M+H]⁺ calcd for C₂₆H₃₁NO, 373.5400; found 374.3000; UV (MeOH) λmax (nm): 342 and 235 nm.

3,5-Bis ((E)-3-trifluoromethylbenzylidene)-N-methyl-4-piperidone [10]. Generated using 3-trifluoromethylbenzaldehyde (2.08 g, 5 mmol) and N-methyl-4-piperidone (0.697 mL, 5 mmol) in 26 mL of ethanol-water 1:1 mixture. To this, NaOH (1.28 g, 30 mmol) was added and the mixture was stirred for 24 h at room temperature. Recrystallized from pet ether-chloroform to give yellow colored crystals; yield: 51.6%. mp: 143-145.2 °C. HPLC purity and retention time: 99.0%, 15.56 min. ¹H NMR (400 MHz, CDCl₃): δ 7.81 (H-7, 2H, s), δ 7.64-7.62 (H-4’, H-6’, 4H, two overlapping doublets), δ 7.57 (H-5’, 2H, t), δ 7.56 (H-2’, 2H, s) δ 3.75 (H-2, H-6, 4H, s), δ 2.47, (NCH₃, 3H, s). ¹³C NMR (100 MHz, CDCl₃): δ 186.31 (C-4), δ 135.82 (C-5), δ 134.92 (C-7), δ 134.3 (C-1’), δ 133.22 (C-6’), δ 131.61 (C-3’), δ 129.2 (C-5’), δ 126.76 (C-2’), δ 125.6 (C-4’), δ 123.82 (C-F), δ 56.73 (C-6) and δ 45.7 (N-C); IR (KBr, cm⁻¹): 3050 (sp² C-H stretch), 2950 (sp³ C-H stretch), 1680 (C=O stretch), 1575 and 1495 (Ar skeletal bands), 1280 and 1155 (C-O stretch), 980 (C-H alkene out of plane bend), 830 and 800 (mono substituted aromatic ring); Mass: (m/z):[M+H]⁺ calcd for C₂₂H₁₇F₆NO, 425.3744; found 426.1000; UV (MeOH) λmax (nm): 315 and 228.
3,5-Bis ((E)-3-nitro benzylidene)-N-methyl-4-piperidone [11]. Generated using 3-nitrobenzlidene 3.02 g, 20 mmol) and N-methyl-4-piperidone (1.162 mL, 10 mmol) in 110 mL of ethanol-water 1:1 mixture. To this, NaOH (2 g, 50 mmol) was added and the mixture was stirred for 8 h at room temperature. Recrystallized from chloroform-methanol to give yellow colored crystals; yield: 97.8%. mp: 185-188.5 °C. HPLC purity and retention time: 98.4%, 14.80 min. \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta 8.25\) (H-2’, 2H, d, J=8.84 Hz), \(\delta 8.24\) (H-4’, 2H, s) \(\delta 7.82\) (H-7, 2H, m), \(\delta 7.72\) (H-6’, 2H, d, J=8.84 Hz), \(\delta 7.64\) (H-5’, 2H, t, J=8.84 Hz), \(\delta 3.79\) (H-2, H-6, 4H, s), \(\delta 2.50\), (N-CH, 3H, s). \(^1^3\)C NMR (100 MHz, CDCl\(_3\)): \(\delta 185\) (C-4), \(\delta 148.37\) (C-3’), \(\delta 136.56\) (C-1’), \(\delta 136.03\) (C-7), \(\delta 135.05\) (C-5), \(\delta 133.84\) (C-2’), \(\delta 129.76\) (C-4’), \(\delta 124.4\) (C-6’), \(\delta 123.8\) (C-5’), \(\delta 56\) (C-6) and \(\delta 45\) (N-C); IR (KBr disc) (cm\(^{-1}\)): 3080 (sp\(^2\) C-H), 2935 (sp\(^3\) C-H), 1670 (C=O), 1610 (C=C), 1590 (aromatic skeletal stretch), 1550 and 1350 (NO\(_2\) stretch), 935 (C-H alkene out of plane bend), 895, 800 and 750 (aromatic C-H out of plane bend); Mass: (m/z):[M+H]\(^+\) calcd for C\(_{20}\)H\(_{17}\)N\(_3\)O\(_5\), 379.3720; found 381.3000; UV (MeOH) \(\lambda_{max}\) (nm): 311, 274 and 221 nm.

3,5-Bis ((E)-4-methylthiobenzylidene)-N-methyl-4-piperidone [12]. Generated using 4-methylthiobenzaldehyde (1.52 g, 10 mmol) and N-methyl-4-piperidone (0.581 mL, 5 mmol) in 20 mL of ethanol-water 1:1 mixture. To this, NaOH (1 g, 25 mmol) was added and the mixture was stirred for 15 h at room temperature. Recrystallized from chloroform-methanol to give yellow colored solid; yield: 82.8%. mp: 172-174 °C; HPLC purity and retention time: 98.2%, 11.94 min. \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta 7.75\) (H-7, 2H, s), \(\delta 7.32\) (H-3’, H-5’, 4H, d, J=7.60 Hz) \(\delta 7.25\) (H-2’, H-6’, 4H, d=7.60 Hz), \(\delta 3.75\) (H-2, H-6, 4H, s), \(\delta 2.47\), (N-CH\(_3\), 3H, s). \(^1^3\)C NMR (100 MHz, CDCl\(_3\)): \(\delta 186.6\) (C-4), \(\delta 140.59\) (C-4’), \(\delta 135.81\) (C-5), \(\delta 132.81\) (C-7), \(\delta 131.7\) (C-1’), \(\delta 130.89\) (C-2’), \(\delta 125.69\) (C-3’), \(\delta 57.15\) (C-
6) and $\delta$ 45.90 (N-C), $\delta$ 15.13 (S-C); IR (KBr disc) (cm$^{-1}$): 3014 (sp$^2$ C-H), 2920 (sp$^3$ C-H), 1669 (C=O), 1587 and 1546 (aromatic skeletal stretch), 1607 (C=C), 821 (para-substitution); Mass: (m/z): [M+H]$^+$ caleed for C$_{22}$H$_{23}$NOS$_2$ 381.5520; found 381.9000; UV (MeOH) $\lambda_{max}$ (nm): 382 and 260 nm.

3,5-Bis ((E)-2-chloro-5-(trifluoromethyl) benzylidene)-N-methyl-4-piperidone [13].
Generated using 2-chloro-5-trifluoromethylbenzaldehyde (1.56 g, 10 mmol) and N-methyl-4-piperidone (0.436 mL, 5 mmol) in 20 mL of ethanol-water 1:1 mixture. To this, NaOH (1 g, 25 mmol) was added and the mixture was stirred for 24 h at room temperature. Recrystallized from chloroform-methanol to give yellow colored crystals; yield: 81%. mp: 154.4-157.2 °C. HPLC purity and retention time: 95.8%, 19.68 min. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.94 (H-7, 2H, s), $\delta$ 7.59 (H-6', 2H, s), $\delta$ 7.58 (H-3', 2H, d, overlapping signals) $\delta$ 7.48 (H-4', 2H, d, overlapping signals), $\delta$ 3.6 (H-2, H-6, 4H, s), $\delta$ 2.39, (N-CH$_3$, 3H, s).
$^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 185.51 (C-4), $\delta$ 138.70 (C-2'), $\delta$ 135.51 (C-5), $\delta$ 134.56 (C-1'), $\delta$ 132.72 (C-7), $\delta$ 130.58 (C-3'), $\delta$ 129.14 (C-5'), $\delta$ 126.98 (C-6'), $\delta$ 126.69 (C-4'), $\delta$ 123.52 (C-F), $\delta$ 56.25 (C-6) and $\delta$ 45.48 (N-C); IR (KBr disc) (cm$^{-1}$): 3000 (sp$^2$ C-H), 2950 (sp$^3$ C-H), 1685 (C=O), 1610 (C=C), 1480 (aromatic skeletal stretch), 1175 and 1120 (C-F), 1090 (aromatic C-Cl), 1000 and 910 (C-H alkene out of plane bend), 825 and 710 (aromatic C-H out of plane bend); Mass: (m/z): [M+H]$^+$ caleed for C$_{22}$H$_{15}$Cl$_2$F$_6$NO, 494.2584; found 494.0000; UV (MeOH) $\lambda_{max}$ (nm): 301 nm.

3,5-Bis ((E)-2,5- dimethoxybenzylidene)-N-methyl-4-piperidone [14]. Generated using 2,5-dimethoxybenzaldehyde (3.32 g, 20 mmol) and N-methyl-4-piperidone (1.162 mL, 10 mmol) in 50 mL of ethanol-water 1:1 mixture. To this, NaOH (2 g, 50 mmol) was added
and the mixture was stirred for 92 h at room temperature. First recrystallized using chloroform-methanol, after which a second recrystallization using pet ether-acetone was performed to give yellow colored crystals; yield: 75.2%. mp: 135-137.3 °C; HPLC purity and retention time: 96.2%, 10.93 min. \(^1\)H NMR (400 MHz, CDCl\(_3\)): δ 8.0 (H-7, 2H, s), δ 6.88 (H-4’, 2H, s), δ 6.84 (H-3’, 2H, d, J=8.96 Hz), δ 6.76 (H-6’, 2H, d, J=8.96 Hz), δ 3.79 (O-CH, 3H, s), δ 3.66 (H-2, H-6, 4H, s), δ 2.38, (N-CH\(_3\), 3H, s). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): δ 186.81 (C-4), δ 152.86 (C-2’), δ 152.84 (C-5’), δ 133.34 (C-5), δ 132.34 (C-7), δ 125.23 (C-1’), δ 116.34 (C-6’), δ 114.72 (C-4’), δ 111.73 (C-3’), δ 57.05 (O-CH), δ 56.08 (C-6) and δ 45.60 (N-C); IR (KBr disc) (cm\(^{-1}\)): 3007 (sp\(^2\)C-H), 2940 (sp\(^3\)C-H), 1663 (C=O), 1578 and 1524 (aromatic skeletal stretch), 1604 (C=C); Mass: (m/z):[M+H]\(^+\) calcd for C\(_{24}\)H\(_{27}\)NO\(_5\), 409.4820; found 410.2000; UV (MeOH) λ\(_{\text{max}}\) (nm): 286 and 310 nm.

**3,5-Bis ((E)-3,4-dimethoxybenzylidene)-N-methyl-4-piperidone [15].** Generated using 3,4-dimethoxybenzaldehyde (2.12 g, 20 mmol) and N-methyl-4-piperidone (1.162 mL, 10 mmol) in 50mL of ethanol-water 1:1 mixture. To this, NaOH (2 g, 50 mmol) was added and the mixture was stirred for 90 h at room temperature. Recrystallized from chloroform-methanol to give bright yellow colored crystals; yield: 56.4%. mp: 167.2-169.7 °C; HPLC purity and retention time: 99.6%, 9.56 min. \(^1\)H NMR (400 MHz, CDCl\(_3\)): δ 7.76 (H-7, 2H, s), δ 7.02 (H-6’, 2H, d, J=8.16 Hz), δ 6.94 (H-2’, 2H, s), δ 6.92 (H-5’, 2H, d, J=8.16 Hz), δ 3.93 (O-CH\(_3\) (3’), 3H, s), δ 3.91 (O-CH (4’), 3H, s), δ 3.79 (H-2, H-6, 4H, s), δ 2.48, (N-CH\(_3\), 3H, s). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): δ 186.68 (C-4), δ 149.91 (C-4’), δ 148.75 (C-3’), δ 136.31 (C-5), δ 131.54 (C-7), δ 128.25 (C-1’), δ 123.7 (C-6’), δ 113.85 (C-2’), δ 110.93 (C-5’), δ 111.73 (C-3’), δ 57.14 (O-CH), δ 55.94 (C-6) and δ 45.62 (N-C); IR (KBr disc) (cm\(^{-1}\)): 3063 (sp\(^2\) CH), 2985 (sp\(^3\) C-H), 1660 (C=O), 1600 (C=C), 1576 and 1514
(aromatic skeletal band), 1147 and 1022 (C-O); Mass: (m/z): [M+H]$^+$ calcd for C$_{24}$H$_{27}$NO$_5$, 409.4820; found 410.2000; UV (MeOH) $\lambda_{max}$ (nm): 256 and 379 nm.

3,5-Bis ((E)-3,5-dichlorobenzylidene)-N-methyl-4-piperidone [16]. Generated using 3,5-dichlorobenzaldehyde (2.12 g, 20 mmol) and N-methyl-4-piperidone (1.162 mL, 10 mmol) in 80 mL of ethanol-water 1:1 mixture. To this, NaOH (2 g, 50 mmol) was added and the mixture was stirred for 48 h at room temperature. Recrystallized from chloroform-methanol to give yellow colored solid; yield: 75%. mp: 186.3-188.6 °C. HPLC purity and retention time: 97.3%, 18.00 min. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.58 (H-7, 2H, s), $\delta$ 7.30 (H-4’, 2H, t, J=1.44 Hz), $\delta$ 7.158 (H-6’, 2H, d, J= 0.416 Hz), $\delta$ 7.155 (H-2’, 2H, dd, J=1.44 and 0.416 Hz), $\delta$ 3.73 (H-2, H-6, 4H, s), $\delta$ 2.47, (N-CH$_3$, 3H, s). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 185.75 (C-4), $\delta$ 137.83 (C-7), $\delta$ 135.32 (C-3’and 5’), $\delta$ 134.09 (C-3), $\delta$ 128.99 (C-4’), $\delta$ 128.19 (C-2’), $\delta$ 127.80 (C-1’), $\delta$ 56.42 (C-2 and C-6) and $\delta$ 45.57 (N-C); IR (KBr disc) (cm$^{-1}$): 3070 (sp$^2$ C-H), 2950 (sp$^3$ C-H), 1675 (C=O), 1615 (C=C), 1590 and 1570 (aromatic skeletal stretch), 1095 (aromatic C-Cl), 1005 and 930 (C-H alkene out of plane bend), 870 and 675 (aromatic C-H out of plane bend); Mass: (m/z): [M+H]$^+$ calcd for C$_{20}$H$_{15}$Cl$_4$NO, 427.1460; found 426.0000; UV (MeOH) $\lambda_{max}$ (nm): 311 and 250 nm

3,5-Bis ((E) 4-nitrobenzylidene)-N-methyl-4-piperidone [17]. Generated using 4-nitrobenzaldehyde (3.02 g, 20 mmol) and N-methyl-4-piperidone (1.162 mL, 10 mmol) with 40 mL of acetic acid-hydrochloric acid solution. The mixture was stirred for 160 h at room temperature. Recrystallized from chloroform-methanol to give brown colored powder; yield: 65%. mp 230.5-231.2 °C. HPLC purity and retention time: 99.8%,17.25 min. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 8.29 (H-3’, H-5’, 4H, d, J=8.72 Hz), $\delta$ 7.82 (H-7, 2H,
\(\delta\) 7.55 (H-2', H-6', 4H, d, J=8.72 Hz), \(\delta\) 3.76 (H-2, H-6, 4H, s), \(\delta\) 2.48 (N-CH\(_3\), 3H, s).

\(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \(\delta\) 185.9 (C-4), \(\delta\) 147.63 (C-4’), \(\delta\) 141.3 (C-5), \(\delta\) 135.63 (C-7), \(\delta\) 134.15 (C-1’), \(\delta\) 130.85 (C-3’ and C-5’), \(\delta\) 123.87 (C-2’ and C-6’), \(\delta\) 56 (C-6) and \(\delta\) 45 (N-C).

IR (KBr disc) (cm\(^{-1}\)): 3082 (sp\(^2\) C-H), 2930 (sp\(^3\) C-H), 1670 (C=O), 1615 (C=C), 1589 (aromatic skeletal stretch), 1548 and 1352 (NO\(_2\) stretch), 937 (C-H alkene out of plane bend), 898, 800 and 753 (aromatic C-H out of plane bend); Mass: (m/z): \([M+H]^+\) calcd for C\(_{20}\)H\(_{17}\)N\(_3\)O, 379.3720; found 380.1000; UV (MeOH) \(\lambda_{\text{max}}\) (nm): 331 and 272.

**3,5-Bis ((E) 3-hydroxybenzylidene)-N-methyl-4-piperidone [18]**. Generated using 3-hydroxybenzaldehyde (2.44 g, 20 mmol) and N-methyl-4-piperidone (1.162 mL, 10 mmol) with 20 mL of acetic acid-hydrochloric acid solution. The mixture was stirred for 152 h at room temperature. Recrystallized from chloroform-methanol to give light yellow colored powder; yield: 83.8%. mp: 284-286 °C. HPLC purity and retention time: 98.0%, 10.56 min.

\(^1\)H NMR (400 MHz, CD\(_3\)OH): \(\delta\) 9.55 (O-H) \(\delta\) 7.80 (H-7, 2H, s), \(\delta\) 7.33 (H-5’, 2H, t, J=7.88 Hz), \(\delta\) 6.95-6.87 (H-2’, H-4’, H-6’, 6H, overlapping signals), \(\delta\) 3.30 (H-2, H-6, 4H, s), \(\delta\) 3.07 (N-CH\(_3\), 3H, s). \(^{13}\)C NMR (100 MHz, CD\(_3\)OH): \(\delta\) 180.77 (C-4), \(\delta\) 157.1 (C-3’), \(\delta\) 139.20 (C-5), \(\delta\) 133.99 (C-5’), \(\delta\) 129.36 (C-7), \(\delta\) 126.17 (C-1’), \(\delta\) 120.97 (C-6’), \(\delta\) 116.5 (C-2’), \(\delta\) 116.8 (C-4’), \(\delta\) 52.52 (C-6) and \(\delta\) 41.4 (N-C); IR (KBr disc) (cm\(^{-1}\)): 3600-3200 (O-H), 3050 (sp\(^2\) C-H), 2980 (sp\(^3\) C-H), 1680 (C=O), 1610 (C=C), 1580 and 1480 (aromatic skeletal stretch), 1225 (C-O), 1005 and 995 (C-H alkene out of plane bend), 850 and 780 (aromatic C-H out of plane bend); Mass: (m/z): \([M+H]^+\) calcd for C\(_{20}\)H\(_{19}\)NO\(_3\), 321.3760; found 321.9000; UV (MeOH) \(\lambda_{\text{max}}\) (nm): 328 and 262 nm.
3,5-Bis ((E) 4-hydroxybenzylidene)-N-methyl-4-piperidone [19]. Generated using 4-hydroxybenzylidene (2.44 g, 20 mmol) and N-methyl-4-piperidone (1.162 mL, 10 mmol) with 20 mL of acetic acid-hydrochloric acid solution. The mixture was stirred for 150 h at room temperature. Recrystallized from chloroform-methanol to give orange colored powder; yield: 84%. mp: 230.5-231.2 °C. HPLC purity and retention time: 98.4%, 8.85 min. $^1$H NMR (400 MHz, CD$_3$OH): δ 10.09 (O-H) δ 7.55 (H-7, 2H, s), δ 7.36 (H-2’, H-6’, 4H, d, J=8.6 Hz), δ 6.87 (H-3’, H-5’, 4H, d, J=8.6 Hz), δ 3.33 (H-2, H-6, 4H, s), δ 2.50, (N-CH$_3$, 3H, s). $^{13}$C NMR (100 MHz, CD$_3$OH): δ 184.7 (C-4), δ 158.26 (C-4’), δ 134.6 (C-5), δ 132.12 (C-2’ and 6’), δ 129.20 (C-1’), δ 124.9 (C-7), δ 115.16 (C-3’ and 5’), δ 55.6 (C-6) and δ 44.5 (N-C); IR (KBr, cm$^{-1}$): 3600-3200 (O-H stretch), 3050 (sp$^2$ C-H stretch), 2990 (sp$^3$ C-H stretch), 1650 (C=O stretch), 1588 and 1572 (Ar skeletal bands), 764 and 693 (mono substituted aromatic ring); Mass: (m/z): [M+H]$^+$ calcd for C$_{20}$H$_{19}$NO$_3$, 321.3760; found 322.1000; UV (MeOH) $\lambda_{max}$ (nm): 373 and 244.

3,5-Bis ((E) 4-hydroxy-3,5-dimethoxybenzylidene)-N-methyl-4-piperidone [20]. Generated using 4-hydroxy-3,5-dimethoxybenzaldehyde (3.64.44 g, 20 mmol) and N-methyl-4-piperidone (1.162 mL, 10 mmol) with 20 mL of acetic acid-hydrochloric acid solution. The mixture was stirred for 140 h at room temperature. Recrystallized from methanol to give red colored powder; yield: 67%. mp: 237.2-238.4 ºC. HPLC purity and retention time: 99%, 9.33 min. $^1$H NMR (400 MHz, DMSO-d6): δ 9.24 (O-H) δ 7.81 (H-7, 2H, s), δ 6.84 (H-2’, H-6’, 4H), δ 3.85 (O-CH$_3$, 12H, s), δ 3.34 (H-2, H-6, 4H, s) δ 2.51, (N-CH$_3$, 3H, s). $^{13}$C NMR (100 MHz, DMSO-d6): δ 181.82 (C-4), δ 148.46 (C-3’, C-5’), δ 140.24 (C-3), δ 138.98 (C-4’), δ 125.42 (C-1’), δ 124.44 (C-7), δ 109.76 (C-2’, C-6’), δ 56.74 (O-CH$_3$), δ 53.91 (C-2, C-6) and δ 43.01 (N-C); IR (KBr disc) (cm$^{-1}$): 3600-3200
(O-H), 3010 (sp² C-H), 2920 (sp³ C-H), 1620 (C=O), 1600 (C=C), 1590 and 1570 (aromatic skeletal stretch), 1275 (C-O), 985 and 900 (C-H alkene out of plane bend), 820 and 790 (aromatic C-H out of plane bend); Mass: (m/z):[M+H]^+ calcd for C_{24}H_{27}NO_{7}, 441.4800; found 442.2000; UV (MeOH) λ_max (nm): 403 and 259 nm.

3,5-Bis((E)-4-hydroxy-3-methoxybenzylidene)-N-methyl-4-piperidone [21].
Generated using 4-hydroxy-3-methoxybenzylidene (1.52 g, 10 mmol) and N-methyl-4-piperidone (0.581 mL, 5 mmol) with 10mL of acetic acid-hydrochloric acid solution. The mixture was stirred for 150 h at room temperature. Recrystallized from methanol to give dark green colored powder; yield: 30%. mp: 217-220.5 °C. HPLC purity and retention time: 98.9%, 9.47 min. ^1H NMR (400 MHz, DMSO-d6): δ 9.69 (O-H) δ 7.60 (H-7, 2H, s), δ 7.08 (H-2', 2H, s), δ 3.83 (O-CH₃, 6H, s), δ 3.34 (H-2, H-6, 4H, s), δ 2.50, (N-CH₃, 3H, s). ^13C NMR (100 MHz, DMSO-d6): δ 185.39 (C-4), δ 148.95 (C-4'), δ 148.09 (C-3'), δ 136.55 (C-3), δ 129.76 (C-1'), δ 126.47 (C-7), δ 124.83 (C-6'), δ 116.24 (C-2'), δ 115.68 (C-5'), δ 56.20 (O-CH₃), δ 49.07 (C-2 and C-6) and δ 45.27 (N-C);IR (KBr disc) (cm⁻¹): 3600-3200 (O-H), 3020 (sp² C-H), 1680 (C=O), 1600 (C=C), 1510 (aromatic skeletal stretch), 1280 (C-O), 990 and 910 (C-H alkene out of plane bend), 830 and 795 (aromatic C-H out of plane bend); Mass: (m/z):[M+H]^+ calcd for C_{22}H_{23}NO_{5}, 381.4280; found 382.1000; UV (MeOH) λ_max (nm): 387, 265 and 257 nm.

3,5-Bis((E)-2-thiophenylmethylene)-N-methyl-4-piperidone [22]. Generated using 2-thiophenecarboxaldehyde (2.24 g, 20 mmol) and N-methyl-4-piperidone (1.162 mL, 10 mmol) in 70 mL of ethanol-water 1:1 mixture. To this, NaOH (2 g, 50 mmol) was added
and the mixture was stirred for 15 h at room temperature. Recrystallized from chloroform-methanol to give light brown colored crystals; yield: 81.5%. mp: 115-118.4 °C; HPLC purity and retention time: 95.2%, 10.22 min. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.93 (H-7, 2H, s), $\delta$ 7.56 (H-5’, 2H, d, J=3.6 Hz), $\delta$ 7.34 (H-3’, 2H, d, J=5.04 Hz), $\delta$ 7.15 (H-4’, 2H, dd, J=3.6 and 5.04 Hz), $\delta$ 3.82 (H-2, H-6, 4H, s), $\delta$ 2.59, (N-CH$_3$, 3H, s). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 186.17 (C-4), $\delta$ 138.72 (C-6), $\delta$ 133.13 (C-7), $\delta$ 130.48 (C-1’), $\delta$ 130.47 (C-2’), $\delta$ 128.36 (C-4’), $\delta$ 127.98 (C-3’), $\delta$ 56.71 (C-6) and $\delta$ 46.08 (N-C); IR (KBr disc) (cm$^{-1}$): 3080 (sp$^2$ C-H), 2968 (sp$^3$ C-H), 1663 (C=O), 1575 and 1505 (aromatic skeletal stretch), 1604 (C=C), 732 and 652 (C-H); Mass: (m/z): [M+H]$^+$ calcd for C$_{16}$H$_{15}$NOS$_2$, 301.42220; found 301.7000; UV (MeOH) $\lambda$max (nm): 256 and 376 nm.

3,5-Bis((E)-2-pyrrolemethylene)-N-methyl-4-piperidone [23]. Generated using 2-pyrrolecarboxaldehyde (1.9 g, 20 mmol) and N-methyl-4-piperidone (1.162 mL, 10 mmol) in 40 mL of ethanol-water 1:1 mixture. To this, NaOH (2 g, 50 mmol) was added and the mixture was stirred for 30 h at room temperature. Recrystallized from chloroform-methanol to give red colored powder; yield: 58%. mp: 214-215.4 °C. HPLC purity and retention time: 98.5%, 8.53 min. $^1$H NMR (400 MHz, DMSO-d$_6$): $\delta$ 7.52 (H-7, 2H, s), $\delta$ 7.09 (H-5’, 2H, d), $\delta$ 6.43 (H-3’, 2H, d), $\delta$ 6.28 (H-4’, 2H, dd), $\delta$ 3.59 (H-2, H-6, 4H, s), $\delta$ 2.46, (N-CH$_3$, 3H, s). $^{13}$C NMR (100 MHz, DMSO-d$_6$): $\delta$ 183.99 (C-4), $\delta$ 127.59 (C-5), $\delta$ 126.50 (C-7), $\delta$ 123.56 (C-2’), $\delta$ 122.10 (C-5’), $\delta$ 113.04 (C-4’), $\delta$ 110.49 (C-3’), $\delta$ 55.80 (C-6) and $\delta$ 45.0 (N-C); IR (KBr disc) (cm$^{-1}$): 2995 (sp$^2$ C-H), 2950 (sp$^3$ C-H), 1635 (C=O), 1600 (C=C), 1550 (aromatic skeletal stretch), 1300 (C-N), 1000 and 920 (C-H alkene out of plane bend), 880 and 720 (aromatic C-H out of plane bend); Mass: (m/z): [M+H]$^+$ calcd for C$_{16}$H$_{15}$N$_3$O, 267.3320; found 267.9000; UV (MeOH) $\lambda$max (nm): 436 and 252 nm.
3,5-Bis((E)-3-thiophenylmethylene)-N-methyl-4-piperidone [24]. Generated using 3-thiophenecarboxaldehyde (1.12 g, 10 mmol) and N-methyl-4-piperidone (0.581 mL, 5 mmol) in 30 mL of ethanol-water 1:1 mixture. To this, NaOH (2 g, 50 mmol) was added and the mixture was stirred for 7 h at room temperature. Recrystallized from methanol to give brown colored crystals; yield: 54%. mp: 125.8-127.8 °C. HPLC purity and retention time: 99.4%, 10.73 min. $^1$H NMR (400 MHz, CDCl$_3$): δ 7.76 (H-7, 2H, s), δ 7.46 (H-2’, 2H, d, J=2.52 Hz), δ 7.38 (H-5’, 2H, dd, J=2.52 and 5 Hz), δ 7.22 (H-4’, 2H, d, J=5 Hz), δ 3.79 (H-2, H-6, 4H, s), δ 2.53, (N-CH$_3$, 3H, s). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 185.87 (C-4), δ 135.75 (C-5), δ 131.48 (C-7), δ 129.28 (C-3’), δ 128.80 (C-2’), δ 127.49 (C-4’), δ 126.45 (C-5’), δ 55.84 (C-6) and δ 44.8 (N-C); IR (KBr disc) (cm$^{-1}$): 3095 (sp$^2$ C-H), 2970 (sp$^3$ C-H), 1675 (C=O), 1600 (C=C), 1515 and 1475 (aromatic skeletal stretch), 1000 and 940 (C-H alkene out of plane bend), 890 and 800 (aromatic C-H out of plane bend); Mass: (m/z): [M+H]$^+$ calcd for C$_{16}$H$_{15}$NOS$_2$, 301.4220; found 301.8000; UV (MeOH) $\lambda_{max}$ (nm): 354 and 212 nm.

3,5-Bis((E)-5-bromo-3-thiophenylmethylene)-N-methyl-4-piperidone [25]. Generated using 5-bromo-2-thiophenecarboxaldehyde (1.9 g, 20 mmol) and N-methyl-4-piperidone (1.162 mL, 10 mmol) in 70 mL of ethanol-water 1:1 mixture. To this, NaOH (2 g, 50 mmol) was added and the mixture was stirred for 46 h at room temperature. Recrystallized from chloroform-methanol to give yellow colored crystals; yield: 87.8%. mp: 204.8-207 °C. HPLC purity and retention time: 95.0%, 13.59 min. $^1$H NMR (400 MHz, CDCl$_3$): δ 7.81 (H-7, 2H, s), δ 7.07 (H-3’, 2H, d, J = 3.12 Hz), δ 7.04 (H-4’, 2H, d, J = 3.12 Hz), δ 3.88 (H-2, H-6, 4H, s), δ 2.61, (N-CH$_3$, 3H, s). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 185.53 (C-4), δ
140.15 (C-7), δ 133.34 (C-3’), δ 130.92 (C-4’), δ 130.29 (C-3), δ 127.97 (C-2’), δ 118.23 (C-5’), δ 56.44 (C-2 and C-6) and δ 45.85 (N-C); IR (KBr disc) (cm⁻¹): 3085 (sp² C-H), 2960 (sp³ C-H), 1630 (C=O), 1600 (C=C), 1575 and 1498 (aromatic skeletal stretch), 1030 and 920 (C-H alkene out of plane bend), 800 and 720 (aromatic C-H out of plane bend); Mass: (m/z): [M+H]^+ calcd for C₁₆H₁₃Br₂NOS₂, 459.2140; found 459.8000; UV (MeOH) λmax (nm): 387 and 272 nm.

3,3’-Dimethyl-2,2’-spirobi[chromene] [26]. Generated using 3-pentanone (0.861 g, 0.01 mol), substituted salicylaldehyde (2.44 g, 0.02 mol), and SiCl₄ (silicon tetrachloride, 5.09 g, 0.03 mol), which were added to absolute ethanol (20 mL) at room temperature. The mixture was stirred under nitrogen atmosphere for 2 h; product obtained as a white solid; yield: 96%, mp: 196.6-197.7 °C; HPLC purity and retention time: 99.2%, 15.01 min. ¹H-NMR (200 MHz, CDCl₃): δ 7.13 (H-10, t, J=8 Hz), δ 7.10 (H-8, d, J=8 Hz), δ 6.94 (H-9, t, J=8 Hz), δ 6.83 (H-11, d, J=8 Hz), δ 6.58 (H-6, s), and δ 1.96 (H-3 and H-4, s); ¹³C-NMR (50 MHz, CDCl₃): δ 148.8 (C12), δ 128.4 (C2, C5), δ 127.7 (C10), δ 125.6 (C8), δ 122.6 (C9), δ 121.2 (C7), δ 118.8 (C6), δ 114.6 (C11), δ 101.2 (C1) and δ 17.6 (C3, C4); IR (KBr disc) (cm⁻¹): 3065 (sp² C-H stretch), 2919 (sp³ C-H stretch), 1614 (C=C stretch), 1572 and 1463 (Ar skeletal bands), 1438 and 1372 (aromatic C-H bend), 1325, 1275, 1117 (C-O stretch), 908, 864, 768 (aromatic C-H out of plane bend); Mass: (m/z): [M+H]^+ calcd for C₁₉H₁₆O₂, 276.1150, found 276; UV (CHCl₃) λmax (nm): 243, 281.

6,6’-Dichloro-3,3’-dimethyl-2,2’-spirobi[chromene] [27]. Generated using 3-pentanone (0.861 g, 0.01 mol), substituted 5-chlorosalicylaldehyde (3.13 g, 0.02 mol), and SiCl₄ (silicon tetrachloride, 5.09 g, 0.03 mol), which were added to absolute ethanol (20 mL) at
room temperature. The mixture was stirred under nitrogen atmosphere for 4 h; product obtained as a yellow solid; yield: 77%, mp: 175.2-176.9 °C. HPLC purity and retention time: 99.5%, 16.70 min. $^1$H-NMR (400 MHz, CDCl$_3$): δ 7.10-7.07 (H-8 and H-10, m), δ 6.75 (H-11, d, J=8Hz), δ 6.50 (H-6, s), and δ 1.94 (H-3 and H-4, s); $^{13}$C-NMR (100 MHz, CDCl$_3$): δ 147.5(C12), δ 128.2 (C9), δ 127.9 (C2, C5), δ 125.9 (C10), δ 125.1 (C8), δ 122.0 (C7), δ 121.3 (C6), δ 117.7 (C11), δ 94.5 (C1) and δ 18.4 (C3, C4); IR (KBr disc) (cm$^{-1}$): 3040 (sp$^2$ C-H stretch), 2920 (sp$^3$ C-H stretch), 1610 (C=C stretch), 1577 and1465 (Ar skeletal bands), 1440 and 1373 (aromatic C-H bend), 1320, 1275, 1110 (C-O stretch), 1079, 1058 (Aromatic -Cl stretch), 910, 866, 770 (aromatic C-H out of plane bend); Mass: (m/z):[M+H]$^+$ calcd for C$_{19}$H$_{14}$Cl$_2$O$_2$, 345.2190, found 344; UV (CHCl$_3$) $\lambda_{\text{max}}$ (nm): 241, 296.

6,6'-Dibromo-3,3'-dimethyl-2,2'-spirobi[chromene] [28]. Generated using 3-pentanone (0.861 g, 0.01 mol), substituted 5-bromosalicylaldehyde (4.02 g, 0.02 mol), and SiCl$_4$ (silicon tetrachloride, 5.09 g, 0.03 mol), which were added to absolute ethanol (20 mL) at room temperature. The mixture was stirred under nitrogen atmosphere for 6 h; product obtained as a pale yellow solid; yield: 73%, mp: 196.6-199.2 °C. HPLC purity and retention time: 97.3%, 17.03 min. $^1$H-NMR (400 MHz, CDCl$_3$): δ 7.25-7.20 (H-8 and H-10, m), δ 6.73 (H-11, d, J=8Hz), δ 6.52 (H-6, s), and δ 1.94 (H-3 and H-4, s); $^{13}$C-NMR (100 MHz, CDCl$_3$): δ 146.5(C12), δ 129.2 (C9), δ 128.9 (C2, C5), δ 126.9 (C10), δ 126.1 (C8), δ 123.0 (C7), δ 122.3 (C6), δ 116.7 (C11), δ 95.5 (C1) and δ 19.4 (C3, C4); IR (KBr disc) (cm$^{-1}$): 3059 (sp$^2$ C-H stretch); 2927 (sp$^3$ C-H stretch), 1633 (C=C stretch), 1476 and1419 (aromatic C-H bend), 1339 (CH$_3$ bend); 1286, 1228, 1179, 1126 (C-O stretch); 1067, 852.
and 682 (aromatic C-H out of plane bend); Mass: (m/z): [M+H]^+ calcd for C_{19}H_{14}Br_2O_2, 434.1270, found 436; UV (CHCl_3) \( \lambda_{\text{max}} \) (nm): 242, 317.

**6,6',8,8'-Tetrachloro-3,3'-dimethyl-2,2'-spirobi[chromene] [29].** Generated using 3-pentanone (0.861 g, 0.01 mol), substituted 5-methoxysalicylaldehyde (3.04 g, 0.02 mol), and SiCl_4 (silicon tetrachloride, 5.09 g, 0.03 mol), which were added to absolute ethanol (20 mL) at room temperature. The mixture was stirred under nitrogen atmosphere for 7 h; product obtained as a pale white solid; yield: 81%, mp = 134.4-136.0 °C. HPLC purity and retention time: 99.8%, 17.23 min. \(^1\)H-NMR(400 MHz, CDCl_3): \( \delta \) 7.25-7.20 (H-8 and H-10, m), \( \delta \) 6.73 (H-11, d, J=8Hz), \( \delta \) 6.52 (H-6, s), and \( \delta \) 1.94 (H-3 and H-4, s); \(^{13}\)C-NMR (100 MHz, CDCl_3): \( \delta \) 146.5 (C12), \( \delta \) 129.2 (C9), \( \delta \) 128.9 (C2, C5), \( \delta \) 126.9 (C10), \( \delta \) 126.1 (C8), \( \delta \) 123.0 (C7), \( \delta \) 122.3 (C6), \( \delta \) 116.7 (C11), \( \delta \) 95.5 (C1) and \( \delta \) 19.4 (C3, C4); IR (KBr disc) (cm\(^{-1}\)): 3038 (sp\(^2\) C-H stretch), 2940 (sp\(^3\) C-H stretch), 1620 (C=C stretch), 1576, 1460 (Ar skeletal bands), 1441 and 1374 (aromatic C-H bend), 1322, 1278, 1111 (C-O stretch), 1080 and 1068 (Aromatic -Br stretch), 912, 867 and 771 (aromatic C-H out of plane bend); Mass: (m/z): [M+H]^+ calcd for C_{19}H_{12}Cl_4O_2, 414.1030, found 418; UV (CHCl_3) \( \lambda_{\text{max}} \) (nm): 242, 298.

**6,6'-Dimethoxy-3,3'-dimethyl-2,2'-spirobi[chromene] [30].** Generated using 3-pentanone (0.861 g, 0.01 mol), substituted 3,5-dichlorosalicylaldehyde (3.82 g, 0.02 mol), and SiCl_4 (silicon tetrachloride, 5.09 g, 0.03 mol), which were added to absolute ethanol (20 mL) at room temperature. The mixture was stirred under nitrogen atmosphere for 10 h; product obtained as a yellow solid; yield: 82%, mp: 191.3-192.6 °C. HPLC purity and retention time: 99.1%, 14.40 min. \(^1\)H-NMR(400 MHz, CDCl_3): \( \delta \) 7.30 (H-11, t, J=8 Hz), \( \delta \)
6.71 (H-10, dd, J = 8Hz and J = 2.4Hz), δ 6.52 (H-6, s), δ 6.40 (H-8, d, J = 2.4Hz), δ 3.76 (s, -OCH3), and δ 1.95 (H-3 and H-4, s); 13C-NMR (100 MHz, CDCl3): δ 147.5 (C12), δ 128.2 (C9), δ 127.9 (C2, C5), δ 125.9 (C10), δ 125.1 (C8), δ 122.0 (C7), δ 121.3 (C6), δ 117.7 (C11), δ 94.5 (C1) and δ 18.4 (C3, C4); IR (KBr disc) (cm⁻¹): 3033 (sp² C-H stretch), 2926 (sp³ C-H stretch), 1615 (C=C stretch), 1578, 1466 (Ar skeletal bands), 1442, 1375 (aromatic C-H bend), 1321, 1276 and 1118 (C-O stretch), 1078 and 1057 (Aromatic -Cl stretch), 918 and 862 (aromatic C-H out of plane bend); Mass: (m/z): [M+H]+ calcd for C21H20O4, 336.3870, found 336; UV-vis (CHCl3) λmax nm: 242, 297.

4.2.2 Crystal Growth and Structural Analysis

The crystals of 3,5-Bis ((E)-2-thiophenyliene)-N-methyl-4-piperidone (22) were grown by slow evaporation technique at ambient temperature. The purified compound was used for growing single crystals using chloroform/methanol mixture (1:1, v/v). Yellow coloured crystals were formed after slow evaporation of chloroform-methanol mixture. Single crystal X-ray diffraction data were collected on Rigaku XtaLABmini X-ray diffractometer equipped with Mercury CCD detector with graphite monochromatic Mo-Kα radiation (λ = 0.71073 Å) at room temperature [298.0 (2) K] using ω scans. The data were analyzed using CrysAlisPro 1.171.39.35c, absorption correction was done by multi-scan method and the space group was determined using ShelXT through Olex2. The crystal structures were solved by using ShelXT and were refined using ShelXL. All of the hydrogen atoms were geometrically fixed and refined using the riding model. All packing and interaction diagrams were generated using Mercury 3.9.40 software.
4.2.3 Computational Analyses/In Silico Studies

The molecules were evaluated for their similarity to drugs (Lipinski parameters) and pharmacokinetic properties (ADME) using the drug design tool from the Supercomputing Facility for Bioinformatics and Computational Biology, Indian Institute of Technology (IIT) Delhi\textsuperscript{55,56} and the free web tool Swiss ADME.\textsuperscript{57}

4.2.4 Water Solubility Studies

1 mg of each compound was added to a 500 mL conical flask. Water at room temperature was then added (with continuous stirring) until the compounds dissolved to obtain saturated solutions. The volumes of water required for dissolution of each compound are as follows: 1 - 100 mL, 3 - 215 mL, 13 - 180 mL, 26 - 180 mL, 27 - 100 mL, and curcumin – 500 mL. Five standard solutions were prepared by serial dilution (dilution factor of 1.111) from each of these stocks. The optical density of the standard solutions was recorded at 328 nm for 1, 322 nm for 3, 301 nm for 13, 281 nm for 26, 296 nm for 27, and 425 nm for curcumin using a Shimadzu 2450 spectrophotometer to generate standard curves. The aforementioned saturated solutions of the compounds were diluted to half of their concentration by taking equal volume of water, and optical density was recorded. Then the solubility of the compounds in water was calculated from the obtained standard curves.\textsuperscript{58}

4.2.5 DPPH Assay

Antioxidant activities of the compounds were determined using a stable free radical DPPH (2,2-Diphenyl-1-picrylhydrazyl) assay.\textsuperscript{58,59} Briefly, 0.15 mM DPPH was dissolved in 20 mL of methanol and separately, 10 µM of each test compound was prepared in 5 mL of DMSO. Then, the DPPH solution was added to the sample in a 2:1 ratio (160 µL DPPH
and 80 µL sample). The mixture was kept in the dark for 30 minutes at room temperature. The absorbance of the solutions was then measured at 517 nm via a Varioskan Lux Multimode Microplate Reader (Thermo Scientific). Ascorbic acid (in methanol) was used as a positive control and prepared similarly. DPPH radical scavenging activity was determined using the following equation: % Inhibition = \( \frac{A_c - A_s}{A_c} \times 100 \), where \( A_c \) = Absorbance of the control and \( A_s \) = Absorbance of the test samples.

4.2.6 Cell Culture

RAW264.7 cells were purchased from American Type Culture Collection (ATCC). Cells were cultured at 37 °C under a humidified atmosphere containing 5% CO\(_2\). Standard growth media consisted of high glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (100 µg/mL penicillin and 100 µg/mL streptomycin). Cells were sub-cultured approximately once every four days and only cells between passages 7 and 15 were used for all experiments.

4.2.7 Cell Viability

RAW 264.7 cells were plated in 24-well plates at a density of 5 x 10\(^4\) cells/well in 500 µL of media. Compounds were dissolved in DMSO to give 10 mM stock solutions. Dosing was performed so that the final concentration per well was 10 µM in 500 µL of media; the final DMSO concentration was 0.4% per sample. Each sample was performed with three biological replicates. 48 hours after treating the cells with the compounds, Alamar Blue Assay was performed by adding Alamar Blue reagent (Thermo Fisher Scientific) to the wells and incubating them at 37 °C for 30 minutes according to the manufacturer’s
protocol. 200 µL of supernatant from each sample was collected and transferred to a 96-well plate, where absorbances at 570 and 600 nm were determined using a SpectraMax M2 plate reader. The data was then normalized to non-treated, DMSO-only control.

4.2.8 RT-PCR Assay

RAW 264.7 cells were plated in 24-well plates at a density of 100,000 cells/well in 500 µL of media. For experiments involving compound-treated pro-inflammatory cells (IFN-γ/LPS), designated cells were then treated with 50 ng/mL each of IFN-γ and LPS for 24 hours. Then, molecules were dissolved in DMSO to give 10 mM stock solutions, which were further diluted in media to give 10 µM with a final DMSO concentration of 0.4% per sample. Media was aspirated from the cells and replaced with media containing designated compounds at 10 µM for 48 hours. Each experiment included three biological replicates per treatment condition. Approximately 1.5 µg RNA was directly harvested from cells using the PureLink RNA Mini Kit (Ambion) following the manufacturer's instructions. SuperScript IV Reverse Transcriptase, RNaseOut, 10mM dNTPs, and 50 µM Random Hexamers were used for the conversion of approximately 150 ng of RNA to cDNA (ThermoFisher), also following the manufacturer's instructions with a sample volume of 20 µL. Briefly, primers were annealed to RNA at 65 °C for 5 min. Then the annealed RNA was combined with the reaction mixture (containing 500 units of reverse transcriptase per sample) and amplified at 53 °C for 10 min and melted at 80 °C for 10 min. cDNA was stored at -20 °C and used for RT-PCR within 1 week. RNA and cDNA were quantified and evaluated using a NanoDrop 2000 spectrophotometer (ThermoFisher). RT-PCR was performed using a CFX Connect real-time system (Biorad) with iTaq Universal SYBR
Green Supermix (Biorad). All DNA primers were purchased from Integrated DNA Technologies. The following primer sequences were used: β-actin (forward) 5’-GATCAG-CAAGCAGGAGTACGA-3’, (reverse) 5’-AAAACGCAGCGCAGTAACAGT-3’; iNOS (forward) 5’-GTTCTCAGCCCAACAATAACAAGA-3’, (reverse) 5’-GTGGACGGGTCTGATGTCAC-3’. 200 nM of forward and reverse primers (1 gene per reaction) was mixed with 1 μL (100 ng) of cDNA, 10 μL SYBR Green and H₂O to a final volume of 20 μL. The thermocycler protocol was as follows: the samples were first activated at 50 °C for 2 min, then 95 °C for 2 min; then denaturing occurred at 95 °C for 30 s followed by annealing at 57 °C; the denature/anneal process was repeated over 40 cycles. Relative gene expression was determined by comparing the Ct value of the gene of interest to that of β-actin control, by the 2ΔΔCt method. Three biological replicates were performed for each treatment condition and three technical replicates were used for each biological replicate. Data was analyzed using the CFX Manager 3.1 software. CQ values were generated by using the point at which the sample fluorescence value exceeded the software’s default threshold value.

4.2.9 Griess Assay

RAW 264.7 cells were plated in phenol red-free DMEM containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in 24-well plates at a density of 1.5 × 10⁵ cells/well 24 hours prior to the experiment. Cells were treated as described in the RT-PCR protocol. Then, cell supernatant was collected from the wells and centrifuged at 5000 rpm for 5 min. Griess reagent (Invitrogen) was freshly prepared according to the manufacturer’s instructions. 60 μL Griess reagent was combined with 60 μL cell supernatant in a clear 96-
well plate. After incubating for 15 min in the dark, absorbance was read using a SpectraMax ID3 plate reader (Molecular Devices) at 548nm. Three biological replicates were performed per treatment condition. Actual NO$_2^-$ concentrations were determined by comparing absorbance values to a standard curve generated using NaNO$_2$ solutions.

4.3 Results and Discussion

4.3.1 Synthesis and Characterization of Curcumin Derivatives
Curcumin, the principal component of Turmeric (Curcuma longa), has been extensively studied due to its anti-inflammatory, antioxidant, and radical scavenging abilities, which can assist in the prevention and treatment of some inflammatory diseases.$^{34}$ Unfortunately, curcumin possesses characteristics that result in its poor biodistribution in vivo,$^{40}$ which is an obstacle for drug delivery. In an effort to harness curcumin’s therapeutic qualities while circumventing its negative aspects, we synthesized two series of curcumin derivatives for further study. A total of 30 compounds, twenty-one diarylidene-N-methyl-4-piperidones (DANMPs; 1-21), four dihetarylidene-N-methyl-4-piperidones (DHANMPs; 22-25), and five spirobibenzopyrans (SBPs; 26-30) were synthesized (Figures 4.3-4.7). The compounds were purified by re-crystallization and characterized using UV-Vis, FT-IR, $^1$H-NMR, $^{13}$C-NMR, and mass spectrometry; purity was assessed via HPLC. As a representative model, one of the compounds (22) was further characterized by single crystal XRD (Figure 4.8, Table 4.1).
Figure 4.7: Molecular structure of curcumin, and three classes of derivatives generated: diarylidene-N-methyl-4-piperidones (DANMPs; 1-21), dihetarylidene-N-methyl-4-piperidones (DHANMPs; 22-25), and spirobibenzopyrans (SBPs; 26-30).
Figure 4.8: Crystallographic data for 3,5-Bis ((E)-2-thiophenylidene)-N-methyl-4-piperidone (22). (a) depiction of asymmetric unit, which contains one molecule of 22; (b) Intermolecular interactions found in the crystal packing of 22. The single crystal of 3,5-Bis ((E)-2-thiophenylidene)-N-methyl-4-piperidone (22) crystallizes in monoclinic system with $P2_1/c$ space group. A weak C-H-O hydrogen bond has been observed in the structure. Other weaker interactions like C-H-π are observed between the two layers of the molecules. These molecules are arranged antiparallel to each other by weak hydrogen bond and van der Waals interaction in crystal packing.
Table 4.1: Crystallographic data for 3,5-Bis ((E)-2-thiophenylidene)-N-methyl-4-piperidone (22).

<table>
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4.3.2 In Silico Evaluations of Compounds for Drug-Like Properties

To assess the drug-like characteristics and potential pharmacokinetic properties of the DANMP and SBP compounds, computational analyses were performed. We found that the majority of the structures are in accordance with Lipinski’s rule of five (Table 4.2). The exceptions to this were 9, 10, and 28, whose partition coefficient (LogP) values slightly exceeded the desired range of -0.4 to +5.6, with 5.91, 5.78, and 6.19, respectively. It is useful to note that curcumin itself has a LogP value of -0.053; the lowest value in the series analyzed was 2.32 (for 23). Analysis of Lipinski’s parameters for the synthesized compounds showed overall improvement and a higher potential to be orally administered.
when compared to curcumin (Table 4.3). Thus, the computationally determined drug-like pharmacokinetic properties of the compounds support that they can be explored as potential anti-inflammatory agents.

Table 4.2: Lipinski parameters for synthesized compounds.

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<th>Compound</th>
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<th>HBA</th>
<th>Molar Refractivity</th>
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Mol. Wt. - Molecular Weight; Log P- partition coefficient; HBD - hydrogen bond donors; HBA - hydrogen bond acceptors.
### Table 4.3: Results of *in silico* ADME studies for evaluated molecules

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<tr>
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<td>-4.78</td>
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ESOL – Estimated Solubility; GI - Gastrointestinal; BBB- Blood–Brain Barrier

### 4.3.3 DPPH Assay

To evaluate the radical scavenging activity of the curcumin-related compounds, a 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay\(^6\) was performed (Figure 4.9, Table 4.4). This method facilitates the evaluation of the antioxidant potential of a compound by exposing it to DPPH, which is a violet, stable free radical solution that loses
its color upon gain of a hydrogen atom. In this assay, ascorbic acid (a.a.) was used as a positive control and benchmark for the new molecules. The majority of the compounds were found to have activity similar to that of a.a, with only one (13, which contained R = 2-Cl-5-CF$_3$) being significantly better (p<0.05). Among the DANMPs, only 12 (R = 4-SCH$_3$) and 20 (R = 3,5-di-OCH$_3$, 4-OH) were significantly worse (p>0.05) than a.a. Overall, most of the best-performing DANMPs contained electron-withdrawing substituents, likely due to improved stabilization of the added electron. Among the DHANMPs and the SBPs, all showed significantly reduced radical scavenging activity versus a.a. All DHANMPs had similar effects to one another with no statistical difference among them, while among the five spiro derivatives, 26 (R = H) and 27 (R = 5-Cl) showed better scavenging activities than the others. Statistical comparison of the DHANMPs showed that there was no significant difference between compounds 26 and 27, or among compounds 26, 28, 29, and 30. Compound 27 was, however, statistically different (p<0.001) when compared to compounds 28, 29, and 30; there was no statistical difference found among 28, 29, and 30.
Figure 4.9: DPPH radical scavenging activity of curcumin derivatives. Thirty synthesized molecules were evaluated and benchmarked against ascorbic acid (a.a.), which was used as a positive control. All compounds were dosed at the same concentration, 10 µM. Error bars represent standard error. Student T-test was performed to compare each treatment to ascorbic acid. (p > 0.05 = not significant (non-marked), p < 0.01 = **, and p < 0.001 = ***).

DPPH Radical Scavenging Activity (%)
Table 4.4: DPPH radical scavenging values of evaluated molecules.

<table>
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<tr>
<th>Compound</th>
<th>Average</th>
<th>Std. Error</th>
<th>Normalized</th>
<th>Normalized Error</th>
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<tr>
<td>a.a.</td>
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<td>0.0116</td>
<td>50.85</td>
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4.3.4 Solubility Studies

From the 30 synthesized derivatives, we selected five compounds for further evaluation based on a combination of results from computational assessments (Tables 4.2 and 4.3), DPPH radical scavenging activity (Figure 4.9 and Table 4.4), and structural diversity. Compounds 1, 3, 13, 26, and 27 were used in additional studies. As described above, a
major challenge preventing the use of curcumin is its lack of bioavailability. In many instances low bioavailability and biodistribution may attributed to poor aqueous solubility resulting in low permeability.\textsuperscript{62} To address whether the newly generated compounds had improved solubilities relative to curcumin, we employed UV absorption spectroscopy. As compared to the water solubility of curcumin (cited in the literature as 0.0006 mg/mL),\textsuperscript{63} experimentally determined by us as 0.00056 mg/mL, all of the tested compounds are substantially improved in this regard (Table 4.5). Strikingly, compounds 1 and 27 showed the greatest solubility (both 0.01 mg/mL), while 3, 13, and 26 were all similar (0.00477, 0.00652, and 0.00693 mg/mL, respectively).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Water solubility (mg/mL)</th>
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<tr>
<td>Curcumin</td>
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<tr>
<td>1</td>
<td>0.01</td>
</tr>
<tr>
<td>3</td>
<td>0.00477</td>
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<td>13</td>
<td>0.00652</td>
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<td>26</td>
<td>0.00693</td>
</tr>
<tr>
<td>27</td>
<td>0.01</td>
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</table>

Lit = literature cited; det = experimentally determined.

4.3.5 Cell-Based Evaluations of Anti-Inflammatory Activity

For cell-based in vitro studies of anti-inflammatory activity of the compounds, the commonly employed RAW264.7 macrophage cell line was used.\textsuperscript{64,65} Prior to additional in vitro studies, we determined whether there were any effects of the five selected curcumin derivatives on RAW264.7 cell viability (Figure 4.10). None of the compounds resulted in dramatic changes. Curcumin has the ability to inhibit COX, LOX, and NF-κB, which in turn reduces the expression of inducible nitric oxide synthase (iNos) and the resulting secretion of nitric oxide (NO).\textsuperscript{66,67} At the same time, enhanced levels of iNos and NO are also markers of inflammation. Therefore, we elected to track iNos and NO to assess the
activities of the curcumin derivatives. To monitor the effects of the selected compounds on iNos expression, Real Time Polymerase Chain Reaction (RT-PCR) was used.

![Figure 4.10: Viability of RAW 264.7 cells treated with curcumin-related derivatives. DMSO = naïve cells treated with 0.4% DMSO (dimethyl sulfoxide) only. Error bars represent standard error. Student T-test was performed to compare each treatment to DMSO, which was used as a control for this experiment (p > 0.05 = not significant (non-marked), p < 0.05 = *).](image)

To assess the abilities of the compounds to resolve inflammation, RAW264.7 cells were first treated with interferon gamma (IFN-γ) and lipopolysaccharide (LPS) to achieve a pro-inflammatory (M1) phenotype. Macrophages were treated with curcumin and molecules 1, 3, 13, 26, and 27 to assess and compare their abilities to reduce iNos expression relative to a non-treated (N.T.), M1-polarized control group (Figure 4.11). Both curcumin and all five derivatives resulted in dramatically decreased levels of iNos expression, within a ten-fold range of levels present in naïve (non-polarized) macrophages. Among the five compounds, compound 1 was the only one that showed a significant decrease in expression (p<0.01) relative to the curcumin-treated group. Compounds 3, 26, and 27, however, showed a statistically significant increase (p<0.05), while compound 13 was not statistically different from the curcumin-treated group. Treatment of naïve (non-polarized) RAW264.7 macrophages with curcumin and the derivative molecules 1, 3, 13, 26, and 27 did not reduce iNos below basal levels, but rather slightly increased it (Figure
In the experiment shown in Figure 4.12, statistical analysis comparing curcumin’s effect on the RAW264.7 cells to the other compounds shows no statistically (N.S.) significant change between curcumin and compound 1, 3 and 13, and statistically significant increase (p<0.001) for compounds 26 and 27.

Figure 4.11: Relative expression of inducible nitric oxide (iNos) following treatment of M1-activated macrophages with anti-inflammatory compounds. M1 phenotype was produced via IFN-γ/LPS-mediated polarization in indicated samples. N.T = non-treated; cur. = Curcumin. Error bars represent standard error. Student T-test was performed to compare each treatment to the M1-polarized, non-compound-treated control (N.T.; p < 0.001 = ***). Inset shows same assay results but without the IFN-γ/LPS N.T. sample; N.I. = not included.

Figure 4.12: Relative expression of inducible nitric oxide (iNos) following treatment of naïve RAW264.7 cells via indicated conditions as determined by RT-PCR. N.T. = non-treated; IFN/LPS = interferon gamma (50 ng/mL) + lipopolysaccharide (50 ng/mL) (yielding pro-inflammatory cells); cur. = Curcumin. Error bars represent standard error. Student T-test was performed to compare each treatment to non-treated control (N.T.; p > 0.05 = not significant (N.S.), p < 0.05 = *, p < 0.01 = **, and p < 0.001 = ***).
To support these results and correlate \(i\text{Nos}\) mRNA transcript levels with NO production, Griess assays were performed to determine the amounts of NO found produced following treatments with curcumin and derivatives versus non-treated (M1-polarized and naïve) controls. Treatment with the each of the compounds resulted in dramatically decreased levels of NO relative to the pro-inflammatory control group (N.T. treated with IFN/LPS; **Figure 4.13**). Statistical analysis indicates that there is no statistical difference comparing curcumin with compound 1, 13, 26, or 27, and a statistically significant decrease when treated with compound 3 (\(p<0.05\)). Altogether, these results demonstrate the ability of the previously described curcumin derivatives to reduce NO secretion in pro-inflammatory, M1 macrophages. It also suggests that compound 3 reduces NO secretion when compared to other compounds, including curcumin. Assessment of NO levels following compound treatment of naïve macrophages showed no substantial changes (**Figure 4.14**). Compound 13 slightly reduced NO levels beyond baseline, while curcumin and molecules 1, 3, 26, and 27 did not yield any significant effects.
Figure 4.14: Griess assay results following treatment of naïve RAW264.7 cells with indicated compounds. N.T. = non-treated; Cur. = curcumin. Student T-test was performed to compare each treatment to non-treated control (N.T.; \( p > 0.05 \) = not significant (N.S.), \( p < 0.05 \) = *).

### 4.4 Conclusion

There is a need for more drug-like molecules that can resolve inflammation and ameliorate associated symptoms in the contexts of chronic inflammation and autoimmune diseases. The use of curcumin has attracted the attention of the immunology field due to its anti-inflammatory properties. While curcumin has shown promising results and has anti-inflammatory properties, its poor biodistribution and solubility have limited its use. Therefore, in this study, we generated curcumin-derivatives aiming to overcome these issues and while maintaining the induction of anti-inflammatory responses in immune cells.

In summary, twenty-one DANMPs (1-21), four DHANMPs (22-25) and five SBPs (26-30) were synthesized and characterized. Using both *in silico* and cell-free experiments, we determined the drug-like properties of the molecules and quantified their radical scavenging activities, respectively. Our results displayed improved Lipinksi and ADME parameters (*Tables 4.2 and 4.3*) and solubilities (*Table 4.5*) compared with those of
curcumin itself. DPPH radical scavenging assay data indicated that all generated compounds largely retained this capability (Figure 4.9).

Using the model macrophage cell line RAW264.7, we assessed the biological effects of five selected compounds (1, 3, 13, 26, and 27). None of these molecules affected cell viability and largely did not influence the presence of basal levels of the inflammation-associated marker \(i\text{Nos}\) or reactive NO in naïve cells (Figures 4.10, 4.12, and 4.14). However, in macrophages activated to pro-inflammatory/M1 phenotypes with LPS and IFN-\(\gamma\), the assessed compounds markedly decreased the expression of \(i\text{Nos}\) and levels of NO with activities largely similar to those of curcumin (Figures 4.11 and 4.13). Altogether, these data demonstrate the potential of curcumin-derived small molecules to reduce inflammation and be used for the treatment of inflammation-associated diseases. Having shown promise here, these molecules should be evaluated in terms of target validation and specificity, and can be tested in other inflammatory models, including disease-specific ones. Structure-activity relationship studies will also be pursued to assist in further optimization to improve upon the compounds’ anti-inflammatory properties and other characteristics.

4.5 References


26. Kanski J, Aksenova M, Stoyanova A, Butterfield DA. Ferulic acid antioxidant protection against hydroxyl and peroxyl radical oxidation in synaptosomal and


CHAPTER 5

MACROPHAGE ACTIVATION BY A SUBSTITUTED PYRIMIDO[5,4- 
B]INDOLE INCREASES ANTI-CANCER ACTIVITY


5.1 Introduction

Cancer is one of the leading causes of death around the world, and fighting it is a focus of research programs globally. However, most cancer therapy regimens continue to rely on non-specific chemotherapy and radiotherapy to eliminate tumors, which have severe side effects. They are also ineffective against many cancer types and can increase disease recurrence. As a result, alternative strategies are sought after, such as immunotherapy. Cancer immunotherapy involves utilizing the immune system to eliminate the disease and is attractive on account of the immune system’s specificity and biocompatibility compared to traditional cancer therapy. Several immunotherapies have been approved clinically, and others have reached the clinical trial stage.

While immunotherapy is alluring, developing immune-based strategies is challenging. Normally, the immune system detects and eliminates pre-oncogenic cells, however, cancer cells can generate cytokines and receptors for immune evasion and reprogramming. In this manner, tumorigenic cells are able to escape detection and disable pro-inflammatory behaviors. An example of the former is overexpression of “don’t eat me” surface marker CD47 by cancer cells, preventing phagocytosis, while in the latter the
tumor releases chemo-attractants and anti-inflammatory signals, such as IL-4, IL-10, CS1, CSF1R, and MFG-E8, to reprogram immune cells to perform protumorigenic roles. These include facilitation of angiogenesis, epithelial to mesenchymal transition (EMT), and microenvironment remodeling. Once this point is reached, the likelihood of patient survival decreases sharply.

Because of the immune system’s role in cancer progression, there is great interest in the re-education of immune elements into anti-cancer entities. Polarization of macrophages into M1 (classically activated) phenotypes is important toward refocusing the immune system for eliminating cancer. In this immune-stimulating phenotype, macrophages attack and phagocytose tumor cells. This facilitates a larger overall attack by the immune system, resulting in tumor elimination. Macrophages also generate reactive oxygen species (ROS) and present tumor antigens, which recruit T cells and B cells to the tumor site. In contrast, immune-suppressing M2 (alternatively activated) phenotypes, or tumor-associated macrophages (TAMs), contribute to tumor progression, joining the tumor mass and microenvironment. TAMs release pro-tumor growth factors, such as vascular endothelial growth factor (VEGF), promote vascularization, remodel the microenvironment, and silence the immune response. Overall, reprogramming macrophages into M1-like states and away from M2/TAM phenotypes has great potential as an anti-cancer immunotherapeutic approach.

The polarization of macrophages toward M1 phenotypes is a well-studied phenomenon, with known pathways identified. Specifically, the interferon gamma (IFN-γ) receptor (IFNGR) and the toll-like receptor (TLR) class are understood to activate
macrophages into pro-inflammatory roles. The most common strategies for \textit{in vitro} M1 macrophage polarization involve treatment with IFN-\(\gamma\) (ligand for IFNGR) and lipopolysaccharide (LPS, a TLR-4 agonist originating from bacterial cell walls). While these agonists are useful as research tools, both IFN-\(\gamma\) and LPS have drawbacks that make them non-viable therapeutically. IFN-\(\gamma\) is a small protein, difficult to consistently modify and incorporate into delivery vehicles, while LPS is a bacterial cell wall component consisting of a mixture of structures and may be contaminated with other bacterial components, resulting in off-target immune effects. Systemic administration of these agents results in immune overstimulation, leading to negative outcomes including septic shock, cytokine storms, and death.

Because of these issues, there is a need to identify alternative macrophage activators. Additionally, therapeutic candidates should be amenable to chemical modifications and association with targeted delivery vehicles. To date, there are few immune-modulating compounds approved clinically. One example is the TLR7 activator imiquimod, which is approved for the topical treatment of genital warts and basal cell carcinoma. However, in terms of general anti-cancer agents, systemically delivered drugs are desirable to provide access to a range of tumor locations and facilitate immune cell recruitment. There have been examples of anti-inflammatory antibody blockades utilized to reprogram macrophages for cancer therapy, but in terms of modifications, antibodies are more difficult to alter than small organic molecules and are also linked to uncontrollable immune-based toxicity.
Here, we utilize a small molecule TLR-4 activator to induce the M1 phenotype and enhance anti-oncogenic properties in macrophages (Figure 5.1). This molecule, a pyrimido[5,4-b]indole referred to as PBI1, was previously identified among a series of compounds that activate TLR-4.\textsuperscript{22} This molecule was shown to bind to TLR-4 and induce expression of various pro-inflammatory cytokines in dendritic cells.\textsuperscript{22} Preclinical studies have shown that structurally similar molecules are effective immune adjuvants for influenza therapy,\textsuperscript{23} however, these compounds have not been evaluated in terms of macrophage activation and anti-cancer activity. Our hypothesis is that this small molecule TLR-4 agonist can activate and polarize macrophages into an anti-cancer phenotype nearly as well as naturally occurring cytokines or existing adjuvants. We demonstrate that PBI1 upregulates pro-inflammatory genes in macrophages and induces M1-associated phenotypic changes and cytokine production. Macrophages treated with PBI1 demonstrate enhanced anti-tumor activity toward B-cell lymphoma cells as determined by phagocytosis assays. We also show that treatment of M2-like macrophages with PBI1 results in their re-education of macrophages toward an M1-like phenotype.

![Figure 5.1. Illustration of pyrimido[5,4-b]indole (PBI1)-mediated macrophage activation. Following TLR-4 stimulation, resulting M1 macrophages have enhanced levels of Tnf-\(\alpha\) and iNos, increased RNS generation, and phagocytosis efficiency.](image-url)
5.2 Materials and Methods

All reagents were purchased from Thermo-Fisher Scientific except where otherwise noted. All DMSO utilized was cell culture grade (Sigma). Confocal microscopy images were obtained on an Eclipse Ti-E microscope (Nikon) using a 63X or 20X objective at room temperature. Images were acquired and processed using NIS-Elements and ImageJ. Flow cytometry analysis was performed on a BD LSRFortessa 5 L flow cytometer equipped with FACSDiva (BD Sciences) at the Flow Cytometry Core Facility at the University of Massachusetts Amherst. RT-PCR data was generated using a CFX Connect Real-Time PCR Detection System (Biorad). For assays requiring absorbance measurements, a SpectraMax M2 plate reader was used (Molecular Devices).

5.2.1 Cell Culture

RAW264.7 cells were purchased from American Type Culture Collection (ATCC). Primary immortalized macrophages were a gift from Prof. Susan Carpenter at the University of California, Santa Cruz. Both types of cells were cultured at 37 °C under a humidified atmosphere containing 5% CO₂. Standard growth media consisted of high glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (100 μg/ml penicillin and 100 μg/ml streptomycin). Daudi cells were a gift from Prof. Vincent Rotello at the University of Massachusetts Amherst and were cultured in Roswell Park Memorial Institute media (RPMI 1640) supplemented with 10% fetal bovine serum and 1% antibiotics (100 μg/ml penicillin and 100 μg/ml streptomycin). Under the above culture conditions, the cells were sub-cultured approximately once every four days and only cells between passages 7 and 15 were used for all experiments.
5.2.2 Synthesis

The synthesis of PBI1 was largely performed as previously described.\textsuperscript{22}

5.2.3 RT-PCR Preparation

RAW264.7 cells were plated in 24 well plates at a density of 100,000 cells/well. Media was aspirated and PBI1-treated cells were dosed with 20 μg/mL compound in fresh media for 24 h. M1 treatment group cells were treated with 50 ng/mL IFN-γ for 24 h after which LPS was added to a final concentration of 50 ng/mL for an additional 24 h. M2 group cells were treated with 50 ng/mL IL-4 for 48 h. For the M2+PBI1 group, the cells were treated with 50 ng/mL IL-4 for 24 h. 24 h after IL-4 dosing, the media was removed and replaced with fresh media containing 20 μg/mL PBI1 for 24 h. Each experiment included three biological replicates per treatment condition. 20 μg/mL of PBI1 was used for this assay and several others as PBI1 demonstrated no toxicity at this concentration. Following treatments, RNA was extracted following the procedure below.

5.2.4 RNA Extraction and cDNA Conversion

Approximately 1.5 μg RNA was directly harvested from cells using the PureLink RNA Mini Kit (Ambion) following the manufacturer's instructions. SuperScript IV Reverse Transcriptase, RNaseOut, 10mM dNTPs, and 50 μM Random Hexamers were used for the conversion of approximately 150 ng of RNA to cDNA (ThermoFisher), also following the manufacturer's instructions with a sample volume of 20 μL. Briefly, primers were annealed to RNA at 65 °C for 5 min. Then the annealed RNA was combined with the reaction mixture (containing 500 units of reverse transcriptase per sample) and amplified at 53 °C for 10 min and melted at 80 °C for 10 min. The cDNA was frozen at -20 °C and then used
5.2.5 Quantitative RT-PCR

RT-PCR was performed on cDNA as prepared above using a CFX Connect real-time system (Biorad) with iTaq Universal SYBR Green Supermix (Biorad). All DNA primers were purchased from Integrated DNA Technologies. The following primer sequences (as determined by NCBI primer-BLAST) were used: β-actin (ACTB, accession number: NM_007393, amplicon length: 86 pairs, exon 6) (forward) GATCAGCAAGCAGGAGTACGA, (reverse) AAAACGCAGCGCAGTAACAGT; iNOS (NOS2, accession number: NM_010927.4, amplicon length: 127 pairs, exon 2,3, splice variants: 1–3) (forward) GTTCTCAGCCCCAACAATACAAGA, (reverse) GTGGACGGGTGATGTCAC; TNF-α (TNF, accession number: NM_013693.3, amplicon length: 145 pairs, exon 3,4) (forward) CCTGTAGCCACTACGTAG, (reverse) GGGAGTCAAGGTACAACCC. 200 nM of each primer was mixed with 1 μl (100 ng) of cDNA, 10 μL SYBR green supermix and H2O to a final volume of 20 μL. Analyses were performed as follows: the samples were first activated at 50 °C for 2 min, then 95 °C for 2 min. Then denaturing occurred at 95 °C for 30 s followed by annealing at 57 °C; the denature/anneal process was repeated over 40 cycles. Relative gene expression was determined by comparing the Ct value of the gene of interest to that of the β-actin housekeeping gene, by the 2ΔΔCt method. Three biological replicates were performed for each treatment condition and three technical replicates were used for each biological
replicate. There was no amplification for the no-template control (NTC). Data was analyzed using the CFX Manager 3.1 software. CQ values were generated by using the point at which the sample fluorescence value exceeded the software’s default threshold value. Each sample was normalized to the untreated control. β-actin was used as a reference gene since it is commonly used and none of the treatments were expected to affect its expression.

5.2.6 Phagocytosis Assay with Flow Cytometry

RAW264.7 and Daudi cells were separately plated in 24 well plates at a density of $2 \times 10^5$ cells/well each. The same day, designated RAW264.7 wells were treated with PBI1 to reach a final concentration of 5 μg/mL in 0.5% DMSO. A slightly lower concentration was utilized in this experiment to demonstrate that profound changes in cell phenotype are possible at low concentrations of PBI1. 24 h following treatment, RAW264.7 cells were trypsinized using TrypLE (ThermoFisher), washed twice in PBS, and labelled with 1 μg/mL PE-F4/80 antibody (BD Biosciences, cat. No: 565410, Clone:T45-2342) for 30 min at 4 °C. All RAW cells were resuspended in culture media and 10 μM cytochalasin D was added to the designated samples. Simultaneously, Daudi cells were removed from the 24 well plate, washed twice with LCIS (ThermoFisher) and labelled with pHRhodo Green according to the manufacturer’s instructions (ThermoFisher). These cells were washed with PBS and then labelled with 10 μg/mL anti-CD47 antibody (BioXcell, cat. No: BE0019-1, Clone: B6.H12) for 30 min at 4 °C. All of the Daudi cells (approximately $4 \times 10^5$) were resuspended in culture media and combined with the RAW264.7 cells for 2 h at 37 °C. The approximate ratio of RAW264.7:Daudi was 1:1. The samples were then washed with PBS, resuspended in FACS buffer (1% FBS in PBS) and transferred to flow
cytometry tubes. The samples were analyzed on an LSRFortessa 5L flow cytometer (BD Biosciences) using 488 nm and 561 nm lasers, counting 30,000 events, at the University of Massachusetts Amherst Flow Cytometry Facility. Three samples per treatment group were evaluated. Phagocytic index was calculated using the following equation:

\[
\frac{[F4/80+pHRhodoGreen^{+} \text{ events}]}{[F4/80+pHRhodoGreen^{-} \text{ events}]}, \text{ normalized to the untreated control group.}
\]

### 5.2.7 Griess Assay

RAW264.7 cells were plated in 24 well plates at a density of $1.5 \times 10^5$ cells/well 24 h prior to the experiment. The following day, the culture media was removed and replaced with serum free Opti-Mem media for 2 h. Cells designated for TLR-4 inhibition were pretreated with 7.2 μg/mL TAK242 (Cayman Chemical) 1 h before additional treatment. Then, media was replaced again with 250 μL phenol red-free DMEM culture media containing either LPS or PBI1 at the indicated concentrations, and cells were incubated for an additional 48 h. Cell supernatant was collected from wells and centrifuged at 5000 rpm for 5 min. Then, Griess reagent (ThermoFisher) was prepared according to the manufacturer’s instructions and 60 μL Griess reagent was combined with 60 μL cell supernatant in a clear 96 well plate. After 15 min in the dark, absorbance was read using a SpectraMax M2 plate reader (Molecular Devices) at 548 nm. Three biological replicates per treatment condition were used. Actual NO$_2^-$ concentrations were determined by comparing absorbance values to a standard curve generated using pure NaNO$_2^-$ solutions.
5.2.8 Confocal Microscopy

For acquisition of representative cell morphology images, RAW264.7 cells were plated in a 4 chamber Lab-Tek II chambered coverglass system (Nunc) at a density of $5 \times 10^4$/well and allowed to adhere overnight. Media was aspirated and cells were then polarized with 20 μg/mL PBI1 in 0.2% DMSO in fresh media for 48 h. After polarization, cells were imaged using an Eclipse Ti-E microscope at 20x magnification. M1 cell images were acquired similarly, using polarization conditions as outlined in the RT-PCR preparation section above. For acquisition of representative phagocytosis images, $5 \times 10^4$ RAW264.7 cells were plated in a 4-chamber Lab-Tek II chambered coverglass system. Designated wells were directly treated with PBI1 to a final concentration of 4.5 μg/mL in 0.4% DMSO for 24 h. The RAW cells were labeled with Cell Tracker Blue according to the manufacturer’s instructions. $1 \times 10^5$ Daudi cells were counted, labeled with phRhodo Green and added to the wells containing RAW cells. After 2 h incubation, wells were imaged using an Eclipse Ti-E microscope at 63x magnification. The final ratio of RAW to Daudi cells was approximately 2:1.

5.2.9 ELISA

RAW264.7 cells were plated in 24 well plates at a density of 60,000 cells/well and allowed to adhere overnight. Media was removed and PBI1-treated cells were dosed with 5, 10, and 20 μg/mL compound in 0.4% DMSO in fresh media for 48 h. M1 treatment group cells were treated with 50 ng/mL IFN-γ for 24 h after which LPS was added to a final concentration of 50 ng/mL for an additional 24 h. M2 group cells were treated with 50 ng/mL IL-4 for 24 h. For the M2+PBI1 group, the cells were treated with 50 ng/mL IL-4 for 24 h. 24 h after IL-4 dosing, the cells were treated with 20 μg/mL PBI1 for 24 h. Each
experiment included four biological replicates per treatment condition. After treatment, supernatant was collected from the wells and then analyzed using a Mouse IL-6 ELISA kit and a Mouse TNF-α ELISA kit (BD Biosciences) according to the manufacturer’s instructions.

Figure 5.2. Effects of PBI1 treatment on cell morphology and M1 marker expression. a) Confocal microscopy images (20x) depicting changes in cell morphology following treatment with 20 μg/ml PBI1. PBI1-treated macrophages (center) resembled those polarized to an M1 phenotype (right), with a “flatter” appearance and more pseudopodia, in comparison with non-treated cells (left). b) RT-PCR analysis of TNF-α (left) and iNOS (right) expression in PBI1 treated cells revealed significantly greater expression of both relative to non-treated controls. M2+PBI1 refers to cells polarized to the M2 phenotype and subsequently treated with PBI1. c) ELISA analysis of IL-6 secretion following treatment. Unpaired, two-tailed student T-tests with equal variance were used to determine significance; *P≤0.05, **P≤0.005, ***P≤0.0005, ****P≤0.00005. n.s. = non-significant.
5.3 Results and Discussion

5.3.1 Confocal Microscopy

RAW264.7 murine macrophages were dosed with the compound, and changes in morphology and gene expression were assessed using confocal microscopy and RT-PCR, respectively (Figure 5.2). Confocal microscopy revealed significant changes in macrophage morphology after 24 h. Treated macrophages acquired a phenotype associated with M1-polarized macrophages (Fig. 5.2a): the cells became flatter and produced longer pseudopodia\textsuperscript{16} compared to non-treated cells.

5.3.2 RT-PCR Analysis

RT-PCR analysis of the mRNA expression of two M1-related markers, tumor necrosis factor-\(\alpha\) (\textit{Tnf-\(\alpha\)}) and inducible nitric oxide synthase (\textit{iNos})\textsuperscript{11}, revealed that PBI1-treated macrophages express significantly higher levels of both genes compared to non-treated cells, in a similar trend as IFN-\(\gamma\)/LPS treatment (Figure 5.2b). This effect was also observed in an immortalized primary macrophage cell line (Figure 5.3). Furthermore, PBI1 treatment was also able to ‘re-educate’ macrophages that had been polarized toward the anti-inflammatory M2 phenotype, resulting in enhanced levels of \textit{iNos} and \textit{Tnf-\(\alpha\)}. Alamar blue assays indicated that no significant toxicity occurred as a result of compound treatment (Figure 5.4).
Figure 5.3: RT-PCR analysis of M1 related genes in primary immortalized macrophages (PIMs) following PBI1 treatment. PIMs treated with PBI1 display increased levels of \( Tnf\alpha \) and \( iNos \) expression as analyzed by RT-PCR. Three biological replicates with three technical replicates each were used in analyzing each treatment condition. Error bars represent standard deviation.

Figure 5.4: Cell viability percentage after treatment with PBI1. Toxicity of PBI1 as evaluated by Alamar Blue Assay RAW264.7 macrophages were treated with increasing concentrations of PBI1; subsequent cellular viability was assessed using Alamar blue reagent. Minimal differences in viability were observed at the utilized concentrations. Three biological replicates were included per treatment condition; error bars represent standard deviation. n.s. = non-significant. \( *P \leq 0.05 \).
5.3.3 ELISA Analysis

ELISA analysis of the protein expression of the M1-related cytokines, interleukin 6 (IL-6) and TNF-α, revealed that cells secreted both cytokines to greater extents when treated with PBI1 or the combination of IFN-γ/LPS compared to untreated cells (Figures 5.2c and 5.5). PBI1 treatment following M2 polarization also resulted in secretion of both cytokines.

![Figure 5.5: ELISA analysis of TNF-α. ELISA analysis of TNF-α secretion following treatment RAW264.7 macrophages were treated with increasing amounts of PBI; subsequent cytokine secretion was assessed via ELISA. Four biological replicates were included per treatment condition; error bars represent standard deviation. Unpaired, two-tailed student T-tests were used to determine significance; *P ≤ 0.05, **P ≤ 0.005, ***P ≤ 0.0005, ****P ≤ 0.00005.](image)

5.3.4 Griess Assay

Generation and release of reactive oxygen and nitrogen species (ROS and RNS, respectively) are important processes of M1 macrophage anti-tumor and pathogen responses. RNS are generally derived from nitric oxide (NO). To evaluate relative NO production by PBI1-treated cells, a Griess assay was performed. This method indirectly measures NO via evaluation of nitrite (NO$_2^-$), one of its two primary stable and non-volatile
breakdown products. The determined NO$_2^-$ concentrations show that PBI1 effectively induced production of NO$_2^-$, achieving significantly higher levels than non-treated samples, and within 2-fold of LPS-treated cells (Figure 5.6). Additionally, pretreatment with the TLR-4 specific inhibitor TAK242 completely eliminated the induction of NO by PBI1 (Figure 5.6).27

Figure 5.6: Supernatant NO$_2^-$ levels are increased following LPS or PBI1 treatment as measured by Griess assay. Pretreatment with TAK242 inhibited TLR-4 activity prior to PBI1 addition (PBI1+TAK242). Unpaired, two-tailed student T-tests with equal variance were used to determine significance; **P≤0.005, ***P≤0.0005, ****P≤0.00005. n.s. = non- significant.

5.3.5 Phagocytosis

A key aspect of the macrophage antitumor response is the identification and phagocytosis, or engulfment, of cancerous cells.6 To assess the phagocytic efficacy of PBI1 treated
macrophage cells, a fluorescent flow cytometry assay was conducted using Daudi (B cell lymphoma) as the target cancer cell line (Figures 5.7 and 5.8). RAW264.7 cells treated with PBI1 had a nearly 5-fold greater average phagocytic efficiency versus non-treated macrophages. This was increased further when macrophage PBI1 treatment was combined with antibody-mediated blocking of Daudi cell CD47, the “don’t eat me” signal involved in phagocytosis inhibition signaling. Cytochalasin D, a potent phagocytosis inhibitor, was used as a negative control.²⁸

![Figure 5.7: Effects of PBI1 on phagocytosis of Daudi (B-cell lymphoma) cells. a) Representative confocal microscopy images illustrating RAW264.7 macrophage (CellTracker Blue) phagocytosis of Daudi cells (phRodo Green). b) Phagocytosis indices generated from flow cytometry data of RAW264.7 macrophages phagocytosing Daudi cells following various treatments.](image-url)
In summary, PBI1 was demonstrated to polarize macrophages toward an anti-cancer phenotype. RAW264.7 macrophages treated with PBI1 adopted an “M1-like” pro-inflammatory morphology, as cells became flattened and produced extended pseudopodia. RT-PCR and ELISA results also corroborated this phenotype via an increase in the expression of M1 inflammatory genes. PBI1 treatment increased the expression of TNF-α, IL-6 and iNOS approximately 2.5, 200, and 500-fold, respectively. Dosing of PBI1 also resulted in the re-education of M2-polarized macrophages, whereupon following treatment, cells expressed higher levels of inflammatory cytokines. Notably, PBI1 treatment after M2 polarization increased TNF-α, IL-6, and iNOS to levels nearly identical to those of M1 polarized macrophages. This is particularly relevant for the conversion of tumor-associated macrophages into tumor-killing macrophages within cancer microenvironments.
We also evaluated the mechanism by which PBI1 promoted macrophage activation. The most common pathways for macrophage stimulation include the TLR family of receptors that recognize a wide variety of substrates. To confirm that TLR-4 is the target of PBI1 for macrophage activation, a competition experiment was performed. Cells were treated with a highly specific TLR-4 inhibitor, TAK242, and activation by PBI1 was determined by Griess assay. This experiment revealed that macrophages treated with either PBI1 or LPS released significant levels of nitric oxide, another confirmation of the activation potential of PBI1. However, with pre-treatment of cells with TAK242, PBI1 did not induce any detectable level of nitric oxide, confirming that TLR-4 is the target of PBI1 and is crucial to resulting macrophage activation.

Having evaluated the pro-inflammatory responses of macrophages to PBI1 treatment, the ability of these cells to subsequently phagocytose cancer cells was investigated. It has been previously shown that activation of local macrophages into an M1 inflammatory phenotype can result in significant anti-tumor macrophage activity, which is of great interest for the generation of cancer immunotherapies. One of the major anti-cancer macrophage mechanisms involves phagocytosis of cancer cells. Inflammatory macrophages can invade tumor tissue, engulf resident tumor cells, and release immune signaling factors that drive further immune responses against the tumor site. A phagocytosis assay revealed that PBI1-treated macrophages engulfed targeted cancer cells with a 5-fold higher efficacy than untreated macrophages, revealing that inflammatory activation by PBI1 does in fact increase anti-tumor activity. It was also demonstrated that pre-treatment of the targeted cancer cells with a CD47 blocking antibody, which blocks the SIRPα-CD47 phagocytosis inhibitory pathway, further increases the efficacy of PBI1-
induced phagocytosis. This effect occurred independently of PBI1-activation as well and could be used to increase the anti-cancer efficacy of PBI1 treatment.

5.4 Conclusion

The immune system and cancer progression have a complex relationship. While effective immune-based strategies are of interest in enervating and eliminating cancer progression, their development can be challenging due to varying macrophage-activating signals.29,30 PBI1 has significant macrophage activation capability. As a potential immune adjuvant for cancer therapy, PBI1 is relatively non-toxic and effectively increases both phagocytic and oxidative burst mechanisms of the macrophage anti-tumor response. While PBI1 has shown promise as a therapeutic on its own, it will likely show the greatest efficacy if used in conjugation with drug delivery vehicles or targeting elements. This is especially relevant in trying to avoid broad immune-activation and runaway immune responses. As a small molecule, it should be fairly straight-forward to attach to a variety of carriers, including nanoparticles, proteins, and cells; further studies will explore these options. Incorporation of PBI1 with more complex therapeutics, as opposed to its use independently, could result in robust combinatorial anti-cancer strategies. Additional studies will evaluate the anti-cancer efficacy of PBI1 in vivo, including in concert with delivery vehicles, and as an adjuvant with other chemotherapeutics. In the future, we will also seek to identify other potential therapeutic targets of PBI1-activated macrophages.

5.5 References


CHAPTER 6

INVESTIGATION OF MACROPHAGE SUBTYPES IN THE CONTEXT OF BREAST CANCER VIA MACROPHAGE-BASED REPORTERS

6.1 Introduction

Macrophages are cells of the innate immune system that play important roles in fighting infections and supporting tissue development, maintenance, and remodeling. They reside in tissues, body cavities, and mucosal surfaces (including but not limited to the lungs, spleen, skin, heart, kidney, and peritoneum), and contribute to both homeostasis and disease. These cells are “plastic,” which refers to their capacity to alter their phenotypes in a process known as macrophage polarization (Figure 6.1). This process is dictated by surrounding pathogens or cytokines that influence macrophage phenotypes and responses.

Figure 6.1: Macrophage polarization. From an undifferentiated, naïve (M0) state, macrophage cells assume a pro-inflammatory (M1) phenotype (lower left, orange) when activated with pro-inflammatory cytokines (e.g., lipopolysacharide (LPS), interferon gamma (IFN-γ), and/or tumor necrosis factor alpha (TNF-α)). When stimulated with anti-inflammatory cytokines such as interleukin 4 (IL-4), interleukin 10, (IL-10), or interleukin 13 (IL-13), macrophages are converted into an anti-inflammatory (M2) subtype (lower right, blue). M2 macrophages have multiple sub-classifications (M2a, M2b, M2c, and M2d/TAMs), which result from different stimuli and have varying characteristics. Once polarized, macrophages can be “re-educated” to a different phenotype, e.g., M2 to M1.

Macrophages can respond to both innate and foreign pro-inflammatory signals, including cytokines, such as interferon gamma (IFN-γ) and tumor necrosis factor alpha
(TNF-α), or lipopolysaccharides (LPS), respectively. These result in what are referred as immune-stimulating, or classically activated macrophage phenotypes, commonly referred to as M1. This subtype of macrophages has pathogen-killing abilities and has been shown to eliminate tumor cells via enhanced phagocytosis and generation of reactive oxygen and nitrogen species (ROS and RNS, respectively). Previous studies in murine primary macrophages showed that M1 macrophages are characterized by enhanced expression of toll-like receptor 2 (Tlr-2), intracellular adhesion molecule 1 (Icam1), Tnf-α, and inducible nitric oxide (iNos), and have decreased expression of mannose receptor (MR/Cd206), early growth response protein 2 (Egr2/Krox20), cluster of differentiation 36 (Cd36) and neuropilin 1 (Nrp1).

At the other end of the spectrum, macrophages can assume roles associated with immune suppression and wound-healing responses. When macrophages are stimulated with interleukin 4 (IL-4), interleukin 10 (IL-10), interleukin 13 (IL-13), or other anti-inflammatory cytokines (described further below), they adopt an immune-suppressive M2 subtype. Compared to the M1 phenotype, these have been shown to exhibit opposite patterns of expression of the aforementioned polarization markers. Due to their complexity, M2 macrophages may be further classified into M2a, M2b, M2c, and M2d/tumor-associated macrophage (TAM) categories. Each results from the presence of specific cytokines, and while some common characteristics are shared, others are unique.

Macrophages can be re-educated from one phenotype to another when the conditions in their surrounding environments change. In the case of cancer, tumor cells possess the ability to secrete anti-inflammatory cytokines, including IL-4, IL-10, IL-13, and others, that can convert undifferentiated (M0) or M1 macrophages into the
M2d/TAM phenotype. TAMs have been shown to aid in multiple aspects of cancer, including tumor growth, angiogenesis, remodeling of the tumor microenvironment, invasion, and establishment and maintenance of metastases. Macrophages have been shown to be involved in multiple types of cancer, including breast, lung, gastric, colorectal, and pancreatic cancers, with these cells sometimes contributing up to 50% of the tumor mass. Studies have also shown that high infiltration of TAMs in tumor tissues are correlated with poor patient prognoses. Due to their implications in cancer and potential to act against it, macrophage re-programming is of interest in immunotherapeutic treatments. Therefore, it is important to gain a better understanding of the interactions between cancers and macrophages, and their outcomes.

To further elucidate their roles in the context of cancer, various techniques have been used for tracking macrophage behavior in *in vitro* and *in vivo* cancer models. Real Time Polymerase Chain Reaction (RT-PCR), for instance, is a technique used to identify macrophage phenotypes *in vitro* based on the expression of phenotype-associated markers. However, this technique is labor intensive and expensive, it requires use or isolation of a single cell type, typically provides average mRNA expression levels, and is difficult to obtain multiple time-points with. Enzyme-linked immunosorbent assays (ELISAs) are similarly time-consuming and expensive, and require single cell types to be able to assess which cells produce given markers, resulting in population-level data. Immuno-staining and flow cytometry are both able to provide data for individual cells, but like RT-PCR, are limited to evaluations at single time points, and macrophage-specific markers must be used to differentiate them from other cell types. In terms of *in vivo* approaches, some of the most commonly used methods for tracking macrophages in animal models are
bioluminescence imaging (BLI) and intravital microscopy, which require fluorescent probes for macrophage labeling (e.g., intrinsic fluorophores (NADH, FAD, and collagen), genetic probes (fluorescent proteins), and commercial chemical probes (fluorescent dye-conjugates)). These techniques facilitate the tracking of macrophages in vivo, but fail to show phenotypic changes in the cells. Therefore, there is a need for methods that allow the tracking of macrophages in multi-cellular environments and the visualization of changes in their phenotype in real time.

Various platforms have been used to study the effects of cancers on macrophages. While some are more physiologically relevant than others, the mode of assessing phenotypic markers is often the dictating factor. The most commonly employed models include exposing macrophages to cancer cell-derived conditioned media, co-culturing them with cancer cells in a monolayer, or more complex experimental designs, such as tumor spheroids or other in vitro 3D models. Conditioned media refers to a collection of secreted signaling proteins (secretome) from cells of interest, and is commonly used to study the effects of cancer on macrophages and other immune cells. While it is compatible with most of the techniques described above (since only a single population of cells is present), it excludes cell-to-cell interactions, which play key roles in diseases like cancer.

Macrophages and other cell types are able to associate and influence one another in both two- and three-dimensional, or 2D and 3D, co-cultures. In the 2D model, cells grow in a monolayer, typically attached to a plastic surface. This method is useful for studying cell-to-cell interactions, simple to maintain, and amenable to functional tests (e.g., phagocytosis of cancer cells by co-cultured immune cells). However, 2D cultures
have limitations that include alterations in cell morphology, polarity, and method of division.\textsuperscript{37,38} 3D cultures, where cells grow in three dimensions,\textsuperscript{40} better represent cell-to-cell and cell-to-extracellular environment interactions, morphology and cell division, and access to oxygen, nutrients, metabolites, and signaling molecules or cytokines.\textsuperscript{37,41,42} The use of 3D co-cultures is also highly relevant, since the characteristics of cells and their responses, including to drugs, can be differ based on whether they are cultured in two versus three dimensions.\textsuperscript{42} In both cases, however, while the cellular environments are more realistic, the means of assessing macrophage polarization becomes more difficult. For this reason, we generated a reporter cell line to track the expression of a phenotype-associated marker in relevant disease models over time and following drug treatment.

In this work, we generated a fluorescent macrophage phenotype reporter cell line (RAW:\textit{iNos}-eGFP) based on polarization-associated marker iNos. We confirmed the fidelity of the RAW:\textit{iNos}-eGFP cells, including following exposure to polarizing cytokines, and established that reporter and parental cells behaved similarly. The reporter cells were then used to monitor macrophage responses in different \textit{in vitro} breast cancer models, and their re-education from anti- to pro-inflammatory phenotypes via a previously reported Tlr4-agonist, PBI1.\textsuperscript{43} Effects of 4T1 and EMT6 cell lines on macrophages were assessed via conditioned media, two-dimensional/monolayer co-culture, and three-dimensional spheroid models. While conditioned media derived from 4T1 or EMT6 cells and monolayer co-cultures of each with RAW:\textit{iNos}-eGFP cells resulted in decreased fluorescence, the trends and extents of effects differed. We also observed a decrease in \textit{iNos}-eGFP signal in the macrophages in 3D culture assays with 4T1- or EMT6-based spheroids. We then show that we are able to induce an increase in \textit{iNos} production, even
in the presence of 3D, M2-polarizing cancer models using PBI1. Taken together, we demonstrate that this reporter-based approach provides an easier and more efficient means to study macrophage responses in more relevant and complex, multicomponent environments. Our results show that this platform has the potential of being used with a variety of \textit{in vitro} models and extended to \textit{in vivo} applications.

### 6.2 Materials and Methods

#### 6.2.1 Cell Culture

Murine RAW264.7 macrophages and 4T1 and EMT6 murine mammary carcinoma cells were purchased from American Type Culture Collection (ATCC). Human embryonic kidney (HEK293) cells were obtained from Prof. D. Joseph Jerry (Veterinary and Animal Sciences, UMass Amherst). All cell lines, including RAW: \textit{iNos}-eGFP, were cultured at 37 °C under a humidified atmosphere containing 5% CO$_2$. Standard growth media consisted of high glucose Dulbecco’s Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Corning), 1% L-Glutamine (200 mM, Gibco) and 1% antibiotics (100 $\mu$g/ml penicillin and 100 $\mu$g/ml streptomycin, Gibco) – herein referred to as complete DMEM. Under the above culture conditions the cells were sub-cultured approximately once every 3-4 days and only cells between passages 7 and 20 were used for all experiments.

#### 6.2.2 Macrophage Polarization

To polarize macrophages for sorting, cells were plated in a T75 culture flask and grown at 37 °C under a humidified atmosphere containing 5% CO$_2$ to confluence prior to polarization. Once confluent, culture media was removed and replaced with media
containing either 50 ng/mL interleukin 4 (IL-4) (BioLegend) for 48 hr to generate M2 macrophages or 50 ng/mL interferon-gamma (IFN-γ) (BD Biosciences) and 50 ng/mL of lipopolysaccharide (LPS) (Sigma Aldrich) for 24 h for M1 macrophages. For confocal microscopy and RT-PCR experiments, cells were plated in three biological replicates in an 8-well Lab-Tek II chambered cover-glass system plate (Nunc) or 24-well plates at a density of 100,000 cells/well in 500 µL of media and incubated at 37 °C under a humidified atmosphere containing 5% CO₂. Non-treated macrophages (M0; grown in complete DMEM only) were used as controls. To generate M2 macrophages, 24 h after plating, the media was removed and replaced with complete DMEM media containing 50 ng/mL IL-4 and incubated for an additional 48 h at 37 °C under a humidified atmosphere containing 5% CO₂. To generate M1 macrophages, culture media was removed and replaced with complete DMEM containing 50 ng/mL IFN-γ and 50 ng/mL of LPS 48 h after plating and incubated for an additional 24 h. 72 h after plating (48 h after treatment with M2 cytokines and 24 h after treatment with M1 cytokines), cells were used further in experiments as indicated.

6.2.3 Isolation and Differentiation of Primary Macrophages from Bone Marrow

Isolation and differentiation of primary macrophages was performed following a previously established procedure. Bones were collected from femurs and tibiae from C57/BL6 mice and put into 0.6 mL micro-centrifuge tubes containing a small hole at the bottom (perforated prior to use with an 18G needle). Each 0.6 mL micro-centrifuge tube (containing one femur and one tibia) was then inserted into a 1.5 mL micro-centrifuge tube and centrifuged for 30 seconds at 10,000 rpm. The 0.6 mL tube with marrow-less bones was then discarded and the pelleted bone marrow (now in the 1.5mL-tube) was re-
suspended in 500 µL of media containing 70% complete DMEM and 30% L929-conditioned media (differentiation media). The contents of each 1.5mL tube were then transferred to a T-175 tissue culture flask, mixed with 12 mL of differentiation media, and grown for 3 days. After 3 days, the media was replaced with 12 mL of fresh differentiation media for another 4 days. Following differentiation, media was replaced with complete DMEM and the cells were kept in the T-175 culture flasks at a high cell density at 37 °C under a humidified atmosphere containing 5% CO₂ until needed for experiments (for a maximum of 3 weeks).

6.2.4 RNA Extraction and cDNA Conversion

Cells were lysed, and approximately 1.5 µg RNA was harvested from each well using the PureLink RNA Mini Kit (Ambion) following the manufacturer’s instructions. To convert RNA to complementary DNA (cDNA), 1 µL of 50 µM random hexamers (Applied Biosystems) and 1 µL of 10 mM dNTPs (Thermo Scientific) were added to 11 µL of RNA and heated at 65 °C for 5 min for annealing. Then, 1 µL/sample of 40 U/µL RNaseOut (Invitrogen), 1 µL/sample of 200 U/µL SuperScript IV Reverse Transcriptase (Invitrogen), 1 µL/sample of 100 mM DTT (Invitrogen), and 4 µL/sample of 5x Super Script IV buffer (Invitrogen) were added and amplification proceeded at 53 °C for 10 min and melting at 80 °C for 10 min. The resulting cDNA was frozen at -20 °C and used for RT-PCR experiments within 1 week. RNA and cDNA were quantified using a NanoDrop 2000 (Thermo Fisher). RNA and cDNA contamination and integrity were assessed by analyzing the A260/A280 ratio, where ratios greater 1.8 for DNA and 2.0 for RNA were considered pure.
6.2.5 Quantitative RT-PCR

RT-PCR was performed on the cDNA generated using a CFX Connect real-time system (Biorad) with iTaq Universal SYBR Green Supermix (Biorad). All DNA primers were purchased from Integrated DNA Technologies. The following primer sequences were used: β-actin (forward) 5'-GATCAGCAAGCAGGAGTACGA-3', (reverse) 5’-AAAACGC-AGCGCAGTAACAGT-3'; iNos (forward) 5'-GTTCTCAGCCCAACAAATACAAGA-3’, (reverse) 5’-GTGGACGGGTGATGTCAC-3’.

The reaction mixtures included 200 nM of each primer, 100 ng of cDNA, 10 μL SYBR green supermix, and H₂O to a final volume of 20 μL. Analyses were performed as follows: the samples were first activated at 50 °C for 2 min, then 95 °C for 2 min. Denaturing occurred at 95 °C for 30 s followed by annealing at 58 °C; the denature/anneal process was repeated over 40 cycles. Relative gene expression was determined by comparing the Ct value of the gene of interest to that of the β-actin housekeeping gene, by the 2ΔΔCt method. Three biological replicates were performed for each treatment condition and three technical replicates were used for each biological replicate. Data was analyzed using CFX Manager 3.1 software (Biorad). Cq values were generated by using the point at which the sample fluorescence value exceeded the software’s default threshold value. Each sample was normalized to the non-treated control.

6.2.6 Molecular Cloning of iNos-eGFP Lentiviral Plasmid

To construct the lentiviral iNos-eGFP reporter construct, a plasmid containing the promoter region of Mus musculus iNos was obtained from Addgene (pGL2-NOS2 Promoter-Luciferase – Plasmid # 19296 from Charles Lowenstein). The following primers were designed and used to amplify the promoter for the gene of interest and incorporate XhoI
and BamHI sites at the 5’ and 3’ ends, respectively, underlined): iNos-XhoI (forward) 5’-
CCGCTCGAGCGGCGAGCTCTTACGCGGACTTT-3’ and iNos-BamHI (reverse) 5’-
CGCGGATCCGCGTTTACCAACAGTACCGGAAT-3’. PCR was performed using
Phusion High Fidelity Master Mix (NEB) using optimized conditions (higher temperatures
for annealing/extension of 72 °C) due to the high GC content of the primers. Following
purification, the resulting ~1.3 kb fragment was subeloned into the pRRLSIN.cPPT.PGK-
GFP.WPRE lentiviral vector (Addgene plasmid # 12252 from Didier Trono). Both the PCR
product and the recipient plasmid were digested with XhoI and BamHI (NEB) according
to manufacturer’s protocols, followed by purification. Ligations were performed using T4
ligase (NEB) according to manufacturer’s protocols. Ligation mixtures were then
transformed into STBL3 bacteria (ThermoFisher) by electroporation and plated for
overnight (approximately 18 h) growth in ampicillin-containing agar plates at 37 °C. Single
colonies were then picked and transferred into 5 mL of LB media with ampicillin for further
expansion in a shaker incubator at 37 °C. 12 h later, the 5 mL culture was diluted to 50 mL
using LB media containing ampicillin, and returned to the incubator for overnight growth.
Sanger sequencing was performed by GeneWiz to confirm the final construct.

6.2.7 Generation of Stable RAW:iNos-eGFP Cells - Lentiviral Transductions

HEK93T cells were seeded in 60 mm culture dishes and transiently transfected with 3 μg
psPAX2 packaging plasmid, 2 μg pMD2G envelope plasmid (both from Prof. D. Joseph
Jerry, Veterinary and Animal Sciences, UMass Amherst), and 5 μg iNos-eGFP reporter
constructs generated above, using Lipofectamine3000 (ThermoFisher Scientific),
according to the manufacturer’s instructions. Lentiviral particles were harvested from the
supernatant 48 h after DNA-lipid complexes were added to cells. The virus-containing
supernatant was passed through a 0.45 µm filter. Equal volumes of lentivirus-containing supernatant and complete DMEM containing 4 µg/mL polybrene (Sigma) were combined. Confluent RAW264.7 cells grown in T25 flasks were treated with 6 mL of lentivirus-containing media. Infections were performed every 12 h over 48 h (total of 4 infections), after which the medium was replaced with complete DMEM, and the cells were allowed to recover, grow, and expand for 2-3 days to ensure a viable population. Cells were then prepared for sorting of positive cells as described below.

6.2.8 Cell Sorting of RAW:iNos-eGFP

To ensure a homogenous population, cells were sorted twice under different polarizing conditions. For the first sorting, cells were exposed to M1-polarizing cytokines for 24 h (described above) to induce an M1 phenotype. After 24 h, cells were detached from the cell culture flask; 5-7 x 10^6 cells were resuspended in 3 mL of FACS buffer (4% FBS in phosphate buffered saline (PBS, Gibco)) and sorted at the University of Massachusetts Amherst Flow Cytometry Core Facility using a BD FACSAria Fusion (Becton Dickinson). The instrument was configured with 4 lasers (405 nm, 488 nm, 561 nm, 640 nm), and a 100 µm nozzle size was used for sorting. Of 2.18 x 10^6 positive cells, the top 20.6% of the highest fluorescing cells (436,000 cells with ~98% purity) were sorted. These were plated in T25 flasks for recovery, expansion, and further sorting. For the second sorting, cells were then treated with M2 cytokines for 48 h. Once polarized, 5-7 x 10^6 cells were resuspended in FACS buffer and sorted using the same instrument and configuration from the first. Of 3.7 x 10^6 positive cells, the bottom 10.5% of the lowest fluorescing cells (370,000 cells with ~98% purity) were sorted and plated in T25 flasks for further expansion and use.
6.2.9 Confocal Microscopy

For acquisition of cell morphology and fluorescence images, RAW:iNos-eGFP cells were plated in a 8-well Lab-Tek II chambered cover-glass system plate (Nunc) at a density of 100,000 cells/mL (50,000 cells/well) and allowed to adhere overnight. All treatments of RAW:iNos-eGFP cells were performed 24 h after being plated. Cells were imaged every 24 h for up to 72 h depending on the experiment. After polarization as described above, cells were imaged using a Nikon Ti-E C2 confocal microscope at 10x magnification. ImageJ/Fiji software was used for the quantification of fluorescence of the confocal images on a per-cell basis via thresholding method. Every experimental group was reproduced with three biological replicates, for which a single image was acquired for each that included between ~100-1000 cells for which the integrated fluorescence intensity (mean fluorescence X area of the cell) was defined.

6.2.10 Generation of Conditioned Media

Cells were cultured and passaged at least once before being used to generate conditioned media. The procedure used to generate 4T1- and EMT6-conditioned media follows a previously established protocol. Briefly, cells were cultured in T175 flasks with complete DMEM until they became >90% confluent. At that point, the media was replaced with complete DMEM media and cells were cultured for an additional 7 days. On day 7, the media was collected and filtered through a 0.45 µm syringe filter and stored at -20 °C. For the experiments described here, it was used within the first six months.
6.2.11 Treatment with 4T1 or EMT6 Conditioned Media

RAW:iNos-eGFP cells were plated in an 8-well Lab-Tek II chambered cover-glass system plate at a cell density of 50,000 cells/well in 500 µL complete DMEM and allowed to adhere overnight at 37 °C, 5% CO₂. After 24 h, the culture media was removed and replaced with 40% conditioned media from 4T1 or EMT6 cells and 60% complete DMEM. Cells were incubated with conditioned media for 48 h at 37 °C, 5% CO₂ and were then assessed via confocal microscopy.

6.2.12 Spheroid Generation

Using the hanging drop technique,49 10 µL-droplets of a 10⁶ cell/mL-solution of 4T1 or EMT6 cells were added to a 60 mm petri dish lid, and 3-4 mL of PBS was added to the bottom of the petri dish. The lids were immediately inverted and placed atop the dishes containing the PBS reservoir. The cells were then incubated at 37 °C at 5% CO₂ for 3-4 days to allow seeds to form. The seeds (typically between 5-15 per plate) were individually transferred from the 10 µL hanging drops using a 100 µL-pipette tip (with the tip cut off) to a 25 mL-round bottom flask containing 5 mL of complete DMEM. Flasks were placed on a platform shaker within a cell culture incubator and grown at 37 °C at 5% CO₂ with shaking at 150 rpm for an additional 3-4 days. Spheroids were subsequently drawn from the flask and used directly in the respective experiments.

6.2.13 Two- and Three-Dimensional Co-Cultures

For two-dimensional (2D) co-cultures, RAW:iNos-eGFP cells were concurrently plated with 4T1, and separately, EMT6 breast cancer cells at a 1:1 ratio, 250 µL of each for a total of 500 µL, in complete DMEM using an 8-well Lab-Tek II chambered cover-glass system
plate. For three-dimensional (3D) co-cultures, 50,000 RAW:iNos-eGFP cells in 500 µL complete DMEM were plated in an 8-well Lab-Tek II chambered cover-glass system, and allowed to adhere overnight. 24 h after plating, a single spheroid in 20 µL complete DMEM was added to the macrophage monolayer using a 100 µL-pipet and imaged immediately following. Images were acquired every 24 h for up to 72 h for both 2D- and 3D co-culture experiments. Experiments were performed in biological triplicates.

6.2.14 Small Molecule (PBI1) Treatment

For experiments involving treatment with pyrimido(5'-4b)indole (PBI1, synthesized as previously described), a 5 mg/mL stock solution of PBI1 dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) was prepared. Cell treatments were then prepared by adding 2 µL of PBI1 stock solution per 500 µL of cell culture media for a final dosing concentration of 20 µg/mL of PBI1 and 0.4% DMSO. Media in 8-well Lab-Tek II chambered cover-glass plate containing cells was removed and replaced with 500 µL of PBI1-containing media (20 µg/mL) for 24 h prior to analysis.

6.3 Results and Discussion

6.3.1 Generation and Validation of RAW:iNos-eGFP Cell Line

Because we sought to generate cell-based reporters that could be easily and consistently used, we elected to use immortalized cells for our system. RAW264.7 cells are an immortalized cell line, commonly used as a model for macrophages. We first confirmed the similarity of the candidate marker, iNos’s expression profiles between RAW264.7 and primary bone derived macrophages (BMDMs). Both cell types were assessed under non-polarized (M0), and immune-activating (M1) and –suppressing (M2) states. We found that
while the ratios of \textit{iNos} levels between phenotypes varied between the primary and immortalized cells, the trends of \textit{iNos} being substantially higher for M1 and slightly lower for M2, respective to M0, were similar (Figure 6.2).

![Figure 6.2: RT-PCR analysis comparing \textit{iNos} levels in immortalized versus primary macrophages. Shown are relative levels of \textit{iNos} expression in bone marrow-derived macrophages (BMDMs; left) versus RAW264.7 (right) cells under polarizing conditions. M0 (gray)=non-treated, M1 (orange)=LPS/IFN-γ, and M2=IL-4. This experiment was performed with three biological replicates. Student T-test was used for statistical analysis (p<0.05 = *, p<0.01 = **, p<0.001 = ***). Error bars represent standard error.](image)

After verifying our marker for use as a reporter, molecular cloning methods were used to generate a lentiviral plasmid for enhanced green fluorescent protein (eGFP) expression under the control of the \textit{iNos} promoter sequence. This construct was stably transfected into RAW264.7 cells to yield the RAW:\textit{iNos}-eGFP reporter cell line. We anticipated that the reporter cell line would increase in fluorescence when adopting a pro-inflammatory M1 phenotype and become dimmer when assuming an anti-inflammatory M2 state. Following transfection and preliminary confirmation of fluorescence via microscopy (data not shown), cells were sequentially sorted via flow cytometry under polarizing conditions. First, transfected cells were polarized to the M1 phenotype via cytokine (LPS and IFN-γ) treatment, and the top 20% of the cells with the highest
fluorescence levels were selected (Figure 6.3, left). Then, this population of cells was polarized to the M2 phenotype using IL-4, and the bottom 10% of the cells with the lowest fluorescence were selected (Figure 6.3, right).

**Figure 6.3: Sorting of RAW:iNos-eGFP cells.** Cytometry plots from the first (top and bottom left) and second (top and bottom right) sortings of RAW:iNos-eGFP cells under M1- followed by M2-polarizing conditions, respectively. For the first sorting under M1 conditions (top and bottom left), selected GFP-positive cells are encased (top left image), from the top 20.6% of positive cells were kept (bottom left image). The cells were then sorted again under M2 conditions (top and bottom right), and the bottom 10.5% of positive cells were kept.

The resulting RAW:iNos-eGFP cell line was evaluated via RT-PCR and confocal microscopy. In both cases, the reporter cells were polarized to M1 and M2 phenotypes (non-treated cells represented M0), and relative iNos and eGFP fluorescence levels were determined and compared. RT-PCR results indicated that the iNos expression patterns of RAW:iNos-eGFP (Figure 6.4a) were similar to those of the RAW264.7 parental cell line (Figure 6.2). This suggests that both iNos expression and macrophage polarization pathways were unaffected by the insertion of the plasmid construct. To confirm fluorescence changes resulting from the promoter-driven reporter, polarized and non-polarized cells were evaluated using confocal microscopy (Figure 6.4b). Significantly
increased fluorescence was observed for cells in the M1 phenotype and diminished levels were observed for the M2 subtype, as determined by quantification on a per cell basis (Figure 6.4c).

Figure 6.4: Effects of cytokine polarization on RAW:iNos-eGFP cells. (a) RT-PCR data showing relative levels of \( iNOS \) mRNA across polarization states (M0 = gray, M1 = orange, and M2 = blue). Error bars represent standard error. (b) Confocal microscopy images acquired following polarization of RAW:iNos-eGFP. BF = bright field, eGFP = enhanced green fluorescent protein. Scale bars on confocal images represent 100 µm. (c) Quantification of fluorescence intensity on a per-cell basis from the figures shown on the left. M0 = gray (n = 1,710), M1 = orange (n = 1,820), and M2 = blue (n = 1,142). The “x” in the box represents the mean; the bottom and top lines of the box represent the median of the bottom half (1\(^{st}\) quartile) and median of the top half (3\(^{rd}\) quartile), respectively; the line in the middle of the box represents the median; the whiskers extend from the ends of the box to the minimum value and maximum value. For all panels, M0 = non-treated; M1 = LPS/IFN-γ; M2 = IL-4. For (a) and (c), Student T-test was used for statistical analysis comparing M1 and M2 macrophages to M0 (\( p<0.05 = * \), \( p<0.001 = *** \)).
6.3.2 RAW:iNos-eGFP Responses to Different Breast Cancer Models

Following validation, the RAW:iNos-eGFP reporter cell line was used to compare macrophage responses to two breast cancer cell types, using different types of models. For this study, we chose to use 4T1\textsuperscript{51} and EMT6\textsuperscript{52} cell lines, both of which represent murine triple-negative breast cancer (TNBC) and are widely used in cancer research. Despite sharing a TNBC background, EMT6 cells are considered to be less aggressive than 4T1, which are highly invasive.\textsuperscript{53} This suggests that these two TNBC cell lines may affect macrophages differently. First, we compared two experimental formats with each cell line: conditioned media (CM) and two-dimensional (2D) mono-layer co-culture. While CM has been used to study macrophage responses, they are not only influenced by cytokines secreted by cancer cells (found in the CM), but also by cell-to-cell interactions and the hypoxic core that forms within the tumor.\textsuperscript{54,55} The use of the macrophage reporter cell line allows us to directly compare simple with more complex experimental models.

The effects of 4T1 and EMT6 cell-derived conditioned media were compared to cancer cell:macrophage co-cultures generated in a 1:1 ratio. Since studies suggest that cancer cells can switch macrophages to the wound-repair (M2) subtype,\textsuperscript{56} we expected to see diminished fluorescence of the RAW:iNos-eGFP reporter following exposure to the cancer cells and CM. Our results indeed showed that the macrophage cells had statistically significant lower levels of fluorescence compared to non-treated macrophages, similarly to the M2-like phenotype, after 48 h of exposure to either conditioned media or the 4T1 or EMT6 cells (Figure 6.5). While both EMT6 models, CM and co-culture, resulted in similar effects, there was a significant difference between them (p < 0.01), with co-culture of the cells having a greater reduction in fluorescence. The 4T1 models’ outcomes were
substantially different from one another. While the 4T1-CM had a statistically significant change versus non-treated cells (p < 0.001), it resulted in the least change from the non-treated control overall. On the other hand, the 4T1 co-culture yielded the lowest levels of fluorescence in the assay. Taken together, the 2D co-cultures produced greater effects than their CM counterparts, however, the trends observed were unexpectedly inconsistent – while co-culture with 4T1 cells yielded the lowest fluorescence, as expected, the CM from EMT6 cells resulted in greater effects than that from 4T1s. These results show the sensitivity of the reporter and highlight the relevance of cell-to-cell interactions.

Figure 6.5: Macrophage exposure to conditioned media (CM) from or co-culture (CC) with 4T1 or EMT6 cells. (a) Confocal microscopy images of RAW:iNos-eGFP cells following exposure to conditioned media from 4T1 or EMT6 cells or subjected co-culturing with each. eGFP=enhanced green fluorescent protein. Scale bars on confocal images represent 100 µm. (b) Per-cell fluorescence intensity quantification of images shown in (a). The “x” in the box represents the mean; the bottom and top lines of the box represent the median of the bottom half (1st quartile) and median of the top half (3rd quartile), respectively; the line in the middle of the box represents the median; the whiskers extend from the ends of the box to the minimum value and maximum value. Student’s T-test was used for statistical analysis versus N.T. (n = 1,422) (p<0.001 = ***). Student T-test comparing 4T1-CM (n = 968) versus 4T1-CC (n = 370) and EMT6-CM (n = 581) versus EMT6-CC (n = 336) shows statistical significance (p<0.001=*** and p<0.01=**, respectively). N.T. = not treated.
While 2D co-culture monolayers are simple to use and allow the study of intracellular signaling cascades and cell behavior, this approach omits the highly complex structural organization found in the three-dimensional TME. Also, solid tumors develop an oxygen-less region within the tumor (hypoxic core), which leads to tumor progression and metastasis and promotes macrophage polarization towards the M2 phenotype. For these reasons, we assessed the impacts of 4T1 and EMT6 spheroids as in vitro three-dimensional (3D) models, on the macrophage reporter cell line. To better represent the TME, only spheroids that were 400 µm or more in diameter were used, since these are known to develop a hypoxic core.

Macrophages exposed to 4T1 or EMT6 spheroids showed reduced levels of iNos-controlled eGFP expression, suggesting that they are adopting a tumor-promoting, M2 phenotype (Figures 6.6 and 6.7). Interestingly, while both 4T1 and EMT6 spheroids seemed to reduce the eGFP signal, the change was greater when macrophages were exposed to 4T1 spheroids, which is consistent with the contrasting aggressiveness of the model cell lines. This also highlights the advantages of using a reporter-based approach to monitor the interactions between macrophages and cancer cells, while keeping track of the macrophage polarization state. The use of our reporter cell line not only facilitates the study of macrophage phenotypes in real time, but also allows the use of more complex and relevant cancer models that could not be used before due to experiment- or technique-associated limitations.
Figure 6.6: Macrophage reporter responses in three-dimensional tumor models. (a) Representative confocal images of RAW:iNos-eGFP macrophages (top row) after 48 h exposure to 4T1 (middle row) or EMT6 (bottom row) spheroids. eGFP = enhanced green fluorescent protein. Scale bars represent 100 µm. Additional spheroids are shown in Figure 6.7. (b) Per-cell fluorescence quantification of confocal images from (a). The “x” in the box represents the mean; the bottom and top lines of the box represent the median of the bottom half (1st quartile) and median of the top half (3rd quartile), respectively; the line in the middle of the box represents the median; the whiskers extend from the ends of the box to the minimum value and maximum value. Student T-test was used for statistical analysis of 4T1 SCC (n = 406) and EMT6 SCC (n = 337) versus N.T. (n = 2,111), (p<0.01 = **, p<0.001 = ***). N.T. = non-treated cells, SCC = spheroid co-culture.

Figure 6.7: Additional 3D co-cultures. To supplement images shown in Figure 6.6, representative images from additional cultures/spheroids are shown here. RAW:iNos-eGFP macrophages (top row) are shown after 48 h exposure to 4T1 (middle row) or EMT6 (bottom row) spheroids. eGFP = enhanced green fluorescent protein. Scale bars represent 100 µm.
6.3.3 Re-Programming of RAW:iNos-eGFP Cells in the Presence of Spheroids via Small Molecule Treatment

To further assess the interactions of macrophages with breast cancer and evaluate their reprogramming capabilities in tumor microenvironments, we used pyrimido(5,4-b)indole (PBI1), a Tlr-4 agonist known to activate macrophages to M1-like phenotypes and enhance their anti-cancer activity.\textsuperscript{43} First, we tested the effects of different PBI1 concentrations (5, 10, and 20 µg/mL) on the viability of RAW:iNos-eGFP cells using Alamar Blue reagent and compared it to non-treated and 0.4% DMSO-treated control cells; no substantial changes were observed (Figure 6.8a). RT-PCR quantifying iNos mRNA transcript levels was also performed to confirm the activation of macrophages by PBI1 (Figure 6.8b), as was an assessment of fluorescence changes in the RAW:iNos-eGFP cells (Figure 6.9). In both cases, PBI1 treatment resulted in expected increases relative to the control groups, confirming that the small molecule’s promotion of macrophage activation persists in the reporter cells.

![Figure 6.8](image-url)  
**Figure 6.8: Evaluation of PBI1 effects on RAW:iNos-eGFP cells.** (a) Viability assay of PBI1-treated cells using three different concentrations (5, 10, and 20 µg/mL). (b) RT-PCR results showing relative iNos mRNA transcript levels in RAW:iNos-eGFP cells following PBI1 treatment at a final concentration of 20 µg/mL. Student T-test comparing DMSO- and PBI1-treated groups to N.T. (p<0.05 = *, p<0.001 = ***) N.T. = non-treated cells, DMSO = cells treated with 0.4% dimethyl sulfoxide vehicle. Error bars represent standard error.
Figure 6.9: Effects of PBI1 on RAW:iNoS-eGFP cells. (a) Confocal images showing changes in fluorescence following PBI1 treatment. Scale bars represent 100 µm. (b) Quantification of fluorescence intensity from images in (a). The “x” in the box represents the mean; the bottom and top lines of the box represent the median of the bottom half (1st quartile) and median of the top half (3rd quartile), respectively; the line in the middle of the box represents the median; the whiskers extend from the ends of the box to the minimum value and maximum value. Error bars represent standard error. Student T-test was performed versus N.T. (p<0.001 = ***). N.T.=non-treated cells, eGFP=enhanced green fluorescent protein.

We then used PBI to affect RAW:iNoS-eGFP macrophages co-cultured with either 4T1 or EMT6 spheroids; controls lacked treatment with the small molecule. As in our earlier experiment (Figures 6.6 and 6.7), reporter macrophages exposed to either 4T1 or EMT6 spheroids in the absence of PBI1 displayed decreased fluorescence (Figure 6.10). Excitingly, a significant increase in iNoS-eGFP signal was observed following treatment with PBI1, suggesting a shift of the macrophages toward a pro-inflammatory, anti-tumor, M1 phenotype. While the change observed was not statistically significant, it is important to note that the mean values for 4T1 SCC (+) and EMT6 SCC (+) groups increased relative to the N.T. (-) group. We also investigated the effects of PBI1 treatment on 4T1 and EMT6 spheroids in the absence of macrophages to verify that the molecule itself did not affect them (Figure 6.11). These results reinforce the idea that macrophages can be reprogrammed at the tumor site, and the reporter can be used to track these changes, even in more complex models of the TME.
Figure 6.10: Re-polarization of RAW:iNos-eGFP macrophages using a small molecule in a spheroid co-culture model. (a) Confocal microscopy images of RAW:iNos-eGFP cells co-cultured with 4T1 or EMT6 spheroids, with (+) or without (-) PBI1 treatment. Scale bars on confocal images represent 100 µm. Images of additional spheroids are shown in Figure 6.11. (b) Student T-test was used for statistical analysis versus PBI1-treated groups (p<0.001 = ***). N.T. (-): n = 2991; N.T. (+): n = 1536; 4T1 SCC (-): n = 703; 4T1 SCC (+): n = 539; EMT6 SCC (-): n = 572; EMT6 SCC (+): n = 276. The “x” in the box represents the mean; the bottom and top lines of the box represent the median of the bottom half (1st quartile) and median of the top half (3rd quartile), respectively; the line in the middle of the box represents the median; the whiskers extend from the ends of the box to the minimum value and maximum value. N.T. = non-treated, SCC = spheroid co-culture.
Figure 6.11: Effects of PBI1 on 4T1 and EMT6 spheroids after 48 h. Confocal microscopy images showing changes in spheroid size after 48 h with or without treatment with 20 µg/mL of PBI, and spheroid co-culture with RAW:iNos-eGFP cells (SCC). Scale bars on images represent 100 µm.

6.4 Conclusion

In summary, our study demonstrates the advantages of using a macrophage phenotype-reporter cell line, RAW:iNos-eGFP to study macrophage interactions with cancer, especially in more complex environments. Following the confirmation of a consistently expressed, phenotype-specific marker, iNos (Figure 6.2), we designed a cell line based on the commonly used macrophage model, RAW264.7 cells, to express eGFP under the
regulation of the iNos promoter. After validating normal functioning of the cells via RT-PCR and reporter fidelity via confocal microscopy (Figure 6.4), we explored the responses of macrophages to different breast cancer models derived from 4T1 and EMT6 TNBC cell lines.

Across the three models examined, conditioned media (CM), 2D co-culture (CC), and 3D/spheroid co-cultures (SCC), both TNBC cell lines resulted in anti-inflammatory M2-like fluorescence profiles (Figures 6.5, 6.6, and 6.7). However, while we expected the 4T1-derived models to demonstrate greater changes than those from the less aggressive EMT6 cells, this was not reflected in the conditioned media experiments. For CM, EMT6 gave a greater response than 4T1; the CC and SCC models showed the opposite – the 4T1 cells resulted in greater changes to iNos-driven eGFP expression than EMT6. These results confirm that there are differences in macrophage responses among models used, even with the same cell line(s), and should be considered in future studies.

The re-education of macrophages in disease states or infections, either from pro- to anti-inflammatory or vice-versa, is a therapeutic strategy of broad interest. In cancer, the polarization of macrophages to the immune-stimulating (M1) phenotype can result in enhanced anti-tumor activity, and is a goal in developing new cancer immunotherapies. Therefore, having assessed the RAW:iNos-eGFP reporters’ functioning in TME models, we decided to test our platform in tracking macrophage re-education in the most disease representative model used here, spheroid co-culture. We used a previously described small molecule Tlr-4 agonist, PBI1. This compound had been shown to induce M1-like polarization and enhance macrophage anti-cancer activity in
vitro; here it was used in the presence of more relevant 3D tumor models. We generated spheroids using 4T1 and EMT6 cells and using only those that were 400 µm or more in diameter, compared non-treated 4T1 or EMT6 spheroid co-cultures with PBI1-treated groups. As expected, the controls (macrophages exposed to spheroids without PBI1), exhibited decreased fluorescence representative of an anti-inflammatory, M2-like phenotype. However, when challenged with the Tlr-4 agonist small molecule, PBI1, macrophages showed increases in fluorescence relative to the non-treated group, indicative of their activation toward a pro-inflammatory state (Figure 6.10). This illustrates the feasibility of using reporters to evaluate therapeutics even in the presence of more relevant and realistic cancer TME models.

In conclusion, we show that the use of the macrophage reporter cell line enables the assessments across different models without needing to apply other probes (e.g., antibodies) for detection, which unless used with fixed cells, could alter cellular characteristics, or segregate or lyse cells. This approach also allows for continuous monitoring of macrophage responses, as conditions change or additional stimuli are presented. Taken together, the use of macrophage reporter cell lines, including the one developed here or others with suitable phenotype-specific markers, can facilitate studies to assess macrophage behavior in response to more complex and accurate models, and high-throughput assessment of drugs for affecting macrophages.

6.5 References


CHAPTER 7

CONCLUSION AND OUTLOOK

Macrophages play important roles in the innate immune system; therefore, they have gained attention as prognostic indicators and therapeutic targets for multiple diseases. While there have been advances in means to assess macrophage polarization, there remains a need for better, more reliable ways to probe their subtypes in biologically relevant contexts. Also, in terms of diseases associated with aberrant macrophage polarization, there is a need for small molecules that can affect macrophage phenotypes, reprogramming them from one to another either as a stand-alone or adjuvant treatment strategy.

In this thesis I have used chemical biology-based strategies for studying and manipulating macrophage phenotypes and behavior, including in the context of breast cancer. To this end, I used a polymer-protein complex as an array-based sensor for the rapid and high-throughput categorization of macrophages, showing that different polarizing stimuli (including cytokines and cancer cell-conditioned media types) result in differing, categorizable signatures. I also used cellular reporters to track circadian rhythm changes, establishing correlations between macrophage phenotypes and oscillations. Here, I showed that modulation of macrophage polarization state to either pro-inflammatory M1 or anti-inflammatory M2 states affected their circadian rhythms differently, as did exposure to conditioned media from more- versus less-aggressive breast cancer cell lines. To enable tracking of macrophage polarization in real time with multi-cell type biological models, I developed a fluorescent phenotypic reporter cell line. I used a consistently expressed marker of macrophage activation, nitric oxide synthetase (iNos), placing the expression of
green fluorescent protein (GFP) under the control of the iNos promoter. Using this construct, I evaluated the effects of different types of breast cancer cell culture-based models on macrophage polarization, finding that macrophages were polarized to an immunosuppressive, M2 phenotype to different extents depending on the breast cancer cell line and the model used. Also, I showed the ability of a TLR-4 agonist, PBI1, to polarize macrophages to a pro-inflammatory, M1 subtype, including in the presence of more relevant breast cancer models. Lastly, I investigated the reprogramming capabilities of novel pro- and anti-inflammatory small molecules, including in the presence of a more realistic and complex version of the TME. Taken together, this thesis offers novel methods for probing macrophage phenotypes in different experimental formats (including in more complex environments) and presents pro- and anti-inflammatory small molecules as modulators of macrophage activity.

While the focus of this thesis was to improve means to study and affect macrophage polarization, there are still many unanswered questions and further progress that can be made. Regarding the generation and use of reporters, future versions should be produced using gene-editing techniques that guarantee the insertion of only one copy of the construct at a pre-determined (or endogenous) site, avoiding the insertion of multiple copies and/or disruption of other genes. Additionally, while I describe two drugs with the ability to alter macrophage phenotypes, these have yet to be evaluated in vivo, which will determine their therapeutic potential. Nonetheless, more target-specific small molecules with potent effects and better biodistribution are needed, since it is likely that those described will require further optimization. Lastly, the interactions between the immune system (macrophages included) and diseases are critical to outcomes and therapeutic efficacy. It is clear that
disease models that include immune cells are critical in order to obtain more accurate and reliable results. Further work should continue their development, including with other reporters and cell types. Hopefully, addressing these limitations will significantly improve current and future macrophage-based immunotherapies.
APPENDIX A

VARIATIONS IN MARKER EXPRESSION AMONG MACROPHAGE CELL TYPES

A.1 Introduction

Macrophages are immune cells of the innate system that protect the host by fighting infections and supporting homeostasis.\textsuperscript{1-3} These cells are considered to be “plastic,” which refers to their capacity to go through phenotypic changes in a process known as macrophage polarization.\textsuperscript{4} The resulting phenotypes broadly range from pro-inflammatory (M1) to anti-inflammatory (M2) subtypes.\textsuperscript{5} Previous studies have confirmed the associations of specific phenotypes with the development and progression of diseases, including sepsis, infection, chronic inflammatory diseases, neurodegenerative disease, and cancer.\textsuperscript{6} The involvement of macrophages in these disorders makes them an attractive therapeutic target.\textsuperscript{7} Therefore, it is of interest to characterize macrophage polarization states, and the phenotypes that result in the context of these diseases.

To this date, there are various macrophage models available that facilitate the study of the polarization process and its implication in different diseases. One of the main macrophage models is primary macrophages, a cell culture of freshly obtained macrophages from an organism. These cells stand as the most accurate representation of macrophage behavior \textit{ex vivo}.\textsuperscript{8} This type of cell culture displays several advantages over other models, including a better representation of the cellular heterogeneity and more realistic functional responses, including to pathogens and drug treatments.\textsuperscript{9,10} Due to accessibility issues associated with obtaining human primary macrophages for the study of immune responses, these primary cells are normally obtained from commonly used mouse
Two common sources of primary macrophages from mice are the peritoneal cavity and bone marrow. However, while being an accurate representation, there are some drawbacks to using primary macrophages, including the need for constant replenishing of the cells, due to their inability to divide, time-consuming protocols, and specific knowledge for handling and culturing of the cells. As a result, the use of immortalized models is generally more feasible for many studies involving macrophages.

An immortalized cell line is a population of cells derived from an organism that would normally not proliferate indefinitely but due to mutation, have evaded normal cellular aging and instead keep dividing. Therefore, the immortalization process allows the culturing of these cells in vitro for prolonged periods. The mutations that give rise to this state can occur spontaneously in some instances, but are typically induced. Immortalized cell lines offer several advantages, including that they are cost-effective, easy to handle, and present a relatively unlimited supply of material, as compared to primary cells. Their use also circumvents ethical concerns associated with the use of animal and human tissue. Cell lines also provide pure populations of cells, which are valuable since they provide consistent samples and reproducible results.

The use of immortalized cell lines has revolutionized the research on macrophages by facilitating the generation of vaccines, drug metabolism and cytotoxicity studies, antibody production, and the study of gene function and immune responses. Among immortalized model macrophages, RAW264.7 and J774 cells are the most frequently used. These murine cell lines have proven to be useful models of macrophages, since there are no human macrophage cell lines. RAW264.7 is a murine macrophage cell line that originates from Abelson leukemia virus-transformed cell linage derived from BALB/c.
mice. This cell line is well-characterized with regard to macrophage-mediated immune, metabolic, and phagocytic functions.\textsuperscript{18} J774 murine macrophages were isolated from an adult female BALB/c mouse with reticulum cell sarcoma.\textsuperscript{17} However, despite being powerful tools, cell lines are genetically manipulated, which can result in altered phenotypes, native functions, and responsiveness to stimuli.\textsuperscript{9,19} Therefore, macrophage cell lines may not accurately represent primary cells in all aspects and can provide misleading results. Thus, it remains important to compare results obtained in immortalized cells with their primary counterparts. Since phenotype-specific markers are used to study the roles and responses of macrophages, it is essential to verify that the model cell line(s) used result in the same patterns as the parental cells.

Previous studies performed with primary bone marrow-derived macrophages (BMDMs) revealed that M1 macrophages are characterized by enhanced expression of toll-like receptor 2 (Tlr-2),\textsuperscript{20} intracellular adhesion molecule 1 (Icam1),\textsuperscript{21} tumor necrosis factor alpha (Tnf-\(\alpha\)),\textsuperscript{22} and inducible nitric oxide (iNos),\textsuperscript{23} and have decreased expression of cluster of differentiation 36 (Cd36),\textsuperscript{24} and neuropilin 1 (Nrp1),\textsuperscript{25} mannose receptor (MR/Cd206)\textsuperscript{26} and early growth response protein 2 (Egr2/Krox20).\textsuperscript{27,28} The opposite patterns of expression of each occurred in M2 macrophages. Despite the prevalence of immortalized macrophages’ use, a comparison between these models and primary cells to immortalized murine macrophage models RAW264.7 and J774 has not been performed. This work aims to compare and correlate the patterns of expression of commonly employed macrophage phenotypic markers between primary and immortalized cells.
A.2 Materials and Methods

A.2.1 Cell Culture

RAW264.7 cells were purchased from American Type Culture Collection (ATCC), J774 cells were obtained from Prof. M. Sloan Siegrist (Microbiology, UMass Amherst), L929 cells were from Prof. Barbara Osborne (Veterinary and Animal Sciences, UMass Amherst), and primary immortalized macrophages (PIMs;\textsuperscript{29} immortalized with J2 virus) were obtained from Prof. Susan Carpenter (Molecular, Cell & Developmental Biology, UC Santa Cruz). Generation and maintenance of primary bone marrow derived macrophages (BMDMs) is described in \textit{Section A.2.3}. Cells were cultured in T75 culture flasks at 37 °C under a humidified atmosphere containing 5% CO\textsubscript{2}. Standard growth media consisted of high glucose Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (100 μg/ml penicillin and 100 μg/ml streptomycin) – herein referred to as complete DMEM. Under the above culture conditions, the cells were sub-cultured approximately once every 3-4 days and only cells between passages 7 and 20 were used for all experiments.

A.2.2 Generation of L929-Conditioned Media

Murine fibroblast L929 cells were cultured and passaged at least once before being used to generate conditioned media, used for macrophage differentiation (described in \textit{Section A.2.3}). The procedure used to generate L929-conditioned media follows a previously established protocol.\textsuperscript{30} L929 cells were cultured in T175 flasks with complete DMEM until they became more than 90% confluent. At that point, the media was replaced with complete DMEM media and cells were cultured for 7 days. On day 7, the media was collected and filtered through a 0.45 μm syringe filter and stored at -20 °C for up to 6 months.
A.2.3 Isolation and Differentiation of Primary Macrophages from Murine Bone Marrow

Isolation and differentiation of primary macrophages was performed following a previously established procedure.30 Bones were collected from femurs and tibiae from C57BL/6 mice and put into 0.6 mL micro-centrifuge tubes containing a small hole at the bottom (perforated prior to use with an 18G needle). Each 0.6 mL micro-centrifuge tube (containing one femur and one tibia) was then inserted into a 1.5 mL micro-centrifuge tube and centrifuged for 30 seconds at 10,000 rpm. The 0.6 mL tube with marrow-less bones was then discarded and the pelleted bone marrow (now in the 1.5 mL-tube) was re-suspended in 500 µL of differentiation media (70% complete DMEM and 30% L929-conditioned media). Once re-suspended, each 1.5 mL tube was transferred to and grown in a T175 with 12 mL of differentiation media for 3 days. After 3 days, the media was replaced with fresh differentiation media for another 4 days. Following differentiation, media was replaced with complete DMEM and the cells were kept in the T175 culture flasks at a high cell density at 37 °C under a humidified atmosphere containing 5% CO₂ until needed for experiments.

A.2.4 Macrophage Polarization

To polarize macrophages, BMDMs, RAW264.7, J774, or PIM cells were plated in triplicates in 24-well plates at a density of 100,000 cells/well (400,000 cells/well for BMDMs) in 500 µL of complete DMEM and incubated at 37 °C under a humidified atmosphere containing 5% CO₂. 24 h after plating, the media was removed and replaced with complete DMEM media containing 50 ng/mL interleukin 4 (IL-4) (BioLegend) and incubated for an additional 48 h at 37 °C under a humidified atmosphere containing 5%
CO₂ to generate M2 macrophages. To generate M1 macrophages, cells were treated with 50 ng/mL interferon-gamma (IFN-γ) (BD Biosciences) and 50 ng/mL of lipopolysaccharide (LPS) (Sigma Aldrich) 48 h after plating and incubated for 24h cytokine-containing media. Non-treated macrophages grown in complete DMEM only were used as M0. 72 h after plating (48 h after treatment with M2 cytokines and 24 h after treatment with M1 cytokines), cells were assayed.

A.2.5 RNA Extraction and cDNA Conversion

Cells were lysed, and approximately 1.5 µg RNA was harvested from each well using a PureLink RNA Mini Kit (Ambion) following the manufacturer’s instructions. To convert RNA to complementary DNA (cDNA), 1µL/sample of 50 μM random hexamers (Applied Biosystems) and 10 mM dNTPs (Thermo Scientific) were added to 11 µL of RNA and heated at 65 °C for 5 min for annealing. Then, 1 µL/sample of 40 U/μL RNaseOut (Invitrogen), 1 µL/sample of 200 U/μL SuperScript IV Reverse Transcriptase (Invitrogen), 1 µL/sample of 100 mM DTT (Invitrogen), and 4 µL/sample of 5x Super Script IV buffer (Invitrogen) were added and incubated at 53 °C for 10 min and followed by 80 °C for 10 min. The resulting cDNA was frozen at -20 °C and used for RT-PCR experiments within 1 week. RNA and cDNA were quantified using a NanoDrop 2000 (Thermo Fisher). RNA and cDNA contamination and integrity were assessed by analyzing the A260/A280 ratio, where ratios greater 1.8 for DNA and 2.0 for RNA were considered pure.

A.2.6 Quantitative RT-PCR

RT-PCR was performed using a CFX Connect real-time system (Biorad) with iTaq Universal SYBR Green Supermix (Biorad). All DNA primers were purchased from Integrated DNA Technologies. The following primer sequences were used:
β-actin (forward) 5’-GATCAGCAAGCAGGAGTACGA-3’,
β-actin (reverse) 5’-AAAACGCAGCGCAGTAACAGT-3’;
iNos (forward) 5’-GTTCTCAGCCCAACAATACAAGA-3’,
iNos (reverse) 5’-GTGGACGGGTCGATGTCAC-3’;
Tnf-α (forward) 5’-CCTGTAGCCCAAGGCTCGAG-3’,
Tnf-α (reverse) 5’-GGGATGCTACGATTTCTCAG-3’;
Cd206 (forward) 5’-GGATGGTGATGCTACTGGA-3’,
Cd206 (reverse) 5’-AGTGACGGGTTCGATGTCAC-3’;
Egr2 (forward) 5’-TGA GAGACGCGGATTGATT-3’,
Egr2 (reverse) 5’-ATAACAGTCAGTGCTCCCC-3’.
Tlr-2 (forward) 5’-GCATCGAATTGATCAACC-3’,
Tlr-2 (reverse) 5’-ACAGCGTTGCTGAAGAGGA-3’;
Cd36 (forward) 5’-TTGAAGAAGGAACCACATTG-3’,
Cd36 (reverse) 5’-AAGCAAGAGGATTTCTCAGC-3’;
Icam1 (forward) 5’-GGACCTTTCGATCTCCAGC-3’,
Icam1 (reverse) 5’-GCCAGGTATATCCGAGCTC-3’;
Nrp1 (forward) 5’-GACCATAACAGGAGTGGCAA-3’,
Nrp1 (reverse) 5’-GTAGCGTAGTTGACCCTCA-3’.

The reaction mixtures included 200 nM of each primer, 100 ng of cDNA, 10 μL SYBR green supermix, and H2O to a final volume of 20 μL. Analyses were performed as follows: the samples were first activated at 50 °C for 2 min, then 95 °C for 2 min. Denaturing occurred at 95 °C for 30 s followed by annealing at 58 °C; the denature/anneal process was repeated over 40 cycles. Relative gene expression was determined by comparing the Ct value of the gene of interest to that of the β-actin housekeeping gene, by the 2ΔΔCt method.31 Three biological replicates were performed for each treatment condition and three technical replicates were used for each biological replicate. No amplification of the no-template control (NTC) samples was confirmed. Data was analyzed using CFX Manager 3.1 software (Biorad). Cq values were generated by using the point at which the sample fluorescence value exceeded the software’s default threshold value. Each sample was normalized to the respective non-treated controls.
A.3 Results and Discussion

In seeking consistently expressed polarization-associated markers among various macrophage types, I analyzed the expression of markers in M0, M1-, and M2-polarized murine bone marrow-derived macrophages (BMDMs) and compared the levels and patterns to those in immortalized macrophage models, RAW264.7, J774, and primary immortalized macrophages (PIMs). Cells were polarized via cytokine treatments, exposing them to 50ng/µL of IFN-γ and LPS to induce an M1-like phenotype, and 50ng/µL of IL-4 to induce an M2 subtype, specifically M2a. As M2a macrophages were used as a general model for M2 in this study, these are referred to as M2 throughout this chapter. RT-PCR results shown in Figure A.1 suggests that Tlr-2, Icam1, Nrp1, and Cd36 were not similarly expressed across the different macrophage types.
Figure A1: Phenotypic markers with inconsistent levels of expression across macrophage models. Shown are results for posited M1-associated markers, Tlr-2 and Icam1 (left two columns), and M2-related genes, Nrp1 and Cd36 (right two columns), in bone marrow-derived macrophage (BMDMs), RAW264.7, J774, and primary immortalized macrophage (PIM) cells. The experiment was performed with three biological replicates. M0 = non-polarized macrophages. Student T-test was used for statistical analysis (p<0.05 = *, p<0.01 = **, p<0.001 = ***, p>0.05 = N.S. (not significant)). Error bars represent standard error.

RAW264.7 and PIM cells displayed opposite patterns of expression for Tlr-2 compared to BMDMs and J774s, but were similar to one another. While BMDMs and J774s had similar trends, the levels observed were more markedly increased for M1 in the primary cells. BMDMs, J774, and PIMs all exhibited the highest levels of Icam1 expression under M1 conditions. However, the levels of Icam observed were more markedly increased for M1 in BMDMs; in J774s and PIMs, the differences were more
subtle (p<0.05) compared to M0 macrophages, and there was no statistical difference in expression under M2 conditions. For PIMs there was no difference between M1 and M2. RAW264.7 cells showed an opposite pattern of expression, with \textit{Icam1} levels decreased in the M1 phenotype. \textit{Nrp1} expression decreased slightly in all four macrophage models under M1 conditions, and only had a statistically significant increase for M2 phenotypes (p<0.05) in J774 macrophages. Expression levels for \textit{Cd36} show a statistical increase (p<0.001) under M1 state for RAW264.7, J774 and PIM cells, but showed a decrease in expression for BMDMs. Under M2 conditions, BMDMs, RAW264.7 and PIM macrophages displayed a statistically significant increase (p<0.001) in expression of \textit{Cd36}, relative to M0, while the change for \textit{Cd36} in J774s was not considered statistically different. However, the greatest differences in levels of \textit{Cd36} between M1 and M2 cells occurred in BMDMs and PIMs.

It is important to mention that BMDMs, RAW264.7, and J774s share a BALB/c genetic background, while the PIM cells had a C57BL/6 background, which can result in different behaviors. Also, the sources of these cells could play different tissue-specific roles that influence the cells’ behavior. For instance, J774 and RAW264.7 macrophages were isolated from ascites, however, BMDMs and PIMs are both bone marrow-derived.\textsuperscript{16-18,29,30} While the cell lines originate from a well-understood tissue type, they have undergone significant mutations to achieve immortality and can result in altered functions and characteristics. Furthermore, cell lines can change genetically over multiple passages, leading to phenotypic differences among isolates and potentially different experimental results depending on when and with what strain isolate an experiment is conducted.\textsuperscript{32}
I then investigated other previously identified polarization markers for BMDMs (iNos, Tnf-α, mannose receptor (Cd206), and early growth response protein 2 (Egr2)). Excitingly, as shown in Figure A2, the expression patterns for these markers were similar among all cell types. These data suggest that iNos, Tnf-α, Cd206, and Egr2 are more reliable phenotype-specific markers across these macrophage types. iNos expression is substantially up-regulated in the M1 phenotype versus the M0 and the M2 subtypes, with the greatest change in the BMDM cells and the least in the J774s. The changes in Tnf-α levels are more subtle across all of the cell lines, with the greatest change again occurring in BMDMs; in this instance, while there is a difference between M1 and M2 in the PIMs, there is none between M0 and M1 (p>0.05). Cd206 is increased in the M2 markers, albeit only slightly in BMDMs, and for PIMs, only relative to M1 cells. The greatest extent of changes occurred in the RAW264.7 and J774 cells. Lastly, Egr2 levels were increased in the M2 phenotype across all four cell lines, although again, the differences were subtle (~3-fold) in all macrophage types, with the exception of the PIMs, where the change was >100-fold.
This study demonstrates that phenotype-associated markers are not necessarily expressed similarly across macrophage models and highlights the necessity of comparing the expression patterns of these markers in immortalized models versus naturally occurring counterparts. Thus, this discrepancy adds another layer of complexity when choosing an adequate model and marker when studying macrophage polarization.

Figure A2: RT-PCR analysis comparing markers with consistent levels of expression across phenotypes and macrophage models. Shown are results for M1-associated markers, iNos and Tnf-α (left), and M2-related genes, Cd206 and Egr2 (right), in bone marrow-derived macrophage (BMDMs; top row), RAW264.7 (second row), J774 (third row), and PIM (bottom row) cells under polarizing conditions. M0 (gray)=non-treated, M1 (orange)=LPS/IFN-γ, and M2=IL-4. This experiment was run with three biological replicates. Student T-test was used for statistical analysis (p<0.05 = *, p<0.01 = **, p<0.001 = ***). Error bars represent standard error.
A.4 References


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APPENDIX B

GENERATION AND VERIFICATION OF A MACROPHAGE-BASED EGR2-MCHERRY REPORTER

B.1 Introduction

While an oversimplification, it is widely accepted that macrophages polarize into two major categories: pro-inflammatory (M1) and anti-inflammatory (M2) macrophages. The M1-like phenotype is mediated by interferon gamma (IFN-γ) and microbial products such as lipopolysaccharide (LPS), and are distinguished by high levels of inducible nitric oxide (iNos) and secretion of potent pro-inflammatory cytokines (such as Il-1β, Il-6, and Tnf-α). The M2-like macrophage phenotype, which is further categorized into M2a, M2b, M2c, and M2d, is induced by IL-4, IL-10 or IL-13 and is characterized by up-regulation of early growth response protein 2 (Egr2). While IL-4 treatment specifically induces an M2a subtype and up-regulation of Egr2, treatment with other cytokines like IL-10 and IL-13 (which lead to other M2 subtypes) also result in up-regulation of Egr2. Therefore, Egr2 serves as a reliable marker of all M2 subtypes. This knowledge of polarization-associated markers facilitates the generation of tools to study macrophage phenotypes in the context of disorders.

Reporter cell lines can be used as efficient ways to probe and monitor cell characteristics in real time. Commonly used immortalized murine macrophages models, such as RAW264.7 and J774, offer a good platform for the generation of macrophage reporter cell lines. As described in Appendix A, Egr2 can serve as a reliable M2-associated marker. Hence, we decided to generate a macrophage reporter cell line that shows changes in fluorescence based on Egr2 expression. This Appendix describes the
approach used to produce macrophages that express mCherry (red fluorescent protein) under the regulation of a murine Egr2 promoter using murine RAW264.7 cells as the parental cell line, and outcomes of the reporter following stable transfection. While the resulting RAW: Egr2-mCherry cells should have shown increased fluorescence intensity under M2-polarizing conditions, and decrease when switching to the M1 state, instead the opposite was found.

**B.2 Materials and Methods**

**B.2.1 Molecular Cloning of Egr2-mCherry Lentiviral Plasmid**

To construct a lentiviral Egr2-mCherry reporter plasmid, a plasmid containing the Mus musculus Egr2/Krox20 promoter region from was obtained from Addgene (Krox20 promoter pGL3-TATA – Plasmid # 21260 from Jerry Crabtree). Primers were designed to amplify out the promoter and gene of interest and included SnabI and BamHI restriction sites (at the 5’ and 3’ ends, respectively) for subcloning the fragment into the pLV-mCherry lentiviral vector (Addgene Plasmid# 36084; http://n2t.net/addgene:36084, from Pantelis Tsoulfas). The PCR primers used to produce the Egr2 promoter product (~1.5kb) and generate matching restriction sites with the backbone vector were purchased from Integrated DNA Technologies (IDT); primer sequences were as follows:

- **Egr2-SnabI** (forward): 5’-ATCTACGTATTACCGCCGGAAGGCGCGCCGC-3’
- **Egr2-BamHI** (reverse): 5’-CGGGATCCCAGGCGCCGGCGTGCAGTGGGA-3’

PCR was performed using Phusion High Fidelity Master Mix (NEB) using optimized PCR conditions due to the high GC content of the primers, which required higher temperatures for the annealing/extension steps (72 °C). Following purification, the Egr2 promoter PCR
product and the pLV-mCherry plasmid were digested with SnaBI and BamHI (NEB) according to manufacturer’s protocols, followed by purification. Ligations were performed using T4 ligase (NEB) according to manufacturer’s protocols. Ligation mixtures were then transformed into STBL3 bacteria (ThermoFisher) and plated for overnight growth in ampicillin-containing agar plates. Single colonies were then picked and transferred into 5 mL of LB media with ampicillin for further expansion in a shaker incubator at 37 °C. 12 h later (with media appearing turbid), the culture was expanded to 50 mL in LB media with ampicillin, and placed back in the incubator for overnight growth. Sanger sequencing was performed by GeneWiz to confirm the final construct.

**B.2.2 Cell Culture**

RAW264.7 cells were purchased from American Type Culture Collection (ATCC). Cells were cultured in T75 culture flasks at 37 °C under a humidified atmosphere containing 5% CO2. Standard growth media consisted of high glucose Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (100 µg/ml penicillin and 100 µg/ml streptomycin) – herein referred to as complete DMEM. Human embryonic kidney (HEK293) cells were obtained from Prof. D. Joseph Jerry (Veterinary and Animal Sciences Department, UMass Amherst), and cultured using the same conditions described for RAW264.7 cells. Under the above culture conditions, the cells were sub-cultured approximately once every 3-4 days and only cells between passages 7 and 20 were used for all experiments.
B.2.3 Generation of Stable RAW:Egr2-mCherry Cells - Lentiviral Transductions

HEK293T cells were seeded in 60 mm culture dishes and transiently transfected with 3 μg psPAX2 packaging plasmid, 2 μg pMD2G envelope plasmid (both from Prof. D. Joseph Jerry, Veterinary and Animal Sciences, UMass Amherst), and 5 μg Egr2-mCherry reporter constructs using Lipofectamine3000 (ThermoFisher Scientific), according to the manufacturer’s instructions. Lentiviral particles were harvested from the supernatant 48 h after DNA-lipid complexes were added to cells. The virus-containing supernatant was passed through a 45 μm filter. Equal volumes of lentivirus-containing supernatant and complete DMEM culture media supplemented with 4 μg/mL polybrene (Sigma) were combined. Confluent RAW264.7 cells were treated with 6 mL of lentivirus-containing media. After performing infections every 12 hours for 48 hours (4 infections total), the medium was replaced with complete DMEM, and the cells were allowed to grow and expand for 2-3 days to ensure a viable population. Cells were then prepared for sorting of positive cells as described below.

B.2.4 Cell Sorting of RAW:Egr2-mCherry

To ensure a homogenous population, cells were sorted twice under different polarizing conditions. For the first sorting, cells were exposed to M1-polarizing cytokines for 24 h (described in Section 6.2) to induce an M1 phenotype. After 24 h, cells were detached from the cell culture flask; 5-7 x 10⁶ cells were resuspended in 3 mL of FACS buffer (4% FBS in phosphate buffered saline (PBS, Gibco)) and sorted at the University of Massachusetts Amherst Flow Cytometry Core Facility using a BD FACSaria Fusion (Becton Dickinson). The instrument was configured with 4 lasers (405 nm, 488 nm, 561 nm, 640 nm), and a 100 μm nozzle size was used for sorting. Of 2.45 x 10⁶ positive cells, the ERW%W2% of
lowest fluorescing cells (494,900 cells with ~98% purity) were sorted. These were plated in T25 flasks for recovery, expansion, and further sorting. For the second sorting, cells were then treated with M2 cytokines for 48 h. Once polarized, 5-7 x 10⁶ cells were resuspended in FACS buffer and sorted using the same instrument and configuration from the first. Of 1.1 x 10⁶ positive cells, the top 10% of fluorescing cells (110,000 cells with ~98% purity) were sorted and plated in T25 flasks for further expansion and use.

**B.2.5 Macrophage Polarization**

To polarize macrophages for sorting, cells were plated in a T75 culture flask and grown at 37 °C under a humidified atmosphere containing 5% CO₂ to confluence prior to polarization. Once confluent, culture media was removed and replaced with media containing either 50 ng/mL interleukin 4 (IL-4; BioLegend) for 48 hr to generate M2 macrophages or 50 ng/mL interferon-gamma (IFN-γ; BD Biosciences) and 50 ng/mL of lipopolysaccharide (LPS; Sigma Aldrich) for 24 h for M1 macrophages. For confocal microscopy and RT-PCR experiments, cells were plated in three biological replicates in an 8-well Lab-Tek II chambered cover-glass system plate (Nunc) or 24-well plates at a density of 100,000 cells/well in 500 µL of media and incubated at 37 °C under a humidified atmosphere containing 5% CO₂. Non-treated macrophages (M0; grown in complete DMEM only) were used as controls. To generate M2 macrophages, 24 h after plating, the media was removed and replaced with complete DMEM media containing 50 ng/mL IL-4 and incubated for an additional 48 h at 37 °C under a humidified atmosphere containing 5% CO₂. To generate M1 macrophages, culture media was removed and replaced with complete DMEM containing 50 ng/mL IFN-γ and 50 ng/mL of LPS 48 h after plating and incubated for an additional 24 h. 72 h after plating (48 h after treatment
and 24 h after treatment with M1 cytokines), cells were used further in experiments as indicated.

**B.2.6 Confocal Microscopy**

For acquisition of representative cell morphology images and fluorescence intensity, RAW: Egr2-mCherry cells were plated in 4- or 8-well Lab-Tek II chambered cover-glass system (Nunc) at a density of 100,000 cells/mL in 500 μL of complete DMEM (50,000 cells/well) and allowed to adhere overnight. Cytokine treatments of RAW: Egr2-mCherry cells were performed starting 24 h after being plated (see Section B.2.5). Cells were imaged every 24 h for up to 72 h depending on the experiment. After polarization, cells were imaged using an Eclipse Ti-E microscope at 10x magnification.

**B.2.7 RNA Extraction and cDNA Conversion**

Cells were lysed, and approximately 1.5 μg RNA was harvested from each well using the PureLink RNA Mini Kit (Ambion) following the manufacturer’s instructions. To convert RNA to complementary DNA (cDNA), 1 μL of 50 μM random hexamers (Applied Biosystems) and 1 μL of 10 mM dNTPs (Thermo Scientific) were added to 11 μL of RNA and heated at 65 °C for 5 min for annealing. Then, 1 μL/sample of 40 U/μL RNaseOut (Invitrogen), 1 μL/sample of 200 U/μL SuperScript IV Reverse Transcriptase (Invitrogen), 1 μL/sample of 100 mM DTT (Invitrogen), and 4 μL/sample of 5x Super Script IV buffer (Invitrogen) were added and amplification proceeded at 53 °C for 10 min and melting at 80 °C for 10 min. The resulting cDNA was frozen at -20 °C and used for RT-PCR experiments within 1 week. RNA and cDNA were quantified using a NanoDrop 2000 (Thermo Fisher). RNA and cDNA contamination and integrity were assessed by analyzing
the A260/A280 ratio, where ratios greater 1.8 for DNA and 2.0 for RNA were considered pure.

B.2.8 Quantitative RT-PCR

RT-PCR was performed on the cDNA generated using a CFX Connect real-time system (Biorad) with iTaq Universal SYBR Green Supermix (Biorad). All DNA primers were purchased from Integrated DNA Technologies. The following primer sequences were used:

- β-actin (forward) 5’-GATCAGCAAGCAGGAGTACGA-3’
- β-actin (reverse) 5’-AAAACGCAGCGCAGTAACAGT-3’
- iNos (forward) 5’-GTTCCTCAGCCCCAAATAACAAGA-3’
- iNos (reverse) 5’-GTGGACGGGTCGATGCAC-3’
- Egr2 (forward) 5’-TGA GAGAGCAGCGGATTGATT-3’
- Egr2 (reverse) 5’-ATAACAGTCAGTGTTCC-3’

The reaction mixture included 200 nM of each primer, 100 ng of cDNA, 10 μL SYBR green supermix, and H2O to a final volume of 20 μL. Analyses were performed as follows: the samples were first activated at 50 °C for 2 min, then 95 °C for 2 min. Denaturing occurred at 95 °C for 30 s followed by annealing at 58 °C; the denature/anneal process was repeated over 40 cycles. Relative gene expression was determined by comparing the Ct value of the gene of interest to that of the β-actin housekeeping gene, by the 2ΔΔCt method. Three biological replicates were performed for each treatment condition and three technical replicates were used for each biological replicate. There was no amplification for the no-template control (NTC). Data was analyzed using the CFX Manager 3.1 software (Biorad). Cq values were generated by using the point at which the sample fluorescence value exceeded the software’s default threshold value. Each sample was normalized to the untreated control.
B.3 Results and Discussion

B.3.1 Molecular Cloning

In pursuit of a macrophage reporter cell line that could reflect polarization state based on changes in fluorescence, I generated a lenti-viral plasmid that places the regulation of the expression of a red fluorescence protein (mCherry) under the control of the Egr2 promoter (Figure B.1).

Figure B.1: Molecular cloning approach for of Egr2-mCherry construct. The Egr2 promoter was amplified from the promoter/donor plasmid (Addgene #21260) to generate matching restriction sites with the lentiviral recipient plasmid. The Cytomegalovirus (CMV) promoter, originally present up-stream from the mCherry sequence was removed using restriction digest and replaced with the Egr2 promoter to produce the reporter construct.

B.3.2 Stable Transfection and Sorting

The Egr2-mCherry plasmid was stably transfected into the parental cell line RAW266.7 as described in the Section B.2.3. Multiple strategies for sorting were used. Initially, in an attempt to obtain a homogenous culture of RAW:Egr2-mCherry cells, I sorted for those
that were positive for the mCherry fluorophore (Figure B.2). However, preliminary confocal experiments suggested that the sorted cells did not have the expected fluorescence intensities following polarization (Figure B.3).

Figure B.2: Sorting of RAW:Egr2-mCherry cells for mCherry-positive cells. Scatterplot for selected RAW:EGR2-mCherry cells.

Figure B.3: RAW:Egr2-mCherry cells sorted for mCherry unexpectedly increase in fluorescence in M1 state and decrease in M2. Microscopy images following polarization of RAW:Egr2-mCherry for validation purposes. Scale bars on confocal images represent 100 µm.
In another sorting approach to attempt to isolate cells with desired responses, cells were polarized before sorting. First, the RAW:Egr2-mCherry cells were polarized to the M1 phenotype, and the bottom 0% of the cells with the lowest fluorescence were sorted (Figure B.4, left). After allowing them to expand, I then polarized these cells to the M2 phenotype via cytokine treatment (IL-4), and selected the top 0% of the cells with the highest fluorescence (Figure B.4, right).

**Figure B.4: Sorting of RAW:Egr2-mCherry cells under phenotype-specific conditions.** Sorting images representing the first (left panels) and second (right panels) sortings of the RAW:EGR2-mCherry cells under M1- and M2-polarizing conditions, respectively.

**B.3.3 Fluorescence Microscopy and Analyses**

I then assessed the abilities of the RAW:Egr2-mCherry cells polarized prior to sortings to alter their fluorescence based on polarization states. To do so, we carried out cytokine-mediated polarizations of the RAW:Egr2-mCherry cell line and imaged the cells using a confocal microscope. Based on previous findings on marker expression shown in *Appendix*.
A, the reporter cell line should have increased fluorescence intensity under M2 conditions, and decreased under M0 and M1. Unpredictably, RAW:Egr2-mCherry showed significantly increased fluorescence when the M1 phenotype was induced, and decreased intensities when polarized to the M2 subtype (Figure B.5).

![Figure B.5](image)

**Figure B.5:** RAW:Egr2-mCherry cells sorted under polarizing conditions increase in fluorescence in M1 state, decrease in M2. Microscopy images following polarization of RAW:Egr2-mCherry for validation purposes. Scale bars on confocal images represent 100 µm.

### B.3.4 RT-PCR

Lastly, I verified the expression of *iNos* (M1-associated) and *Egr2* (M2-associated) markers in polarized RAW:Egr2-mCherry cells via RT-PCR. The expression of these genes is well known in M1 and M2 macrophages (see *Appendix A*). Unexpectedly, but consistent with microscopy evaluations, RT-PCR results show that the RAW:Egr2-mCherry cell line did not express *Egr2* in the same manner as before the lentiviral transfection (higher in M2 and lower in M0/M1; *Figures A.2* and *B.6*, left), suggesting
that cell behavior was likely affected by random insertion of the lentiviral plasmid. Similarly, \textit{iNos} expression was also altered: while it should have been increased in M1 states (\textbf{Figure A.2}), it is now higher in M2 (\textbf{Figure B.6}, right) This data supports the microscopy results and suggests that while the mCherry reporter accurately represents the \textit{Egr2} status of the RAW: \textit{Egr2}-mCherry cells, the behavior of these cells no longer aligns with that of the parental RAW264.7 cell line. Thus, this reporter cell line cannot be used for reliably tracking macrophage phenotypes.

\textbf{Figure B.6: RT-PCR analysis of \textit{iNos} and \textit{Egr2} expression under polarizing conditions.} Shown are results for M2-associated marker, \textit{Egr2} (left) and M1-associated marker, \textit{iNos} (right). M0 (gray) = non-treated, M1 (orange) = LPS/IFN-γ, and M2 (blue) = IL-4. This experiment was run with three biological replicates. Student T-test was used for statistical analysis (p<0.001 = ***, p>0.05 = N.S. (not significant)) relative to M0. Error bars represent standard error.

In conclusion, using a different gene editing tool that ensures the insertion of the gene/sequence of interest at a specific location in the cells’ genome could overcome the outcome here, which was likely related to use of lentiviral transfections. Therefore, further optimization of the gene-editing process is needed in order to use \textit{Egr2} as a reporting gene for macrophage polarization.
B.4 References

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APPENDIX C

COMPARISON OF MACROPHAGE RESPONSES TO FILTERED VERSUS NON-FILTERED CONDITIONED MEDIA

C.1 Introduction

Macrophages are cells of the innate immune system that participate in tissue homeostasis and phagocytosis of pathogens and abnormal cells.\textsuperscript{1} In order to perform these roles, macrophages undergo phenotypic changes, adopting either pro-inflammatory (M1) or anti-inflammatory (M2) behaviors. This process is commonly referred to as macrophage polarization.\textsuperscript{2} In some instances, specific polarization states are associated with the progression of disease, including cancers.\textsuperscript{3} To study macrophage polarization and their responses to stimuli, many studies have used conditioned media (CM) derived from diseased cells, or models thereof.\textsuperscript{4} CM typically includes signaling molecules secreted by cells, including enzymes, growth factors, cytokines/chemokines, and hormones, many of which can influence the behaviors of other cell types.\textsuperscript{5} Additionally, CM from diseased cells contains proteins shed from the cell surface, intracellular proteins released through non-classical secretion pathways, and exosomes that may also influence other cells.\textsuperscript{5} CM generated from cancer cells is commonly used to study the effects of cancer-derived secretomes (secreted factors) on other cell types, such as macrophages.\textsuperscript{6} However, while proven to be a useful tool/model for the study of diseases like cancer, there are some disadvantages to using CM, such as the lack of cell-to-cell interactions.

There are a variety of protocols for the generation of CM from cancer cells. Many of these recommend CM centrifugation and filtration to specifically isolate the cancer cells’ secretomes.\textsuperscript{4} In doing so, remaining tumor cells and cell debris are removed from the
media, and only soluble signaling entities remain. This is likely a limitation when studying macrophages in the context of cancer. Cell-to-cell interactions and the presence of cell debris are known to influence macrophage polarization at the tumor site, and their removal could result in a less realistic version of the tumor micro-environment (TME). Previous studies suggest that the presence of dying cells (either tumor cells and/or other immune cells) in the TME induces an M2-like phenotype in macrophages. This tumor-promoting subtype then aides in generating an immuno-suppressive environment that facilitates cancer growth and metastasis.

To my knowledge, there are no studies that directly compare the effects of filtered (containing no dead cells or cell debris) versus non-filtered (containing dead cells or cell debris) breast cancer-derived CM on macrophage polarization. Thus, this study takes advantage of a RAW:\textit{iNos}\textsuperscript{-}eGFP reporter cell line (described in \textit{Chapter 6}) to investigate macrophage responses to different types and preparation means of conditioned media. The results obtained demonstrate that macrophage responses to the CM differ, and that the means by which it is prepared should be considered in study design and analysis. This work may also help establish appropriate conditions for the generation of conditioned media and its subsequent use to assess macrophage polarization in the context of breast cancer or other diseases.

C.2 Materials and Methods

C.2.1 Cell Culture

4T1 and EMT6 murine mammary carcinoma cells were purchased from the American Type Culture Collection (ATCC). The generation of RAW:\textit{iNos}\textsuperscript{-}eGFP cells is described in
Chapter 6. All cell types were cultured in T75 culture flasks at 37 °C under a humidified atmosphere containing 5% CO₂. Standard growth media consisted of high glucose Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (100 μg/ml penicillin and 100 μg/ml streptomycin) – herein referred to as complete DMEM. Under the above culture conditions, the cells were subcultured approximately once every 3-4 days and only cells between passages 7 and 20 were used for all experiments.

C.2.2 Generation of Conditioned Media from Cells

RAW:iNos-eGFP, 4T1, and EMT6 cells were (separately) cultured using complete DMEM at 37 °C under a humidified atmosphere containing 5% CO₂ until they became confluent. At this time, the media was replaced with complete DMEM, and cells were incubated for an additional 7 days. The supernatant from each cell line was then collected and stored at -20 °C (for non-filtered CM). Alternatively, the supernatant from each cell line was filtered using a 0.45 μm syringe filter then stored at -20 °C (for filtered CM).

C.2.3 Treatments with RAW:iNos-eGFP, 4T1, and EMT6 Conditioned Media

RAW:iNos-eGFP cells were plated in an 8-well plate at a cell density of 50,000 cells/well in 500 μL complete DMEM and allowed to adhere overnight at 37 °C, 5% CO₂. After 24 h, the culture media was removed and replaced with 60% complete DMEM and 40%-conditioned media from RAW:iNos-eGFP, 4T1, or EMT6 cells (see Section C.2.2). A 40% PBS-60% complete DMEM treatment (referred to as PBS) was used as control. Cells were incubated with the conditioned media for 48 h and were then assessed via confocal microscopy, described below.
C.2.4 Confocal Microscopy and Fluorescence Intensity Quantification

For acquisition of representative cell morphology and fluorescence images, RAW:iNos-eGFP cells were plated in an 8-well Lab-Tek II chambered cover-glass system (Nunc) and treated according to the experimental conditions. They were then imaged every 24 h for up to 72 h using a Nikon Eclipse Ti-E microscope at 10x magnification. ImageJ/Fiji software was used for the quantification of fluorescence of the confocal images on a per-cell basis via thresholding method. Each replicate for each experimental group (each image) included between 300-2000 cells for which the integrated fluorescence intensity (mean fluorescence X area of the cell) was defined.

C.3 Results and Discussion

To compare the effects of conditioned media (CM) generated using different approaches, I prepared both filtered and non-filtered CM from mammary carcinoma cell lines 4T1 and EMT6, and the RAW:iNos-eGFP cell line. We then exposed the macrophage reporter cell line RAW:iNos-eGFP cells to the different CM treatments for 48 h and tracked changes in fluorescence. Cells cultured with complete DMEM only were used as a non-treated (N.T.) control group. A 40% PBS-60% complete DMEM (“PBS” group) was used as a control to account for the removal of nutrients from the CM in the other treatments, where 40% of the solution is CM and 60% is fresh complete DMEM. Filtered and non-filtered CM from RAW:iNos-eGFP cells was used to ensure that the changes observed in the experimental groups treated with cancer cell line-derived CM were specific to effects of the cancer cells.

Interestingly, confocal microscopy images (Figures C.1a and C.1b) and their per-cell fluorescence quantification (Figure C.1c) suggest that filtered CM did not affect the
polarization state of the RAW: iNos-eGFP cells significantly, except for the EMT6 (filtered) group, which appeared to produce a pro-inflammatory response. However, when the RAW: iNos-eGFP cells were exposed to non-filtered CM from any the cell types, including the RAW: iNos-eGFP cells themselves, the iNos-eGFP signal decreased significantly relative to the non-treated (N.T.) group, similar to an M2-like subtype (see Figure 6.4b). These results question the filtration step and indicate that many relevant factors, like cell debris, that influence macrophage polarization process are being removed when using a syringe filter. Intriguingly, RAW: iNos-eGFP cells exposed to CM derived from themselves displayed a greater decreased in fluorescence, signifying a more immunosuppressive phenotype than in the other experimental groups.

This data suggests that the presence of cell debris, especially from other dying macrophages, in the unfiltered media could be a major driver of M2-like polarization. This possibility is particularly relevant in the context of diseases like cancer, where there is an increased number of apoptotic/dying cells in the tumor microenvironment that could promote the polarization of macrophages toward a tumor-helping subtype. These findings offer the possibility of an alternate therapeutic strategy in which interference with the sensing, targeting and removal of cell debris and dying cells could prevent the polarization of macrophages toward a tumor-helping M2 subtype.
Figure C.1: RAW:iNos-eGFP responses to filtered or non-filtered conditioned media derived from different cell types. (a) Representative confocal images of RAW:iNos-eGFP macrophages after 48 h of exposure to filtered (F) or non-filtered (U) conditioned media derived from RAW:iNos-eGFP (RAW), 4T1 cells (4T1), EMT6 cells (EMT6), or a control PBS-treated group. Top panel shows brightfield images, while bottom panel displays the eGFP channel. Scale bars represent 100 µm. (b) Per-cell fluorescence quantification of confocal images from (a). N.T.=non-treated cells, PBS=40% PBS/60% DMEM. Student T-test was used for statistical analysis (p<0.01 = **, p<0.001 = ***, p>0.05 = N.S. (not significant)), relative to N.T. Error bars represent standard error.
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