

# Navigating Macrophage Polarization by Tumors

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## Abstract:

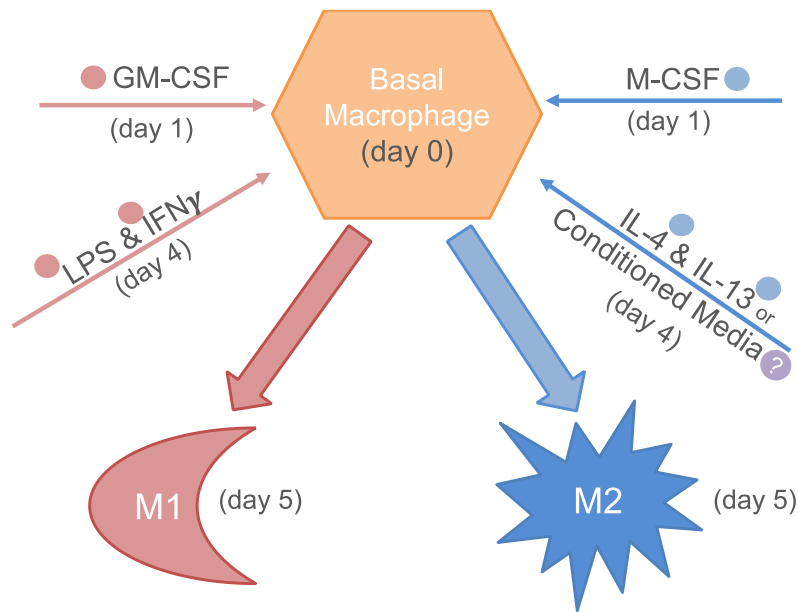
Macrophages are able to polarize from a basal, naïve state to an M1 or M2 phenotype. M1 pro-inflammatory macrophages are characterized by recognizing and eliminating abnormal cells, while M2 anti-inflammatory macrophages are characterized by tissue repair and healing. Polarization toward these phenotypes is induced by the cellular release of specific cytokines. In a tumorigenic environment, cancer cells manipulate the polarization of macrophages by directly releasing cytokines to stop the M1 attack response, and instead induce the M2 phenotype. These hijacked macrophages are called tumor-associated macrophages (TAMs) and are used to sustain an environment that promotes angiogenesis and tumor growth and development. Polarization toward M1 is induced by LPS and IFN- $\gamma$  cytokines, while polarization toward M2 is induced by IL-4 and IL-13. Experiments were conducted using a murine macrophage cell line (RAW264), murine bone marrow-derived primary macrophages, and a human macrophage cell line (THP-1) to observe cytokine-induced macrophage polarization. Additionally, human glioblastoma cell lines (U87 and T98G) were used to observe the effects of tumor-conditioned media on macrophage polarization in the murine bone marrow-derived primary macrophages and human THP-1 macrophage line. Real-time PCR confirmed the accurate polarization of macrophages treated with M1 and M2-polarizing cytokines, as well as the polarization of macrophages treated with tumor-conditioned media by measuring the mRNA expression of M1 and M2-specific target genes.

## Introduction:

Glioblastomas are the most common primary adult human brain tumor, with 17,000 new diagnoses per year<sup>[4]</sup>. They are classified as grade IV malignant tumors and are very difficult to treat due to their location in the brain and their ability to infiltrate other regions of the brain with their elongated projections<sup>[8]</sup>. Consequently, patients diagnosed with glioblastomas usually have a dismal prognosis and poor quality of life as the disease progresses<sup>[1]</sup>. Treatments for glioblastomas involve a combinatorial approach of surgery, radiation, chemotherapy, and angiogenesis inhibitors. Still, the complete removal of glioblastomas is often unsuccessful because tumor growth and extraction occurs in regions of the brain that are vital for everyday life, including language, coordination, essential reflexes, vision hearing, etc. Furthermore, the average survival period is 12-14 months after diagnosis and treatment<sup>[1]</sup>, with less than 10% of patients surviving 2 years after diagnosis<sup>[2]</sup>. Additionally, patients who survive glioblastomas encounter neurological deficits, impairment of cognition, psychological distress, reduced social function, and future uncertainty<sup>[2]</sup>. The pressing need for a successful treatment has led to the rapid research and development of immunotherapy treatments, in which immune cells are activated to eliminate cancer cells without or in combination with other current cancer treatments<sup>[3]</sup>.

The immune system has many types of leukocytes, white blood cells, that protect the body from pathogen-related infections. Macrophages are a type of leukocyte which, when activated, are responsible for the engulfment of pathogen and cell debris and the overall repair and growth of tissues. Before the macrophage becomes activated, the macrophage is in a basal state, also known as a naïve macrophage. Macrophages have the unique ability to polarize toward two phenotypes, depending on the need of the local environment. Polarization is not fixed, as macrophages are sufficiently plastic to integrate multiple signals, such as those from microbes, damaged tissues, and the normal tissue environment<sup>[6]</sup>. In the presence of antigen,

macrophages travel to the site of an infection and become activated toward the M1 phenotype. M1 macrophages induce a pro-inflammatory response in which foreign cells and cell debris are recognized and eliminated by the M1 macrophages [5]. The polarization of basal macrophages toward M1 is essential to the immune system, as they defend the body from infection and sustain a healthy internal environment. Subsequent to M1 activation, macrophages must be polarized toward an alternative phenotype, M2, to promote healing in localized tissues where M1 macrophages previously acted. An anti-inflammatory response is induced by M2 macrophages, allowing for an increased recruitment of blood vessels to provide the oxygen and nutrients needed to repair the tissue [5]. The body's dependence on both M1 and M2 phenotypes are essential for the overall health of the body and functionality needed to respond to the various conditions of the tissues.



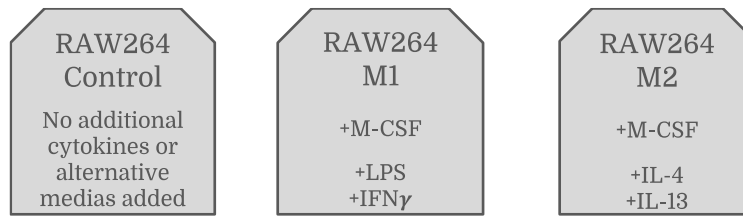
**Figure 1:** Methods used to induce macrophage polarization toward M1 or M2.

Cytokines are small substances released by immune cells for signaling or communication with nearby cells. Macrophages utilize this form of communication, as it directly influences macrophage polarization and affects the way macrophages respond to the environment. Experiments involving macrophage polarization have demonstrated that granulocyte-colony-stimulating factor, GM-CSF, primes macrophage polarization toward the M1 phenotype, while macrophage colony-stimulating factor, M-CSF, primes the macrophages toward the M2 phenotype. Additional cytokines such as LPS and IFN- $\gamma$  further promote the polarization toward M1, while IL-4 and IL-13 further promote the polarization toward M2 [5] (**Figure 1**). In a tumorigenic environment, cancer cells subvert the polarization of macrophages by directly releasing cytokines, resulting in the conversion of M1 macrophages to tumor-hijacked macrophages. These hijacked macrophages are called tumor-associated macrophages, TAMs, and are the most abundant immune cells present within the tumor. The polarization toward TAMs enable tumor survival by suppressing M1 attack and positively correlate with tumor growth [7]. TAMs are characteristically similar to M2 macrophages, supplying the tumor with an abundance of nutrients and an oxygen-rich environment in which the tumor can live and grow indefinitely [7].

From the previous research done involving cytokine-induced polarization of various macrophage lines, experimentation began *in vitro* with a murine macrophage cell line, RAW264, to confirm it whether macrophage polarization could be replicated in our lab. Trends regarding M1 and M2 polarization from the undifferentiated RAW264 cells were analyzed, as well as trends observed in the mRNA expression levels of M1 and M2-specific target genes from each of the experimental groups. Once M1 and M2-specific trends were observed, additional macrophage models were selected to determine which model was most optimal for studying cytokine-induced macrophage polarization. To observe whether macrophage polarization was attainable in primary macrophages, murine bone marrow-derived cells were extracted, cultivated *in vitro*, and treated with M1 and M2-specific cytokines. Moreover, to observe whether macrophage polarization was attainable in human macrophages, a human THP-1 cell line was cultivated *in vitro* and treated with M1 and M2-specific cytokines. Additionally, glioblastoma cell lines, U87 and T98G, were used to observe the effects of human tumor-conditioned media on macrophage polarization of the murine bone marrow-derived primary macrophages and human THP-1 macrophage line; tumor-conditioned media being the proper cultivation of media-specific tumor cells followed by an induced stressful low-glucose environment with the expectation that cytokines will be released into the media. The conditioned media is then collected and placed on the macrophage cell lines, allowing macrophage polarization to occur. It was hypothesized that the addition of tumor-conditioned media, in place of M2-polarizing cytokines, would polarize macrophages toward the M2 phenotype due to the tumor's increased need for angiogenesis. In determining these specific aims, innate macrophage polarization and tumor-induced macrophage polarization can be better understood. Ultimately, this can lead to advancements in modern knowledge of tumor behavior, as it pertains to the immune system, and create a baseline in which immunotherapy can be further studied.

#### Methods:

*In expanding on previous research, experimentation with the murine RAW264 cell line was done to confirm the accurate polarization of macrophages toward M1 and M2, with the use of M1 and M2-specific cytokines. To further this, a thorough literature search was done to find additional macrophage models that are susceptible to cytokine-induced polarization, as well as if macrophage polarization could be induced via treatment with conditioned media. To do so, murine bone marrow-derived cells were selected for experimentation to further study the differentiation of primary macrophages toward M1 or M2, with the use of cytokines or conditioned media. Furthermore, the human THP-1 cell line was selected for experimentation to better understand cytokine and conditioned media-induced macrophage polarization in a human macrophage model, with the use of human glioblastoma lines U87 and T98G. Challenges did arise from working with the human THP-1 cell line, as these cells were propagated and grown entirely in suspension.*

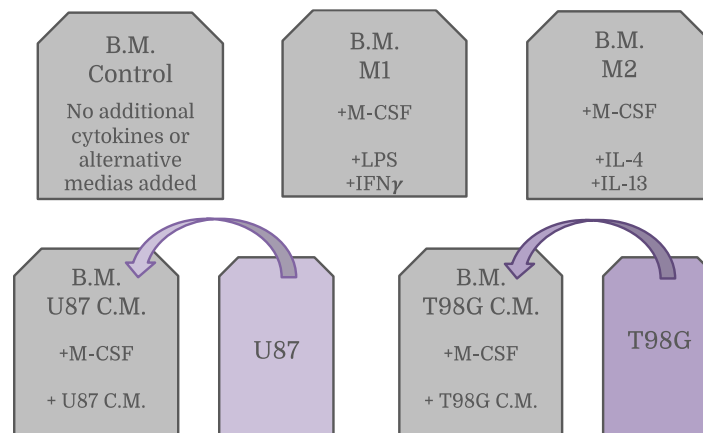


**Figure 2:** Graphic depiction of methodology used for RAW264 cell line.

### RAW264 Experiment – Murine Macrophage Cell Line

(Figure 2) To test the polarizing effects of cytokines on macrophages, a murine macrophage cell line was used, RAW264. Three experimental groups were used: control, M1, and M2.

Propagation, feedings, splits were done on the same days for each of the three groups. The media used for each group consisted of 4.5 g/L glucose DMEM media, 10% FBS, and 1% ANTI-ANTI. For the control flask, no additional cytokines or media were added to the control flask. For the M1 flask, the macrophages were treated with cytokines GM-CSF on day 1, LPS and IFN- $\gamma$  on day 4, and then harvested on day 5. For the M2 flask, the macrophages were treated with cytokines M-CSF on day 1, IL-4 and IL-13 on day 4, and then harvested on day 5. Real-time PCR was performed to measure the gene expression of specific M1 or M2 genes: INOS (M1), CD68 (M1), MMR (M2), CD163 (M2), Arg-1 (M2).

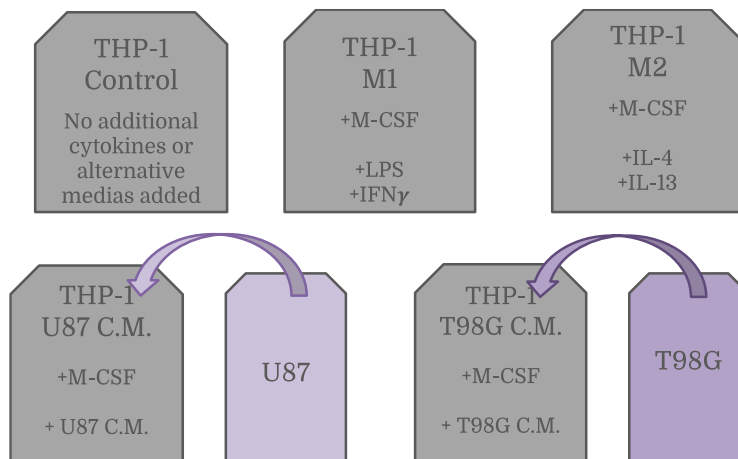


**Figure 3:** Graphic depiction of methodology used for bone marrow-derived primary macrophages.

### Bone Marrow Experiment – Murine Bone Marrow-Derived Primary Macrophages

(Figure 3) Murine bone marrow-derived primary macrophages were used to further evaluate the polarizing effects of cytokines or conditioned media on macrophages. Five experimental groups were used: control, M1, M2, U87 Conditioned Media, T98G Conditioned Media. The media used for each group consisted of DMEM/F12 media, 10% FBS, and 1% ANTI-ANTI. For the control flask, no additional cytokines or media were added to the control flask. For the M1 flask, the macrophages were treated with GM-CSF on day 1, LPS and IFN- $\gamma$  on day 4, and then harvested on day 5. For the M2 flask, the macrophages were treated with M-CSF on day 1, IL-4 and IL-13 on day 4, and then harvested on day 5. The U87 and T98G flasks were each grown up

with EMEM media, 10% FBS, and 1% ANTI-ANTI, but on day 3 they were fed with 1 g/L glucose DMEM, 10% FBS, and 1% ANTI-ANTI, to mimic a nutrient-poor environment. Cells were incubated overnight, to allow for cytokine release from the tumor, and on day 4 the media and cytokines were transferred to each appropriate macrophage flask, U87 C.M or T98G C.M. For the U87 C.M or T98G C.M flasks, the macrophages were treated with M-CSF on day 1 and then treated with their appropriate tumor's media and cytokines on day 4; harvesting was done on day 5. A real-time PCR was performed to measure the gene expression of specific M1 or M2 genes: INOS (M1), CD68 (M2), MMR (M2), CD163 (M2).



**Figure 4:** Graphic depiction of methodology used for human THP-1 cell line.

#### **THP-1 Experiment – Human Macrophage Cell Line**

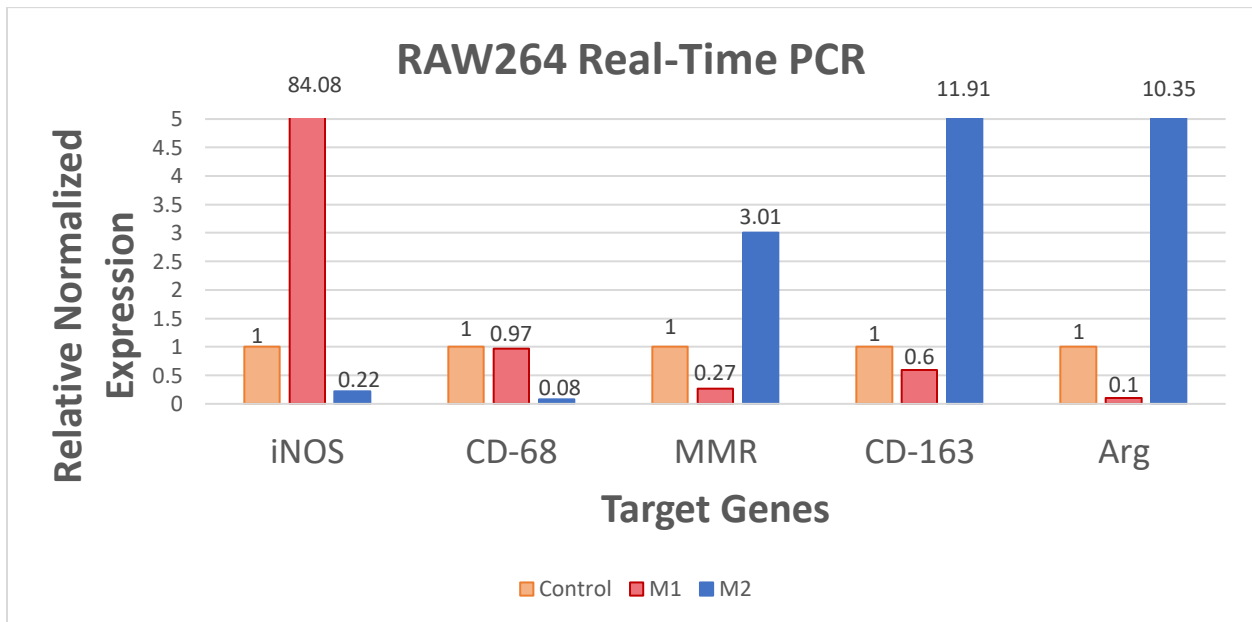
**(Figure 4)** Human THP-1 cells were used to further evaluate the polarizing effects of cytokines or conditioned media on macrophages. Five experimental groups were used: control, M1, M2, U87 C.M, T98G C.M. The media used for each group consisted of RPMI media, 10% FBS, and 1% ANTI-ANTI. For the control flask, no additional cytokines or media were added to the control flask. For the M1 flask, the macrophages were treated with GM-CSF on day 1, LPS and IFN- $\gamma$  on day 4, and then harvested on day 5. For the M2 flask, the macrophages were treated with M-CSF on day 1, IL-4 and IL-13 on day 4, and then harvested on day 5. The U87 and T98G flasks were each grown up with EMEM media, 10% FBS, and 1% ANTI-ANTI, but on day 3 they were fed with 1 g/L glucose DMEM, 10% FBS, and 1% ANTI-ANTI, to mimic a nutrient-poor environment. Cells were incubated overnight, to allow for cytokine release from the tumor, and on day 4 the media and cytokines were transferred to each appropriate macrophage flask, U87 C.M or T98G C.M. For the U87 C.M or T98G C.M flasks, the macrophages were treated with M-CSF on day 1 and then treated with their appropriate tumor's media and cytokines on day 4; harvesting was done on day 5. A real-time PCR was performed to measure the gene expression of specific M1 or M2 genes: INOS (M1), PPAR $\alpha$  (M2), MMR (M2), Arg-1 (M2), CD163 (M2).

**Results:**

**Raw264-** Polarization of murine RAW264 macrophages toward M1 or M2 was observed when treated with M1 or M2-specific cytokines. Real-time PCR confirmed the polarization of these macrophages by measuring the gene expression of M1 and M2 specific target (M1: iNOS; M2: CD68, MMR, CD163, Arg-1). These targets and their increased gene expression for, either, M1 or M2 determined whether the macrophage has been polarized toward an M1 or M2 phenotype. The increase in iNOS for the M1 experimental group signified accurate M1 polarization, while the increase in MMR, CD163, and Arg-1 of the M2 experimental group signified accurate M2 polarization. However, the data from the target gene, CD68, was found to be inconclusive because the gene expression measured for M1 and M2 were less than the gene expression from the control group (**Figure 5 and 6**). Nevertheless, the polarization of RAW264 macrophages using specific cytokines was confirmed with the measurements of M1 and M2- specific target genes.

	Control	M1	M2
iNOS	1	84.08	0.22
CD-68	1.00	0.97	0.08
MMR	1.00	0.27	3.01
CD-163	1	0.6	11.91
Arg-1	1	0.1	10.35

**Figure 5:** Real-time PCR gene expression of M1-specific genes (iNOS) and M2-specific genes (CD68, MMR, CD163, and Arg-1) for RAW264 experimental groups: control, M1, and M2.

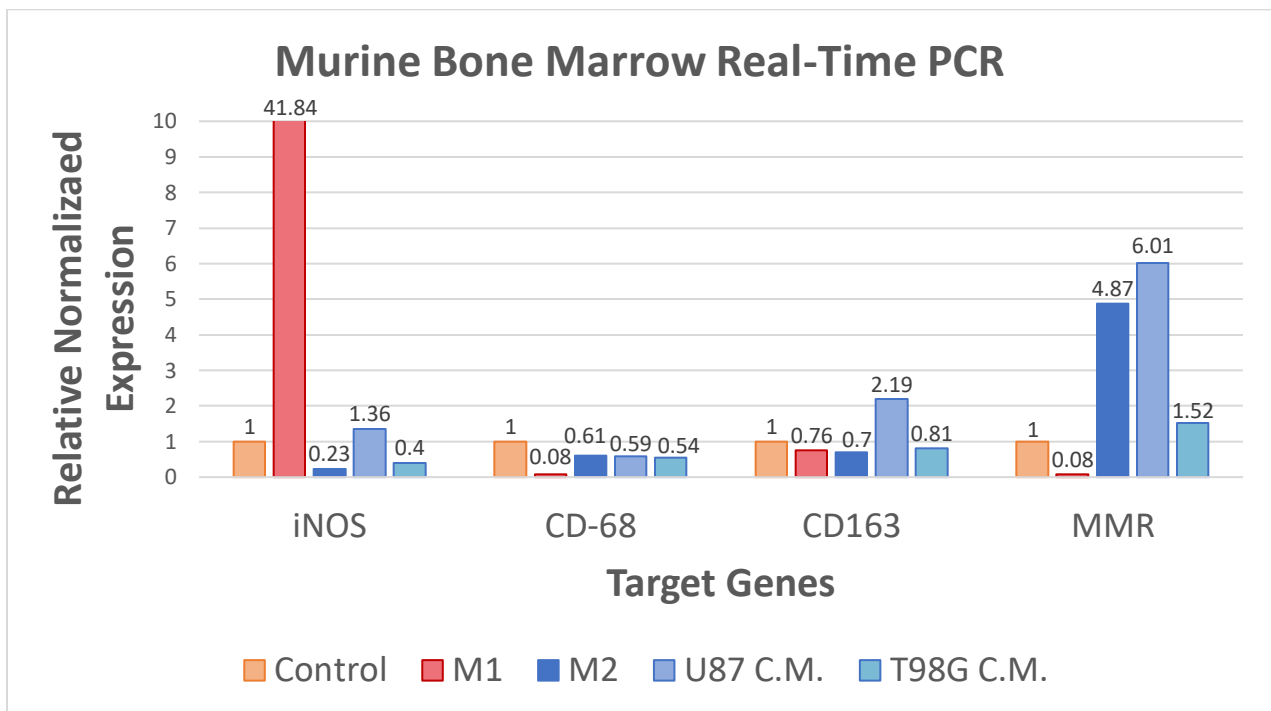


**Figure 6:** Visual comparison of the measured gene expression of specific M1 and M2 target genes from each of the differentiated murine RAW 264 macrophage line's experimental groups: control, M1, and M2.

**Murine Bone Marrow-** The polarization of murine bone marrow-derived primary macrophages toward M1 and M2, using M1 or M2-specific cytokines or tumor-conditioned media, was confirmed via real-time PCR. The increased gene expression of iNOS for the M1 experimental group signified accurate M1 polarization, while the increase in CD68, CD163, and MMR of the M2 experimental group signified accurate M2 polarization. Additionally, the use of tumor-conditioned media, in place of M1 and M2-polarizing cytokines, were able to polarize the murine bone marrow experimental groups, U87 C.M and T98G C.M, to the M2 phenotype. This polarization was confirmed by observing the increase in gene expression of M2-specific genes and the decreased gene expression for the M1-specific gene in the U87 C.M and T98G C.M flasks (**Figure 7 and 8**).

	Control	M1	M2	U87 C.M.	T98G C.M.
iNOS	1	41.84	0.23	1.36	0.4
CD-68	1	0.08	0.61	0.59	0.54
CD163	1	0.76	0.7	2.19	0.81
MMR	1	0.08	4.87	6.01	1.52

**Figure 7:** Real-time PCR gene expression of M1-specific genes (iNOS) and M2-specific genes (CD68, CD163, and MMR) for murine bone marrow experimental groups: control, M1, M2, U87 C.M, and T98G C.M.



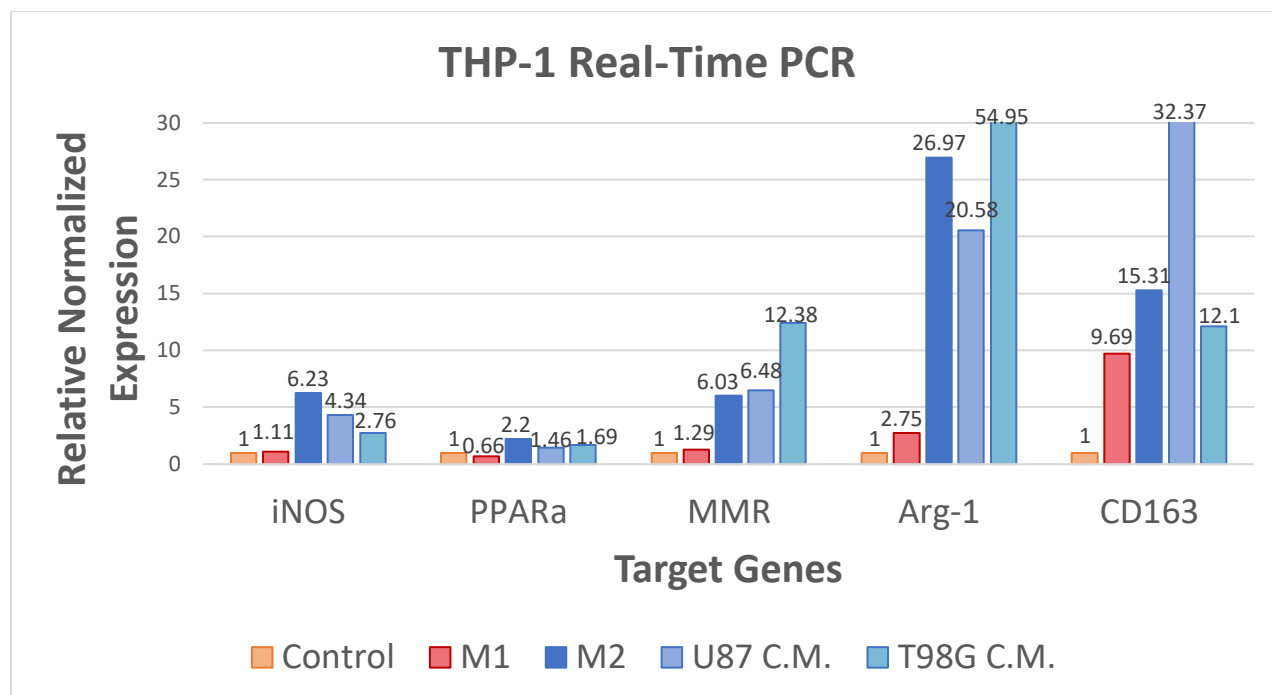
**Figure 8:** Visual comparison of the measured gene expression of specific M1 and M2 target genes from each of the differentiated murine bone marrow primary macrophages' experimental groups: control, M1, M2, U87 C.M, and T98G C.M.



**THP-1-** The human THP-1 macrophage line was successfully polarized toward the M2 phenotype for the experimental groups: M2, U87 C.M, and T98G C.M. Polarization was accurately induced with the addition of M1 or M2-specific cytokines, or the addition of tumor-conditioned media. Real-time PCR revealed the accurate polarization of these experimental groups, with each of them having an increased gene expression for the M2-specific genes (PPAR $\alpha$ , MMR, Arg-1, CD163). Conversely, M1 polarization was not supported by the gene expression of iNOS in the THP-1 experiment. The data from iNOS was found to be inconclusive because the gene expression measured for M1 was similar to the gene expression from the control. Additionally, the iNOS gene expression of M2, U87 C.M, and T98G C.M were measured to be 16 times higher (2<sup>4</sup>) than the gene expression of the control and M1 groups. However, the M2-specific gene expression of the U87 and T98G were most comparable to that of the M2 experimental group, which supported that the macrophages were polarized toward M2 (**Figure 9 and 10**).

	Control	M1	M2	U87 C.M.	T98G C.M.
iNOS	1	1.11	6.23	4.34	2.76
PPAR $\alpha$	1	0.66	2.2	1.46	1.69
MMR	1	1.29	6.03	6.48	12.38
Arg-1	1	2.75	26.97	20.58	54.95
CD163	1	9.69	15.31	32.37	12.1

**Figure 9:** Real-time PCR gene expression of M1-specific genes (iNOS) and M2-specific genes (PPAR $\alpha$ , MMR, Arg-1, and CD163) for human THP-1 experimental groups: control, M1, M2, U87 C.M, and T98G C.M.



**Figure 10:** Visual comparison of the measured gene expression of specific M1 and M2 target genes from each of the differentiated human THP-1 cell line’s experimental groups: control, M1, M2, U87 C.M, and T98G C.M.

Conclusion:

According to previous research of macrophage polarization, experiments were done in our lab using murine RAW264 cells which supported cytokine-induced polarization toward M1 or M2 phenotypes. Because of our lab’s success in polarizing murine RAW264 cells, additional polarizable macrophage models were investigated to obtain an optimal macrophage model for further research in our lab. Herein, two additional macrophage models, murine bone marrow-derived primary macrophages and human THP-1 macrophages, were successfully polarized toward M1 or M2 when treated with M1 or M2-specific cytokines. Additionally, the use and treatment of tumor-conditioned media on macrophages was seen to have successfully polarized the murine primary macrophages and the human THP-1 macrophages toward the M2 phenotype; thus, resembling and supporting the behavior observed within the tissues of a tumorigenic environment. Furthermore, certain M2 target genes had higher mRNA expression levels in both the conditioned media-polarized groups compared to the cytokine-polarized M2 group, indicating that the cytokines directly released by the tumor may drive polarization toward the M2 phenotype more robustly than the addition of commercial M2-polarizing cytokines. Of the two macrophage models, the human THP-1 cells were polarized more robustly by cytokines and conditioned media than the murine bone marrow-derived primary macrophages, making it the optimal macrophage model for further research. However, of the two human tumor cell lines, both U87 and T98G cell lines were equally able to induce macrophage polarization.

These findings provoke questions regarding the mechanisms in which tumors subvert macrophage polarization. To better understand the environment a tumor creates, additional experiments should be done in which pre-existing M1-differentiated macrophages are treated with tumor-conditioned media to observe whether macrophage polarization toward the M2 phenotype is conserved. For this, undifferentiated THP-1 cells would be polarized toward M1

macrophages using GM-CSF, LPS and IFN- $\gamma$ , followed by the treatment of tumor-conditioned media to induce macrophage re-polarization. The results from this would further support the premise that tumors subvert macrophage polarization and would distinguish which macrophages, differentiated or not, are targeted for tumor growth. Next, additional chemistry fragmentation techniques could be done on the cytokines in tumor-conditioned media to elucidate which cytokines are released by the tumor. Once the tumor-secreted cytokines are identified, further experiments can be done to understand the mechanisms by which a tumor affects macrophage polarization and the signaling events which mediate the changes observed, ultimately for the use of immunotherapy in the future.

## Works Cited

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