

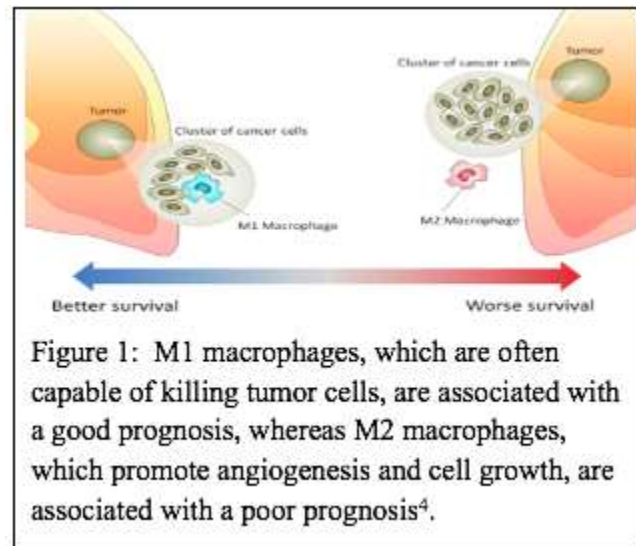
Cancer Proliferation Mediated by Tumor-Associated Macrophages (TAMs)

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Abstract

Glioblastoma multiforme (GBM) is an exceptionally deadly form of brain cancer that consistently evades treatment. Macrophages are a type of immune cell present in the tumor microenvironment that can be polarized by the cancer cells to aid in the growth and proliferation of the tumor. The goal of this study is to decipher the communication between glioblastoma cells by determining the pathway by which macrophages are polarized toward the M2 (pro-tumor) phenotype. Once the pathway is determined, treatments can be developed that inhibit the pathway, which would subsequently halt the growth of the tumor.



Background and Significance

Glioblastoma, also known as glioblastoma multiforme (GMB), is a malignant, grade IV brain tumor with an annual incidence of 3 in 100,000 people in America alone, accounting for over half of all brain tumor cases¹. GBM has a very poor prognosis; less than 5% of people live past five years post-diagnosis¹. Thus, there is a need to better understand the mechanisms associated with progression of GBM. In order to fully understand cancer, it is not sufficient to study the tumor alone. Instead, it is also necessary to consider the microenvironment in which the tumor resides. The tumor microenvironment consists of various non-cancerous cells such as fibroblasts, immune cells, endothelial cells (tumor vasculature), as well as extracellular proteins produced by the cancer cells².

One important constituent of the tumor microenvironment is the macrophage. When macrophages mature, they can be polarized toward one of two phenotypes, M1 or M2, as a result of cell signals or environmental cues. M1 macrophages promote cell death, associated with fighting infection or eliminating abnormal cells, including tumor cells, while M2 macrophages promote tissue healing, including cell growth and proliferation. Thus, tumor infiltration of M1 versus M2 macrophages are associated with a better or worse prognosis, respectively (Figure 1).

In preliminary studies, we demonstrated polarization of THP-1 macrophages. THP-1 cells are an immortalized M0 human cell line that are often used to study macrophages in vitro. THP-1 macrophages were polarized towards the M1 or M2 phenotypes by exposure to cytokines such as LPS and IFN- γ or IL-4 and IL-13, respectively, confirming previous published scientific literature³. Thus, a sufficient method of analysis has already been established. However, in order to study macrophage polarization with regard to cancer, signals released by cancer cells must be used rather than specifically added cytokine cocktails. In the tumor microenvironment, cancer cells release signals that polarize macrophages toward the M2 (pro-tumor) phenotype, resulting in increased cell growth and cancer proliferation⁵. Thus, we wish to determine the signals, and

the resulting signaling pathways by which glioblastoma cells influence the macrophage polarization to promote their own survival and proliferation.

Cancer cells release signals, similar to those tested previously, that polarize macrophages toward the M2 (pro-tumor) phenotype which results in increased cell growth and cancer proliferation³. Thus, a high proportion of M2 macrophages is typically associated with a poor prognosis⁴. However, further studies are needed regarding the signaling mechanisms by which tumors polarize macrophages to this pro-tumor phenotype. Three signaling pathways are hypothesized to influence this polarization - JAK-STAT6, PPAR Beta and PPAR Gamma.

JAK-STAT is an abbreviation for the Janus kinase (JAK), signal transducer of activation (STAT) pathway, which is involved in gene expression by extracellular factors such as cytokines or interferons (IFNs)⁶. The JAK-STAT pathway allows communication between transmembrane (cytokine) receptors and the nucleus. Janus kinase (JAK) protein is located on some transmembrane proteins, and, upon a ligand binding to the receptor, JAKs are activated and phosphorylated by one another, which activates STATs. STATs are able to directly bind to DNA and regulate gene expression⁶. STAT6 is a specific membrane within the signal transducer and activator of transcription (STAT) family⁷. STAT6 is activated by interleukin-4 (IL-4) and interleukin-13 (IL-13) and has the same ability to bind directly to DNA as the other known STATs⁷. As we previously demonstrated, our THP-1 cell model polarizes towards the M2 phenotype upon the addition of IL-4 and IL-13. Thus, it seems logical that this cytokine induction is mediated, at least partially, by the STAT6 transcription modulator. The JAK-STAT pathway has been linked to cancer proliferation⁸, which makes it a good option as a pathway to study.

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors related to energy metabolism⁹. They serve as transcription factors, which are proteins involved in transcribing DNA to RNA, and are activated by the binding of ligands. PPAR beta (PPAR- β) and PPAR gamma (PPAR- γ) are two main subtypes of the PPAR family. PPAR gamma is linked to the inflammatory response¹⁰, and the role of PPAR beta depends on the environment as to whether it supports cancer proliferation or the inflammatory response¹¹. Macrophages are present at inflammatory sites, and polarized accordingly, which causes PPAR beta and PPAR gamma to be worthy of investigation.

This study aims to extrapolate the role of these pathways - JAK-STAT, PPAR beta, and PPAR gamma - in the basic mechanism of macrophage polarization by glioblastoma cells. Furthermore, novel computer programming will be created to automate the analysis of qPCR data used to determine activation status.

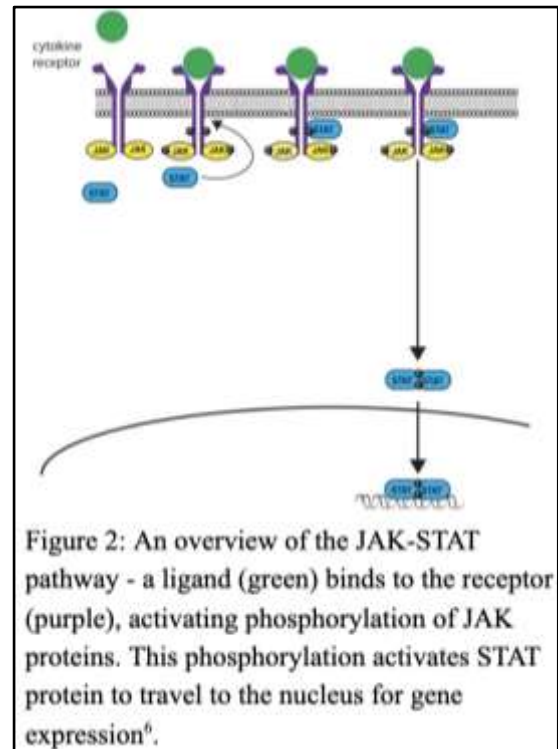


Figure 2: An overview of the JAK-STAT pathway - a ligand (green) binds to the receptor (purple), activating phosphorylation of JAK proteins. This phosphorylation activates STAT protein to travel to the nucleus for gene expression⁶.

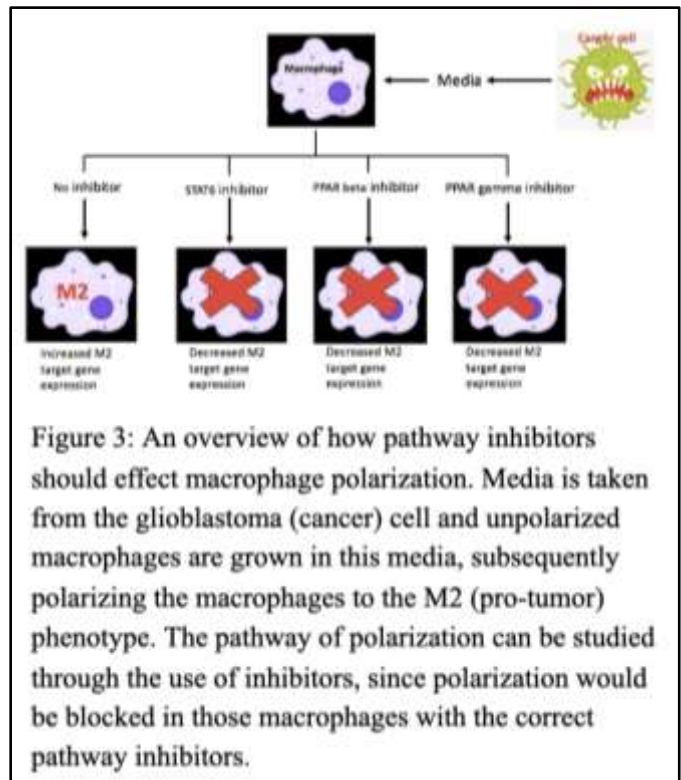
Methods

Cell Culture

Human monocytic THP-1 cells (ATCC TIB-202) were maintained in culture in Roswell Park Memorial Institute medium (RPMI 1640, Gibco) culture medium containing 10% of heat inactivated fetal bovine serum (Invitrogen). T98G glioblastoma tumor cells (ATCC CRL-1690) were cultured in Eagle's minimal essential medium (EMEM, Gibco) containing 10% fetal bovine serum.

THP-1 monocytes were differentiated into macrophages by 24 hour incubation with 150 nm phorbol 12-myristate 13-acetate (PMA, Sigma, P8139) followed by 24 hour incubation in RPMI medium. Macrophages were polarized to M1 macrophages by 24 hour incubation with 20 ng/ml of interferon-gamma, IFN- γ (Sigma Aldrich), and 10 pg/ml of lipopolysaccharide, LPS (Sigma Aldrich). Alternatively, macrophages were polarized to M2 macrophages by 48 hour incubation with 20 ng/ml of interleukin 4, IL-4 (Sigma Aldrich), and 20 ng/ml of interleukin 13, IL-13 (Sigma Aldrich). Conditioned media (CM) refers to the low-glucose media with serum that T98G cells were maintained in for a period of 3 days.

In the co-culture experiments, THP-1 monocytes were differentiated in a 6 well plate, unadhered using trypsin, and transferred to the top membrane of 6 Transwell inserts (membrane pore size of 0.4 μ m, Corning, #3450). The 6 Transwell inserts containing THP-1 macrophages were transferred to a 6 well plate containing T98G cells in EMEM media containing 10% fetal bovine serum. 300nM of STAT6 antagonist (Sigma Aldrich AS1517499), 1 μ M of PPAR Beta antagonist (Sigma Aldrich GSK3787), or 1 μ M of PPAR Gamma antagonist (Sigma Aldrich T0070907) were added. Cells were co-cultured, with or without antagonists, for 24 hours.



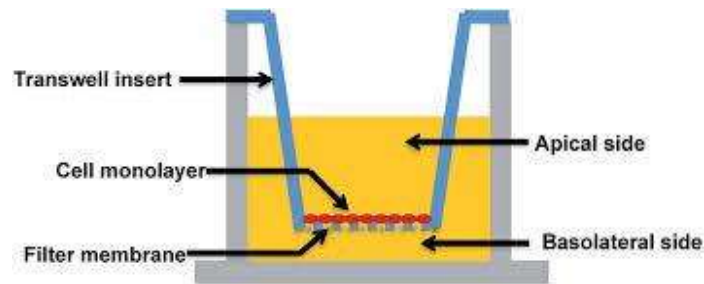


Figure 4: A visual of the transwell setup. The cell monolayer is comprised of THP-1 macrophages, and the T98G cells are located under the transwell insert. The filter membrane allows cell signaling molecules (cytokines) to freely travel, but is too small for whole cells to pass through.

qPCR

Any experiment run between August - October 20th, 2019 used the RNeasy Mini Kit (Qiagen 74104) and the method associated with that kit's protocol to isolate mRNA from the THP-1 macrophages for analysis. After October 20th, 2019, the protocol for RNA isolation/cDNA synthesis was switched to the use of TRIzol Reagent (Thermo Fisher 15596018) and protocol. A total of 2 μg of RNA was reverse transcribed using Transcriptor First Strand cDNA Synthesis Kit (Thermo Fisher 18091050).

Amplification reaction assays contained SYBR Green PCR Master Mix (Thermo Fisher 4309155) and primers. Cyclophilin was used as a reference gene, and gene expression was quantified using the threshold cycle method.

RStudio

The programming language R was used to develop code that automated the cleaning process of qPCR data. After the qPCR is finished cycling, a raw and unorganized Excel file is outputted. The raw Excel file has unnecessary columns and "N/A" values. No calculations are computed, and the data is not visualized in any way. RStudio was the specific developing environment used to create this script due to RStudio being more comprehensive than simply using R. The programming language R is very good at visualization, which was the end goal of the calculations.

When a raw qPCR Excel file is uploaded and run through the developed script, data cleaning and visualization are done automatically. The code allows the user to upload the raw excel file and clean the data by clearing unnecessary columns as well as removing rows with an "N/A" Cq value, using the R package "tidyverse". Cq, short for quantitation cycle, refers to the cycle in which the sample went above the threshold and fluorescence was able to be detected. The code averages Cq values for each target and sample (normally in triplicates), and normalizes this result to the housekeeping gene (cyclophilin). This calculation is then normalized within targets, using the package "dplyr". The code then calculates the final value (gene expression), and visualizes the calculated data into a bar graph using the packages "RColorBrewer" for aesthetic and "ggplot2" for graphing. The final graph displays data from the experimental groups, separated by target gene, and their subsequent levels of gene expression for each target gene.

Well	Fluor	Target	Content	Sample	Cq	Cq Mean	Cq Std. Dev
C08	SYBR	CD209	Unkn	2	28.00	28.00	0.000
C09	SYBR	CD209	Unkn	2	28.16	28.16	0.000
C10	SYBR	CD209	Unkn	3	23.58	23.58	0.000
C11	SYBR	CD209	Unkn	3	30.03	30.03	0.000
C12	SYBR	CD209	Unkn	3	23.65	23.65	0.000
D01	SYBR	CCL18	Unkn	1	36.85	36.85	0.000
D02	SYBR	CCL18	Unkn	1	33.62	33.62	0.000
D03	SYBR	CCL18	Unkn	1	34.60	34.60	0.000

	Target	Sample	average	DeltaCq	DeltaDeltaCq	Expression
1	CCL18	1	35.0253	14.96147	0	1.00E+00
2	CCL18	2	32.5196	12.66669	-2.2947748	4.91E+00
3	CCL18	3	30.86567	11.60207	-3.3594025	1.03E+01
4	CCR7	1	32.35811	12.29427	0	1.00E+00
5	CCR7	2	31.50039	11.64748	-0.64679	1.57E+00
6	CCR7	3	36.54931	17.2857	4.9914253	3.14E-02

Figure 5: An example of raw data outputted from PLNU’s qPCR (top) compared to the cleaned qPCR data through R programming from 10/15/19. Calculations must be done on each target, taking the housekeeping gene into account, to ultimately derive gene expression levels. The code cleans/analyzes the data and outputs a visualization, bar graph, of target gene expression for each group automatically. This saves up to an hour of time and prevents human error. Sample 1 refers to THP+PMA, sample 2 refers to THP+PMA+M1 cytokines, and sample 3 refers to THP+PMA+M2 cytokines.

Results

We first checked to see if the addition of PMA to promote differentiation of THP-1 cells from monocytes to macrophages was necessary to prepare the macrophages for polarization. THP-1 cells were allowed to incubate in 2 uL of 150 nm PMA for 24 hours before the addition of cytokines. This made a large difference in the physical characteristics of each of the two phenotypes (M1 and M2), as seen in figures 6 and 7. Next, we analyzed gene expression of polarized macrophages to assess upregulation of key factors known to be expressed by M1 versus M2 macrophages.

RNA isolation for four experimental groups was run on 10/1/19, including THP-1 cells (control), THP-1 cells and M1 cytokines (LPS and IFN- γ), THP-1 cells and M2 cytokines (IL-4 and IL-13), and finally THP-1 cells with conditioned media (CM) from the T98G cells. Additionally, PMA (phorbol 12-myristate 13-acetate) was added to all experimental groups. Replicate wells were combined when collecting for RNA isolation. 600,000 cells were plated per well, so each of the four experimental groups had at least 1 million cells. CXCL-10, iNOS, CD163, and CD209 were the target genes tested for the four groups (Figure 8).

The M1 genes, CXCL-10 and iNOS, were greatly upregulated in the THP-1 cells that had been exposed to M1 cytokines. Exposing the THP-1 cells to M2 cytokines resulted in downregulation of the M1 factors, and a 4-fold increase in CD209 expression, but no increase (and a possible decrease) in CD163 expression. The THP-1 cells exposed to tumor-conditioned media had a similar reduction in expression of M1 target genes, but, in contrast to the M2 positive controls, had an increase in CD163 and a decrease in CD209 (M2 markers) (Figure 8).

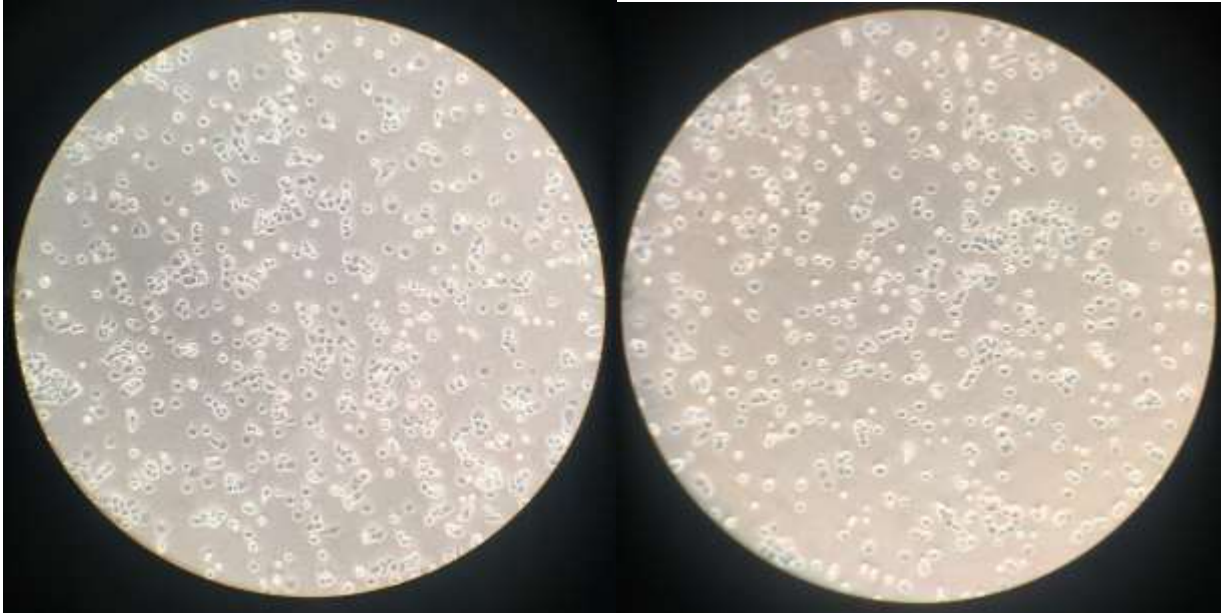


Figure 6: Without the addition of 150 nm PMA to passage 5 THP-1 cells and a subsequent 24 hour incubation period, macrophages appear similar in phenotype even after cytokine differentiation. The image on the left shows M1 macrophages by 24 hour incubation with 20 ng/ml of IFN- γ and 10 pg/ml of LPS, whereas the image on the right shows M2 macrophages by 48 hour incubation with 20 ng/ml of IL-4 and 20 ng/ml of IL-13.

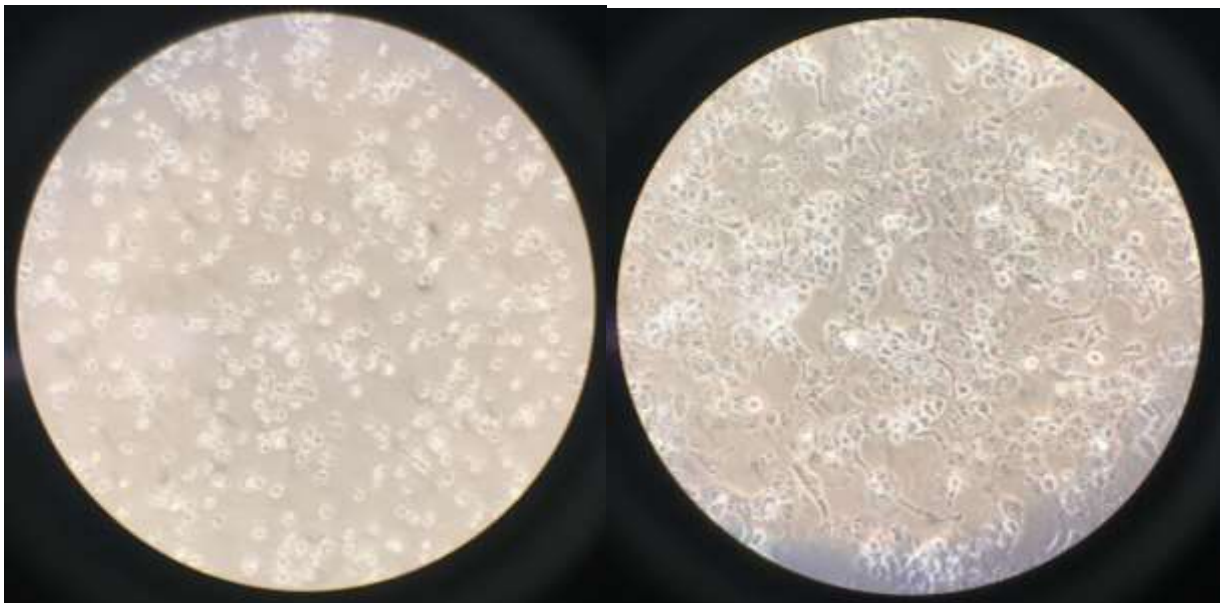


Figure 7: After a 24 hour incubation period with 150 nm PMA, and then the addition of either M1 (left) or M2 (right) cytokines, passage 6 THP-1 cells become significantly different in phenotype. The left image shows M1 macrophages by 24 hour incubation with 20 ng/ml of IFN- γ and 10 pg/ml of LPS, whereas the right image shows M2 macrophages by 48 hour incubation with 20 ng/ml of IL-4 and 20 ng/ml of IL-13. M1 macrophages are more circular in shape, whereas M2 macrophages have a more elongated shape, displaying their differentiation.

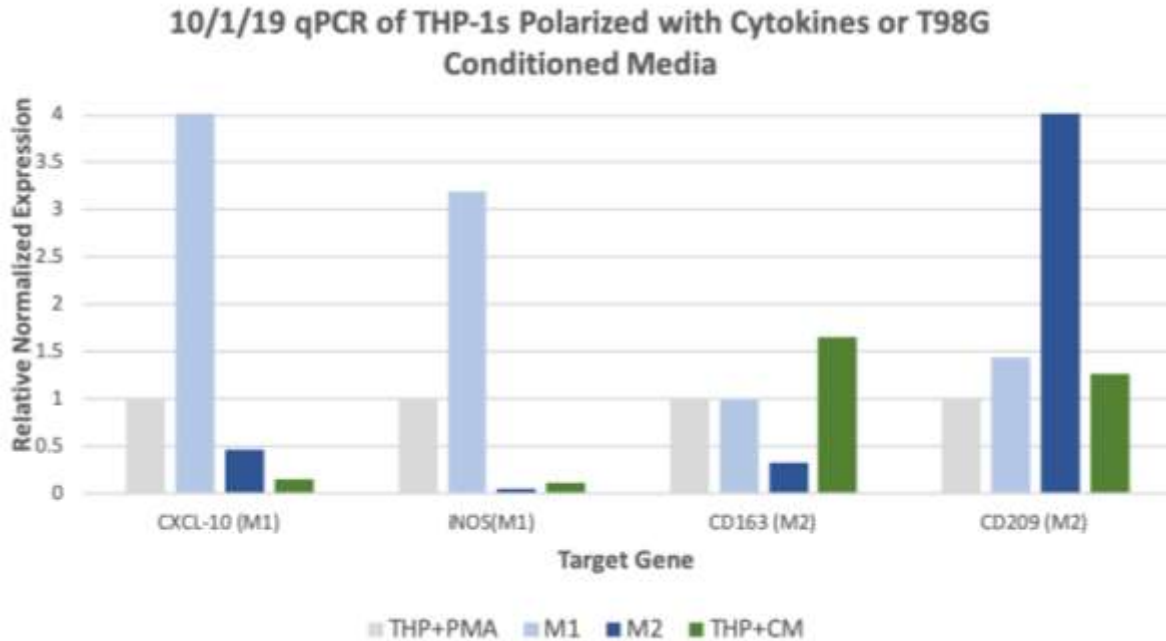


Figure 8: Data obtained from a qPCR run on 10/1/19. Gene expression levels are shown for the four groups for the target genes CXCL-10, iNOS, CD163, and CD209. In the legend, “THP+PMA” refers to THP-1 cells with 150 nm PMA, “M1” refers to THP-1 cells with 150 nm PMA and M1 cytokines (20 ng/ml of IFN- γ and 10 pg/ml of LPS), “M2” refers to THP-1 cells with 150 nm PMA and M2 cytokines (20 ng/ml of IL-4 and 20 ng/ml of IL-13), and “THP+CM” refers to THP-1 cells with 150 nm PMA and conditioned media from T98G cells. Parentheses following the target gene indicate whether the gene is associated with the M1 or M2 macrophage phenotype.

A follow-up experiment was run on 10/15/19, as a way to confirm the results seen on 10/1/19. The exact same protocol was used, except this experiment omitted the group of THP-1 cells with conditioned media from the T98G cells, as well as adding more target genes to measure in the qPCR, such as CCL18, and CCR7 (Figure 9). At least two target genes must be identified that effectively measure M1 or M2 polarization. Figure 9 shows results for expression of six target genes. Because the same experimental protocol was used on 10/1/19 and 10/15/19, the target gene expression should be identical if the target genes are reliable.

The only M1 gene that showed upregulation in the THP-1 cells that had been exposed to M1 cytokines was CCR7. CXCL-10 and iNOS, other M1 markers, did not show any M1 upregulation in comparison to the other tested groups. Cyclophilin, the housekeeping gene, showed an even expression across all three groups, further verifying its effectiveness as a control gene. The M2 target genes CCL18 and CD209 showed upregulated expression for THP-1 cells exposed to M2 cytokines. However, CD163, an M2 marker, barely showed upregulation (Figure 9). One outlier for the group “THP-1 + PMA + M1 cytokines” was observed for the target gene CXCL-10. This point was omitted from the data visualization since the gene expression was 1.36×10^{-2} , which highly skews the visuals of the other values.

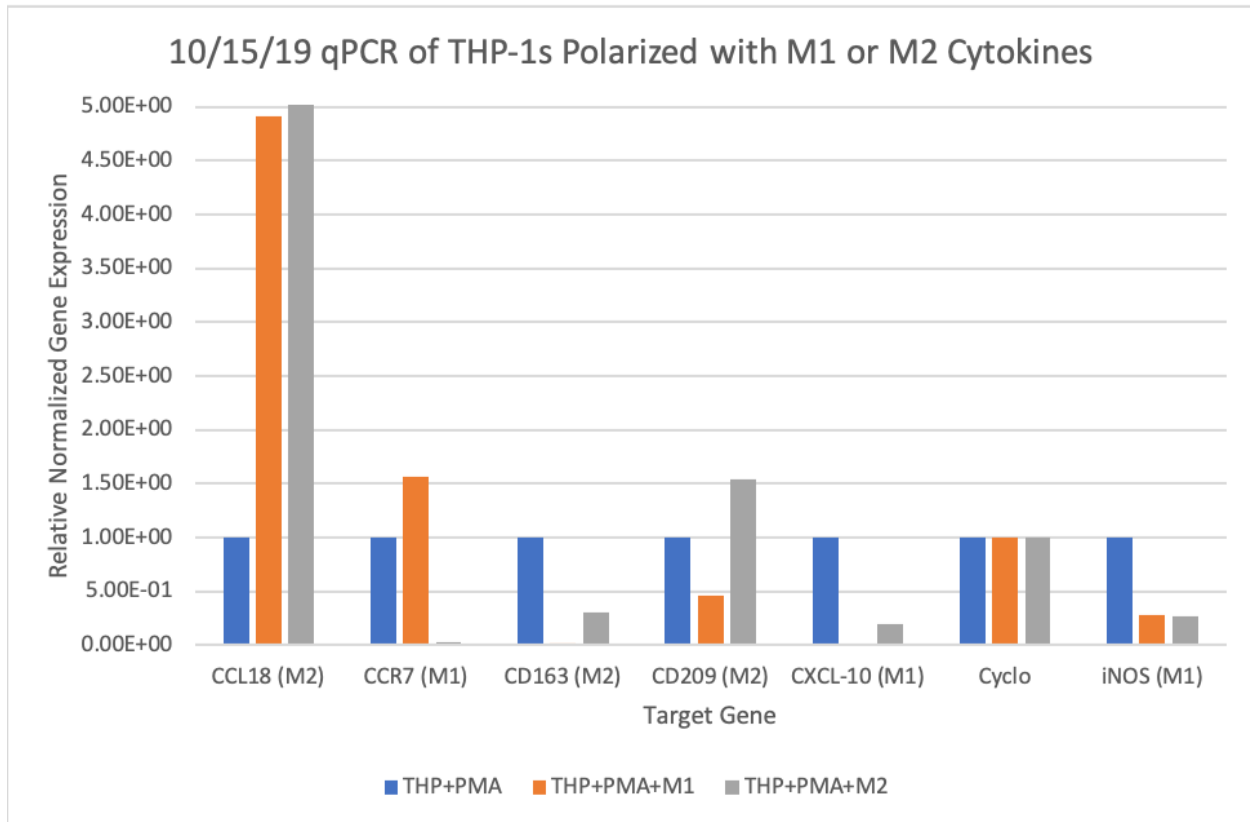


Figure 9: Data obtained from a qPCR run on 10/15/19. THP-1 cells were polarized with M1 or M2 cytokines, and subsequent levels of gene expression for the target genes CCL18, CCR7, CD163, CD209, CXCL-10, and iNOS were measured. In the legend, “THP+PMA” refers to THP-1 cells with 150 nm PMA, “THP+PMA+M1” refers to THP-1 cells with 150 nm PMA and M1 cytokines (20 ng/ml of IFN- γ and 10 pg/ml of LPS), and “THP+PMA+M2” refers to THP-1 cells with 150 nm PMA and M2 cytokines (20 ng/ml of IL-4 and 20 ng/ml of IL-13). “Cyclo” is an abbreviation for the target gene, cyclophilin. Parentheses following the target gene indicate whether the gene is associated with the M1 or M2 macrophage phenotype. The y-axis was truncated to 5 (instead of 12) for ease of viewing the other data points.

Due to the relative success of the previous two qPCR datasets, co-culture membranes were first tested on 10/23/19. Co-culture membranes allowed a better simulation of the tumor microenvironment because, rather than us adding cytokines to the wells, glioblastoma cells would be releasing signals directly to the THP-1 cells in transwell plates (Figure 4). This was also the first experiment that used TRIzol reagent in place of the RNeasy Mini Kit. The same target genes were tested for: CD163, CD209, CCL18, CXCL-10, CCR7, and iNOS. The experimental groups were the same with the addition of the THP-1 cells co-cultured with T98G cells, which should display an upregulation in M2 (pro-tumor) target genes.

THP-1s exposed to M1 cytokines had a significant upregulation of M1 target genes CXCL-10 and CCR7 (Figure 8, 9, 10), although iNOS remained inconclusive. THP-1s exposed to M2 cytokines did not have an upregulation of M2 target genes CD163, CD209, or CCL18.

However, the other M2 target gene, CD209, was significantly upregulated for the co-culture group.

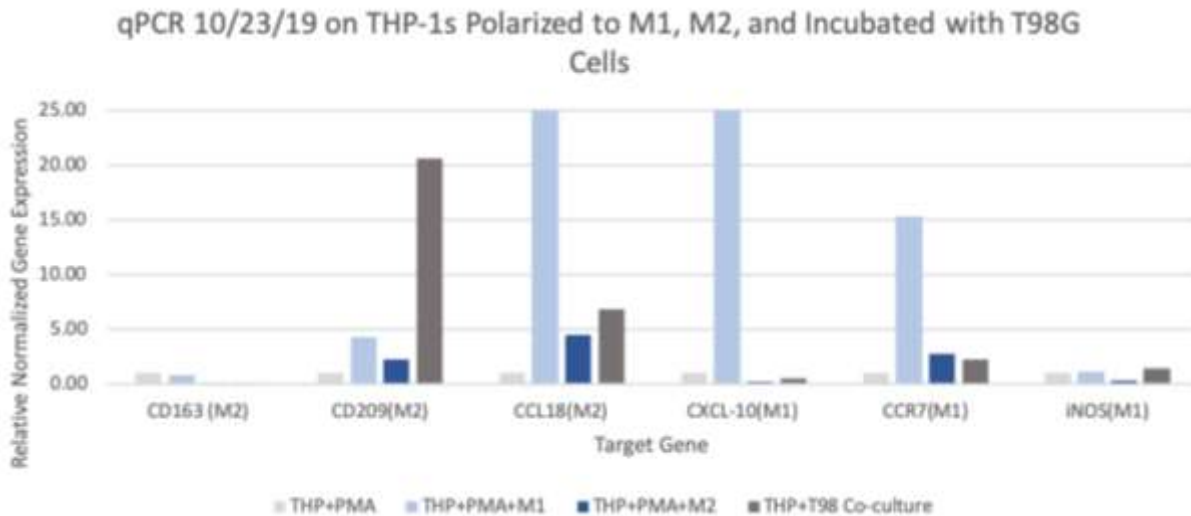


Figure 10: Data obtained from a qPCR run on 10/23/19. “THP+PMA” refers to THP-1 cells with 150 nm PMA, “THP+PMA+M1” refers to THP-1 cells with 150 nm PMA and M1 cytokines (20 ng/ml of IFN- γ and 10 pg/ml of LPS), “THP+PMA+M2” refers to THP-1 cells with 150 nm PMA and M2 cytokines (20 ng/ml of IL-4 and 20 ng/ml of IL-13), and “THP+T98 Co-culture” refers to THP-1 cells with 150 nm PMA grown in co-culture with T98G cells. Target genes tested include: CD163, CD209, CCL18, CXCL-10, CCR7, and iNOS. Parentheses following the target gene indicate whether the gene is associated with the M1 or M2 macrophage phenotype.

Because of the inconclusive results of the previous experiment, a new experiment was run to determine the cause of the inconsistency. This experiment tested expression levels of the housekeeping gene, cyclophilin, of THP-1 cells with only PMA. 6 wells of 500,000 THP-1 cells per well were plated in a 6-well plate - essentially 6 biological replicates. The purpose of this was to see if RNA levels and gene expression was consistent between biological replicates, as well as to verify our procedure using TRIZOL reagent. TRIZOL reagent and protocol was used to isolate the RNA from these samples. The same samples were run on 11/13/19 and 11/19/19. However, on 11/13/19, only ran samples labeled 1, 4, and 5, whereas on 11/19/19, the samples labeled 1-6 were run (Table 1). Target gene expression of cyclophilin, a housekeeping gene, was consistent within the same sample, meaning that the RNA isolation qPCR protocols are consistent between people, but inconsistent between biological replicates. This inconsistency between biological replicates is likely due to our cell culture protocol. In order to reduce variability between groups, it was determined that THP-1s should be matured together before being separated into groups. So, for all consequent experiments, THP-1s were exposed to PMA in the same T75 flask and separated into different groups in a six well plate the next day rather than splitting cells prior to the use of PMA.

Sample	Cq (11/3/19)	Cq (11/19/19)
1	23.73	23.62
1	23.42	23.66
1	23.46	23.37
2		33.95
2		33.95
2		34.14
3		32.76
3		33.57
3		33.47
4	30.29	30.96
4	30.37	30.68
4	30.25	31.39
5	25.35	25.79
5	25.29	25.61
5	25.04	25.16
6		22.93
6		23.36
6		23.37

Table 1: Cq values obtained from the same samples run on 11/13/19 and 11/19/19. All 6 samples were biological replicates of just THP-1 cells and 150 nm PMA, but only samples 1, 4, and 5 were run on 11/13/19, and then all 6 samples were run on 11/19/19. Cq, short for quantitation cycle, refers to the cycle in which the sample went above the threshold and fluorescence could be detected. Lower Cq values mean higher initial copies.

Due to time constraints, we decided to proceed with the antagonist experiment, despite the inconsistencies in data. On 12/10/19, a qPCR was run, now with the addition of pathway antagonists. The final groups were as follows, all with 150 nm PMA added for 24 hours: 2 groups of THP-1 cells, a double concentration of THP-1 cells, THP-1 cells and M1 cytokines, THP-1 cells and M2 cytokines, THP-1 cells and conditioned media from T98G cells, THP-1 cells with conditioned T98G media and a STAT6 antagonist, THP-1 cells with conditioned T98G media and a PPAR Beta antagonist, and finally THP-1 cells with conditioned T98G media and a

PPAR Gamma antagonist (Figure 11). The final RNA concentration of these experimental groups were approximately 100 ng/uL except for the group with twice as many cells. This indicates that higher concentrations of cells produce better RNA yields. A co-culture group of THP-1 cells with PMA with T98G cells, STAT6, PPAR Beta, or PPAR Gamma were also run; however, no pellet was isolated during the TRIzol protocol, so these groups were omitted and only the above groups grown in the 6-well plates were used.

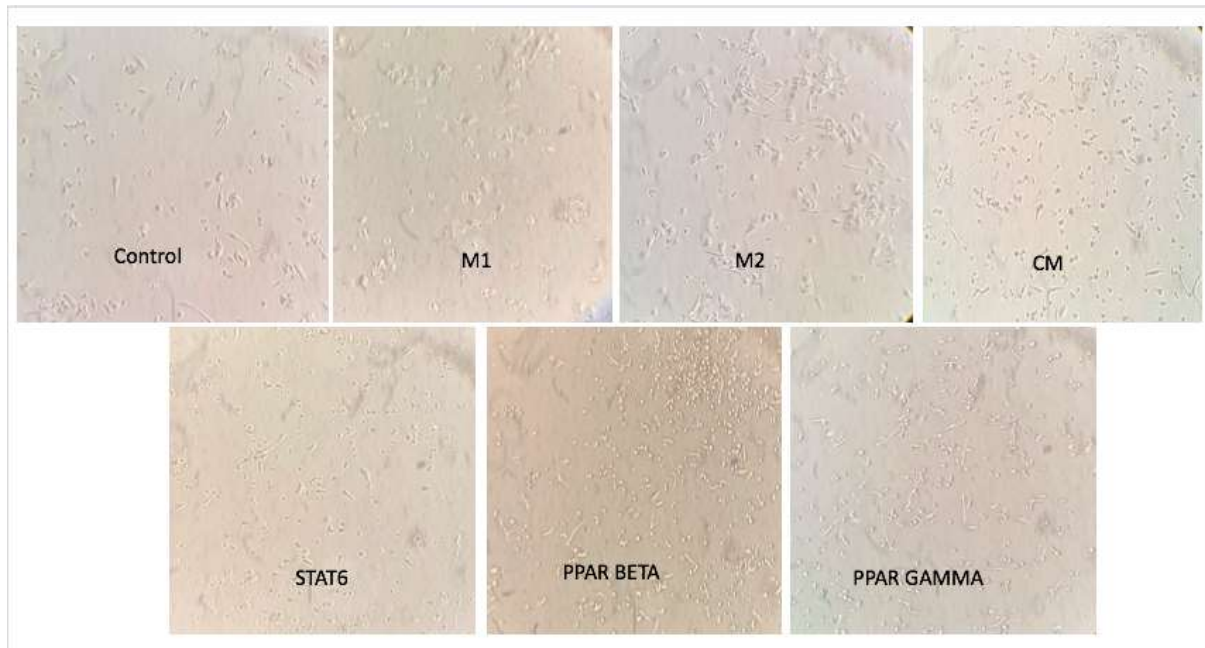


Figure 11: Images taken before 12/10/19 RNA isolation of passage 9 THP-1 cells with PMA (“Control”), THP-1 cells with PMA and M1 cytokines (“M1”), THP-1 cells with PMA and M2 cytokines (“M2”), THP-1 cells with PMA and conditioned media from T98G cells (“CM”), THP-1 cells with PMA and a STAT6 antagonist (“STAT6”), THP-1 cells with PMA and a PPAR Beta antagonist (“PPAR BETA”), and THP-1 cells with PMA and a PPAR Gamma antagonist (“PPAR GAMMA”),

Conclusion

The goal of this study was to analyze the influence of three pathways, PPAR Beta, PPAR Gamma and JAK-STAT, on macrophage polarization by cancer cells. A human bone marrow derived macrophage cell line, THP-1, and a human glioblastoma cell line, T98G, were used. Cytokines were used to induce polarization of THP-1s to the M1 (anti-tumor) and M2 (pro-tumor) phenotypes, which served as positive controls for polarization, and polarization was confirmed / analyzed by assessing expression levels of genes known to be correlated to M1 vs. M2 polarization using qPCR analysis. Prior to this project, there was no effective protocol to stimulate macrophage polarization using cytokines. We found that phorbol 12-myristate 13-acetate (PMA) is necessary to prime THP-1s for polarization by cytokines. Without the addition of PMA, the morphology of M1 and M2 is similar, meaning that the cytokines had little to no effect on polarization (Figure 6). However, with the addition of PMA prior to the exposure to cytokines, the morphology of the M1 and M2 groups was significantly different (Figure 7). The

cells primed with PMA and exposed to M2 cytokines were flattened and had small extensions when compared to the cells primed with PMA and exposed to M1 cytokines (Figure 7). The ideal concentration of PMA is 10 pg/uL for 24 hours followed by media without PMA for 24 hours, which successfully primes the macrophages without demonstrating cytotoxic effects. Based on this, we conclude that the THP-1 monocytes must be differentiated into macrophages using PMA in order to respond to M1 and M2 polarization factors. This makes sense because monocytes are the circulating version, which become mature, differentiated macrophages upon efflux from the blood into the tissue, something which PMA simulates.

After PMA differentiation into macrophages, followed by a 24 hour growth in the absence of PMA, cytokines were used to polarize the macrophages towards M1 or M2. LPS and IFN- γ were used to polarize the THP-1s to the M1 (anti-tumor) phenotype and IL-4 and IL-13 were used to polarize the THP-1s to the M2 (pro-tumor) phenotype. However, the time of exposure to cytokines varied. After 24 hours of exposure to M1 cytokines, the cells began to die, whereas 24 hours of exposure was not enough for the M2 cytokines to polarize the THP-1 cells. Therefore, M1 cytokines were added for 24 hours and M2 cytokines were added for 48 hours. The cells were then collected using the RNEasy Mini Spin Kit, cDNA was synthesized and the expression of target genes were measured using qPCR. These parameters were necessary in order to effectively polarize THP-1 cells to the M1 and M2 phenotype (Figure 8).

By performing the experiment stated above again with the same conditions, it was found that the protocol is reproducible and the target genes chosen effectively represent macrophage polarization (Figure 9). Then, we wanted to determine if the experiment is reproducible within biological replicates and person-to-person. The experiment was performed again with six replicates of only PMA primed THP-1 cells. The cDNA synthesis and qPCR was performed once by Allison (11/13/19) and then once by Bridget (11/19/19). The target gene expression was similar from run-to-run, which indicates that the experiment is reproducible from person-to-person; however, there was a significant difference, almost ten-fold, between target gene expression within the biological replicates (Table 1). This means that the variability is not due to the cDNA synthesis or qPCR protocol, but rather to some aspect of the cell culture procedure. As a result, PMA was added to the cells prior to splitting into different groups which should reduce variability.

In order to determine how cancer cells influence macrophage polarization, the protocol discussed above was used with the addition of an experimental group containing the PMA primed THP-1 cells exposed to T98G glioblastoma conditioned media rather than cytokines. Conditioned media contains all cellular secretions including signals that have the ability to polarize macrophages. To obtain the conditioned media, T98G cells were grown in the same media for three days after which the media was collected and spun. The primed THP-1s were grown in this media for 24 hours. The results of this experiment show a downregulation of M1 target genes, CXCL-10 and iNOS, and an upregulation of M2 target genes, CD209 and CD163, meaning that the conditioned media polarized the macrophages to the M2 (pro-tumor) phenotype (Figure 10).

After determining that T98G glioblastoma cells release signals into the media that polarize THP-1 macrophages to the M2 (pro-tumor) phenotype, we aimed to decipher which pathway was being used. The same protocol was used with the addition of inhibitors, rather than cytokines, against each of the hypothesized pathways, PPAR Beta, PPAR Gamma and JAK-STAT. If M2 target gene expression was downregulated, the pathway is important for M2 (pro-tumor) polarization by T98G glioblastoma cells. However, when this experiment was performed,

it was found that the M2 controls did not behave as expected. After extensive literature searches, it was found that spontaneous differentiation to M1 increases as the passage number of the THP-1 cells increases. This is consistent with the results in Figure 10 which shows significant upregulation of M1 target genes for all groups.

Due to time constraints, we were not able to conclude how influential PPAR Beta, PPAR Gamma or JAK-STAT are for macrophage polarization by glioblastoma cells. However, we can make conclusions based on the results obtained. PMA has proven to be necessary for priming THP-1 macrophages for polarization. In order to reduce variability between groups, PMA should be added to cells in a T75 flask prior to splitting into separate groups. Another method of RNA collection, TRIzol, was attempted but failed to produce quality RNA yields, and thus the RNeasy Mini Spin Kit is preferred for RNA collection. Additionally, plating less than 600,000 cells/mL significantly reduces RNA yields. Finally, in order to prevent spontaneous macrophage polarization to M1, the passage number should be kept below 10.

Finalized Protocol

Stock and dilutions of cytokines/reagents

- PMA (Sigma, P8139): want 150 nM stock at 150uM
- LPS (M1 cytokine, Sigma): want 10 pg/mL stock at 50 ug/mL
- IFN (M1 cytokine, Sigma): want 20 ng/mL stock at 0.2 mg/mL
- IL4 (M2 cytokine, Sigma): want 20 ng/mL stock at 100ug/mL
- IL13 (M2 cytokine, Sigma): want 20 ng/mL stock at 100ug/mL
- STAT6 (antagonist, Sigma Aldrich AS1517499):
want 300nM (1:1000 dilution) → 2uL for 2mL stock at 300uM
- PPAR beta (antagonist, Sigma Aldrich GSK3787):
want 1uM (1:1000 dilution) → 2uL for 2mL stock at 1mM
- PPAR gamma (antagonist, Sigma Aldrich T0070907):
want 1uM (1:500 dilution) → 4uL for 2mL stock at 500uM

A low passage of THP-1s, less than passage ten, should be utilized for M0 polarization studies.

Day 1: to THP-1s in a T75 flask, add PMA for 24 hours

if using conditioned media, grow 70-80% confluent T98 or U87 cells in T75 for three days in regular media + 10% FBS to condition the media + ANTI-ANTI

Day 2: aspirate the media with PMA from wells (cells should be adhered to bottom of flask), trypsinize THP-1s and plate 600,000 cells/mL in a six-well plate, add fresh warm THP-1 media for 24 hours

Day 3: add M2 cytokines (IL4 and IL13) to M2 wells for 48 hours

Day 4: add M1 cytokines (LPS and IFN) to M1 wells for 24 hours, co-culture or add CM for 24 hours, add antagonists for 24 hours

-if co-culturing, trypsinize cells and transfer 600,000 cells/mL to bottom of co-culture membrane, trypsinize T98 or U87 cells and transfer 300,000 cells/mL to top of co-culture membrane, add fresh warm THP-1 media to well

-if using conditioned media, collect conditioned media from T98 or U87 in 15mL conical and spin for 5 minutes at 1000 rpm, aspirate media from THP-1 CM wells and add conditioned media supernatant

Day 5: RNA isolation, cDNA synthesis, qPCR *see protocols below*

Qiagen RNA Isolation Kit (Qiagen 74104): trypsinize cells then follow protocol at link below <http://www.bea.ki.se/documents/EN-RNeasy%20handbook.pdf>

extremely important to wait a full minute with Rnase-free water on membrane before spinning on step 10

SuperScript IV First-Strand Synthesis System

<https://www.thermofisher.com/order/catalog/product/18091050#/18091050>

qPCR: three step amp + melt

see TAMs summer notebook for plate setup and dilutions

Target genes: CXCL-10 (M1), iNOS (M1), CD209 (M2), CD163 (M2) but others can be used as well if determined to be accurate/reproducible result

Code

<https://drive.google.com/a/pointloma.edu/file/d/1hi6o5CdALCqvj6hCk-OtgsWQyocoOkaU/view?usp=sharing> (shared with anyone in PLNU with link)

Key Findings and Future Directions

- PMA is necessary to mature THP-1s for polarization
 - 10 pg/uL for 24 hrs then PMA-free media for 24 hrs
- Plating more than 600,000 cells/mL produces higher RNA yields
- Should keep the passage number below 10 in order to prevent spontaneous M1 differentiation
- Repeat experiment outlined in Table 1 after exposure to PMA prior to splitting THP-1s into separate groups (determine if less variability than PMA exposure after splitting THP-1s into separate groups)
- RNeasy mini spin kit is best for isolating RNA (TRIzol is difficult to use)
- Should try to plate THP-1s on bottom and T98 on co-culture membrane (easier to isolate THP-1s), unlike what is stated in literature

From extensive literature searches, it was found that qPCR is not used as the sole determinant of macrophage polarization. This is likely due to the unreliability of target gene expression and macrophage polarization. It was found that, after around ten passages of THP-1s, spontaneous differentiation to the M1 phenotype was seen. This complicates our experimental protocol because cell stocks must be thawed frequently in order to keep the passage number low. It also introduces a factor of uncertainty because the longer we grow the THP-1s, the less reliable our data is. Instead of relying solely on qPCR, most scientists use three or four experimental techniques to confirm macrophage polarization. For example, qPCR would be performed in addition to flow cytometry and Western Blotting or immunohistochemistry to measure gene expression and protein expression, respectively. Therefore, if we were to continue this experiment, we would like to use multiple methods to confirm macrophage polarization. We would also test T98 conditioned media on an Antibody Based Cytokine Membrane Array in order to determine what cytokines are present in the media that could influence macrophage polarization. Finally, during our culture protocol, we wish we would have cultured THP-1s on

the bottom of the co-culture well and T98 on the membrane in order to make collection of THP-1s easier.

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