Characterization of the Interaction Between Glioblastoma Cells and Tumor-Associated Macrophages (TAMs) Within the Tumor Microenvironment

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Abstract

This project seeks to better understand the cellular interactions within the tumor microenvironment that lead to polarization of macrophages towards the M2 phenotype. The glioblastoma cell line T98G and monocyte cell line THP-1 were cultured, and the primed monocytes were exposed to conditioned media from the T98Gs to model the tumor microenvironment. QPCR was used to quantify biomarkers of the tumor-associated macrophages, and a cytokine array was performed to analyze the signaling factors in the conditioned media. The cytokine array revealed that glioblastomas secrete numerous factors, including IL-6, IL-8, MCP-1, VEGF, Osteoprotegerin, TIMP-1, and TIMP-2. During the qPCR, the genes CCR7, CXCL10, and CD80 were identified as reliable biomarkers for the M1 phenotype, while MMR and CD209 indicated the M2 phenotype. The protocol to produce tumor associated macrophages was optimized by exposure to PMA rather than MCSF, and separate cultures rather than coculture. This protocol will be used in the future so the lab can confidently produce tumor-associated macrophages to send to our collaborators at the La Jolla Institute of Immunology for analysis of the resulting TAMs' surface protein profile. These studies may yield further insights into the signaling pathway that glioblastoma tumors use to polarize macrophages and evade the immune system, and characterization of tumor induced TAMs. Ultimately, a better understanding of this mechanism could lead to therapeutic treatments that prevent TAM activation, and perhaps repolarize TAMs into M1 macrophages to retain the body's natural immune system to fight cancer.

Introduction

In the United States, cancer is a leading cause of death, second only to heart disease[5]. Half of women and a third of men will develop cancer during their lifetime. Though some cancers have favorable prognoses, for the particularly aggressive brain cancer glioblastoma, only a quarter of patients survive the first year and less than 5% live more than 5 years[20]. Cancer is difficult to treat because of the way it hijacks normal cells and secretes factors to create its own microenvironment. The immune system often overlooks cancerous cells because they identify as "self".

Macrophages are derived from monocyte precursors and serve as the first responders of the innate immune response. They are well known for their recognition of antigens, activation of neutrophils, and phagocytic ability. However, macrophages function on a spectrum of polarization (Figure 1). In circumstances such as injury, macrophages also promote tissue growth. Macrophages' response to pathogens is known as their classical phenotype, also referred to as the M1 polarization phenotype, whereas their growth-promoting function is considered the alternative, or M2, phenotype. In tumor microenvironments, it has been observed that macrophages exhibit this alternative activation and thus help the tumor grow[10].

The purpose of this project is to identify how glioblastoma polarizes tumor-associated macrophages with the goal of inhibiting and reversing this transition. The model system is cell culture of human glioblastoma cell lines U87 and T98Gs, and the human monocyte cell line THP-1s. Conditioned media from the cancer cells should contain secreted factors that may play a role in signaling macrophages to shift towards the M2 phenotype. This chemical signaling would be similar to the natural mechanism by which normal cells communicate to the macrophages to convert from the classical (M1) phenotype to the cell growth-promoting alternative (M2) phenotype after a wound has been cleaned and is ready to start the healing process. Amongst the other mutations and cellular changes that cause cells to become cancerous, aggressive tumor cells can also activate similar signaling pathways to prevent macrophage killing and convert them to the growth promoting phenotype. However, more needs to be done to characterize how these tumor cells hijack the immune system in this way.

Cell culture is a useful model system because it allows the study of specific cell types and their signaling factors. Because cells are constantly dividing so long as they don't become confluent, and the cells don't need to be isolated from an organism, cell culture is much faster than using an in vivo model. It is also much easier to manipulate the growing conditions of the cells; differentiation can be induced by simply adding cytokines to the media. Harvesting the cells to analyze gene expression, or collecting media to analyze secreted factors, is a simple process. The cells' morphology can be observed directly through a microscope, so confirming the success of procedures such as priming the monocytes with PMA only takes a glance.

Despite the benefits, this model system does not account for cell signaling dependent on direct contact. Focusing only on the key players oversimplifies the tumor microenvironment and neglects the roles of other cell types and structures such as blood vessels. During the experiment, the identity of the macrophages was analyzed with genetic markers through polymerase chain reactions. The markers for M1 included CCR7, CXCL10, iNOS, and CD80, and the markers for M2 included MMR, CD209, Arg-1, PD-L1, CCL18, and CD163. As well as playing an important role in activating the adaptive immune response by sending dendritic cells to lymph nodes, CCR7 is expressed on macrophages at the onset of inflammation[17]. CXCL10 is an inflammatory cytokine that activates and attracts cells within the innate immune response[19]. CD80 is known to regulate B cell activity as well as stimulate T cells[15], and iNOS causes angiogenesis and inflammation[8]. Meanwhile, the M2 marker MMR encodes the macrophage mannose receptor, which allows uptake of glycoproteins and can recognize some extracellular pathogens[6]. CD209 is C-type lectin that helps macrophages recognize pathogens and adhere to cells[13]. Arg-1 suppresses T cell activity and decreases nitric acid production[1], and PD-L1 acts as a checkpoint to stop an immune response[16]. CCL18 attracts lymphocytes towards activated macrophages, typically in the lymph nodes[11]. CD163 encodes a receptor that helps break down hemoglobin/haptoglobin complexes, which may protect tissues from oxidative damage[12]. The reference genes were GAPDH, Beta-actin, and cyclophilin. GAPDH plays a critical role in glycolysis and cyclophilin helps protein folding. Beta-actin helps the cell maintain its shape and

move. All three genes serve as reliable baselines because they are always expressed in living cells.

A successful method of reactivating tumor-associated macrophages to the M1 phenotype could be used in conjunction with other immunotherapeutic treatments to fight cancer. By better understanding the factors secreted by glioblastoma cells that influence macrophage polarization, we will be better poised to reverse this polarization and harness the body's natural immune system in the treatment of cancer.

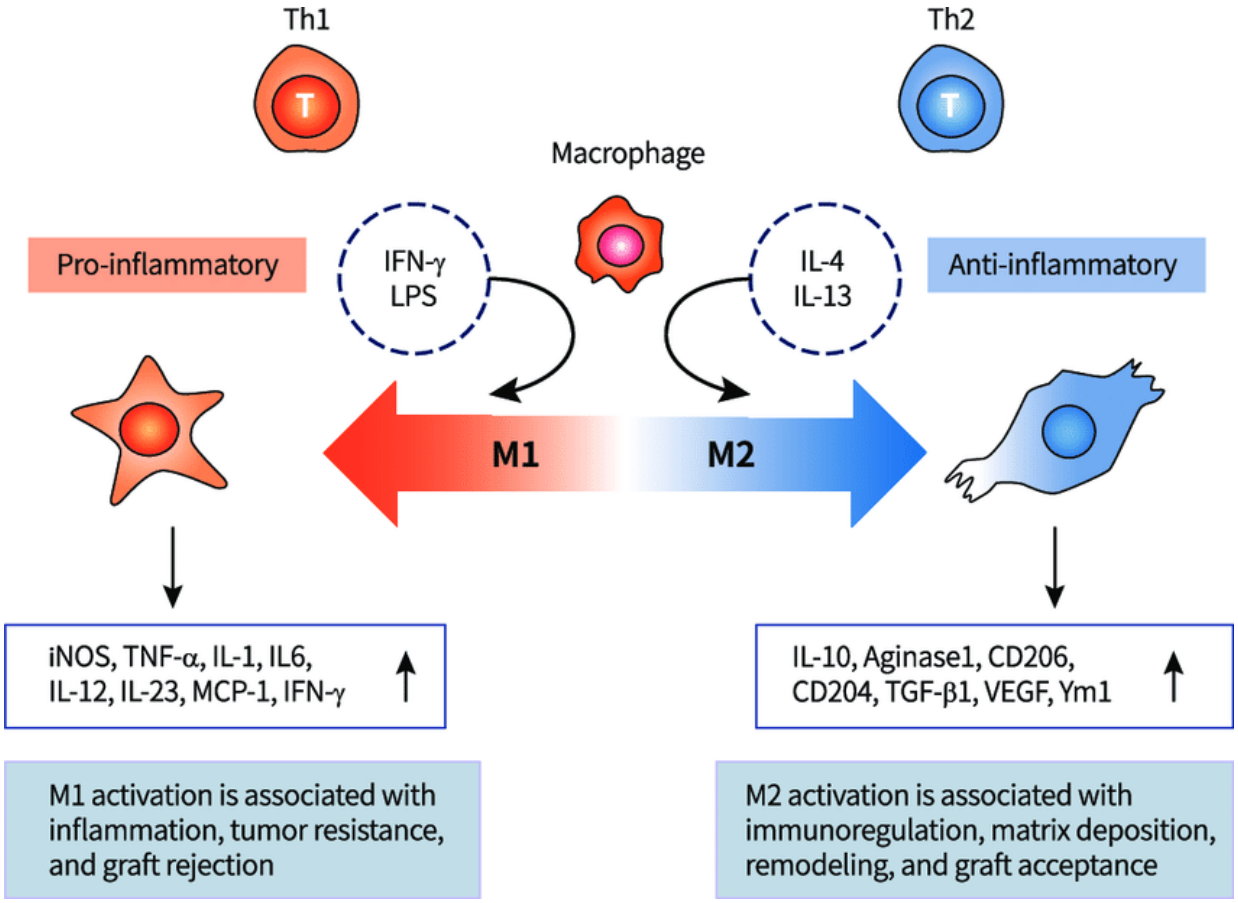

Figure 1. The polarization spectrum of macrophages courtesy of Dr. Kun Lee[10]. This diagram shows the main cytokines added for polarization in cell culture as well as the main functions of M1 and M2 macrophages.

Methods

Cell Culture

Human monocytic THP-1 cells (ATCC TIB-202) were maintained in culture in Roswell Park Memorial Institute Medium with 10% heat-inactivated fetal bovine serum (hiFBS), 1% antibiotic-antimycotic (anti-anti), and 0.1% β-mercaptoethanol. T98G glioblastoma multiforme

cells (ATCC CRL-1690) and U87 glioblastoma cells (ATCC HTB-14) were maintained in culture in Eagle's Minimum Essential Medium with 10% hiFBS and 1% anti-anti. Glioblastoma cells were also grown in stressed media, which contained no hiFBS.

THP-1 monocytes were differentiated into macrophages in a 6-well plate (seeded 300,000 cells) or 6cm dishes (seeded 900,000 cells) starting with a 24 hour incubation with 150nM phorbol 12-myristate 13-acetate (PMA, Sigma P8139). The PMA was removed, and the THP-1s were rested for 24 hours in RPMI*. Macrophages were polarized to M1 via a 24 hour incubation in the presence of 10pg/mL lipopolysaccharide and interferon gamma . Alternatively, macrophages were polarized to M2 with a 24 hour incubation in the presence of 20ng/mL interleukin four and interleukin thirteen. Macrophages were also exposed to tumor conditioned media (CM) - normal or stressed media which the glioblastoma cells were maintained in for three days.

For co-culture experiments, glioblastoma cells grown in T75 culture flasks were unadhered using trypsin and transferred to the top membrane membrane of a transwell insert. These transwell inserts containing glioblastoma cells were transferred to a 6-well plate containing PMA-treated THP-1 cells in RPMI and incubated in a co-culture for 48 hours.

In the co-culture media experiment, THP-1 cells were cultured in a 6-well plate with one of the following mediums: RPMI, RPMI and EMEM, and EMEM. These conditions were repeated with T98G cells.

*For the macrophage colony stimulating factor (M-CSF) experiments, 10ug/mL M-CSF was added to the RPMI for this incubation.

Quantitative Polymerase Chain Reaction (qPCR)

Any experiment analyzing macrophage gene expression June 2021-December 2021 utilized an RNeasy mini prep kit and its associated protocol to harvest and isolate mRNA from THP-1 cells. 8uL of the mRNA was reverse transcribed into cDNA using Invitrogen's Superscript IV First-Strand Synthesis System. Amplification reactions contained SYBR Green PCR master mix (Bio-Rad) and primers. Cyclophilin, β-actin, and GAPDH were used as reference genes. Gene expression was quantified through comparison of threshold cycle values. Data was analyzed through a double normalization - first to the reference gene, then to the PMA control.

Cytokine Array

Stressed (EMEM without FBS) and non-stressed (EMEM with FBS) conditioned media was collected from T98G cells grown in T75 culture flasks. Using a human antibody cytokine array (Abcam ab133998) and the associated protocol, the cytokine content of 1mL of the following mediums were analyzed: EMEM, low serum EMEM, conditioned media, and stressed conditioned media. ImageJ was used to quantify the density of the blots.

Results

qPCR Primers

In order to analyze macrophage gene expression in future experiments, we decided to test the reliability of various genes of interest. There were four M1 genes of interest: CCR7, CD80, CXCL10, and iNOS. Compared to THP-1 cells exposed to M2 cytokines, those exposed to M1 cytokines exhibited upregulation of CCR7, CD80, and CXCL10 (**Figure 2a**). iNOS was expressed at a lower level in both cytokine groups compared to the PMA control; however, the THP-1 cells exposed to M2 cytokines expressed iNOS at a level about twice that of the group exposed to M1 cytokines (**Figure 2b**). This experiment focused on six M2 genes: Arg-1, CCL18, CD163, CD209, MMR, and PD-L1. CD163, CD209, and MMR exhibited higher levels of expression in THP-1 cells exposed to M2 cytokines than in those exposed to M1 cytokines (**Figure 2c**). MMR expression in the M1 cytokine group was lower than in the PMA control. THP-1 cells exposed to M1 cytokines expressed Arg-1, CCL18, and PD-L1 at a higher level than those exposed to M2 cytokines (**Figure 2d**). β-actin and GAPDH were the two reference genes of focus in this experiment. Across all experimental groups, both β-actin and GAPDH expression levels fall within 0.3 of the PMA control (**Figure 2e**). These data indicate that some primers are more reliable than others for our experimental purposes. CCR7,

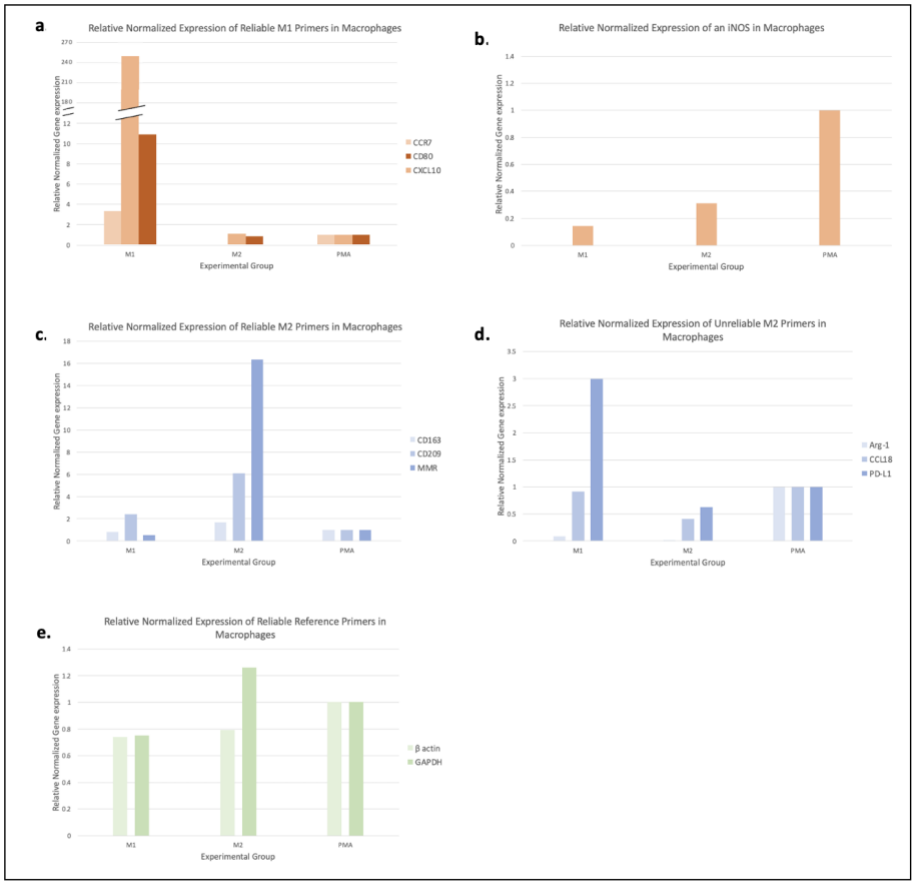

Figure 2. qPCR Primers This figure contains graphical representations of the reliability of primers used in this experiment: **(a)** reliable M1 primers, **(b)** unreliable M1 primer, **(c)** reliable M2 primers, **(d)** unreliable M2 primers, and **(e)** reliable reference genes.

CD80, and CXCL10 are reliable M1 markers (**Figure 2a**). CD163, CD209, and MMR are reliable M2 markers (**Figure 2c**). β-actin and GAPDH are reliable reference genes (**Figure 2e**).

Polarization

To characterize the induction of the M2 phenotype in THP-1 cells by glioblastoma cells, it was necessary to optimize the protocol through which we obtain M2 macrophages. Successful macrophage polarization occurs when phenotypic markers are upregulated in their respective experimental groups. In **Figure 3a**, CCR7 (M1 marker) is expressed at the highest level in the M1 cytokine group and at the lowest level in the M2 cytokine group. Similarly, CD209 (M2 marker) is expressed at a higher level in the M2 cytokine group than in the M1 cytokine group. Our focus in this experiment was whether the addition of M-CSF aids in macrophage polarization. While the addition of M-CSF increased expression levels when only PMA was added, this expression significantly decreased with the addition of M2 cytokines (**Figure 3b**). In the PMA and M-CSF group, all genes of interest except MMR were expressed at a higher level than in the PMA control. When THP-1 cells were exposed to PMA, M-CSF, and M2 cytokines, only CXCL10 was expressed higher than both the PMA and the PMA and M2 cytokine controls. The overall lower expression levels with both M-CSF and M2 cytokine addition indicate M-CSF priming is not a necessary addition to optimize macrophage polarization.

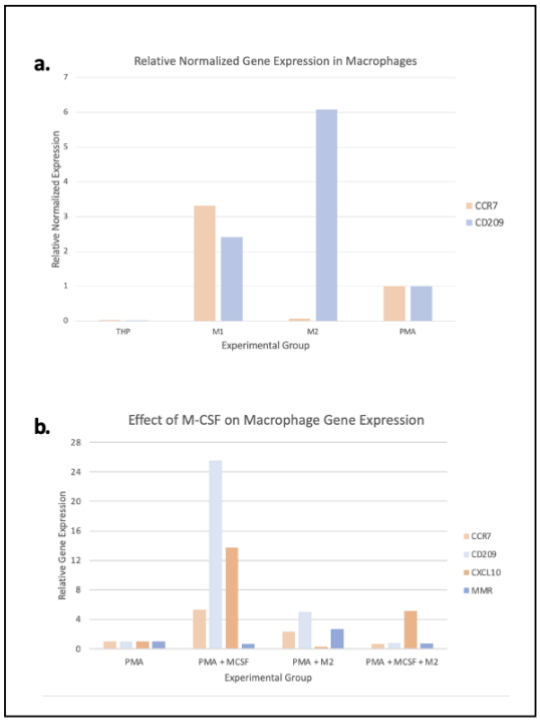

Figure 3. Macrophage Polarization This figure contains graphical representations of gene expression levels following macrophage polarization. **(a)** illustrates a typical readout using one M1 and one M2 marker. **(b)** contains expression levels as a result of M-CSF priming.

Conditioned Media

In order to study how conditioned media from glioblastoma cells affects macrophage polarization, we decided to test various types to determine the optimal conditions. Due to the M2 phenotypic nature of TAMs, conditioned media exposure is expected to result in M2-like gene expression. Conditioned media from T98G cells caused CD80 upregulation compared to the PMA control; however, stressed T98G media (no serum) resulted in upregulation of both CD80 and MMR – CD80 was expressed at a lower level than in the normal T98G conditioned media (**Figure 4a**). Normal U87 conditioned media resulted in upregulation of CD80, CD209, and MMR compared to the PMA control. Removal of serum from the U87 media caused the

upregulation of CD80 and the decreased expression of CD209 and MMR compared to the PMA control and normal U87 conditioned media groups. The upregulation of genes because of T98G conditioned media exposure indicates that T98G conditioned media is more reliable.

To determine the effect glioblastoma cells have on macrophage polarization, we analyzed differences in gene expression that occurred as a result of exposure to normal T98G conditioned media. In the conditioned media group CCR7 and CD80 were expressed at levels more similar to the M2 cytokine group, while CXCL10 was expressed at a level most similar to that of the M1 cytokine group (**Figure 4b**). On the other hand, CD163 and MMR expression in THP-1 cells exposed to conditioned media was most similar to that of the M2 cytokine group, while CD209 expression was more similar to the M1 cytokine group (**Figure 4c**). This indicates that conditioned media is not completely polarizing the macrophages toward one phenotype.

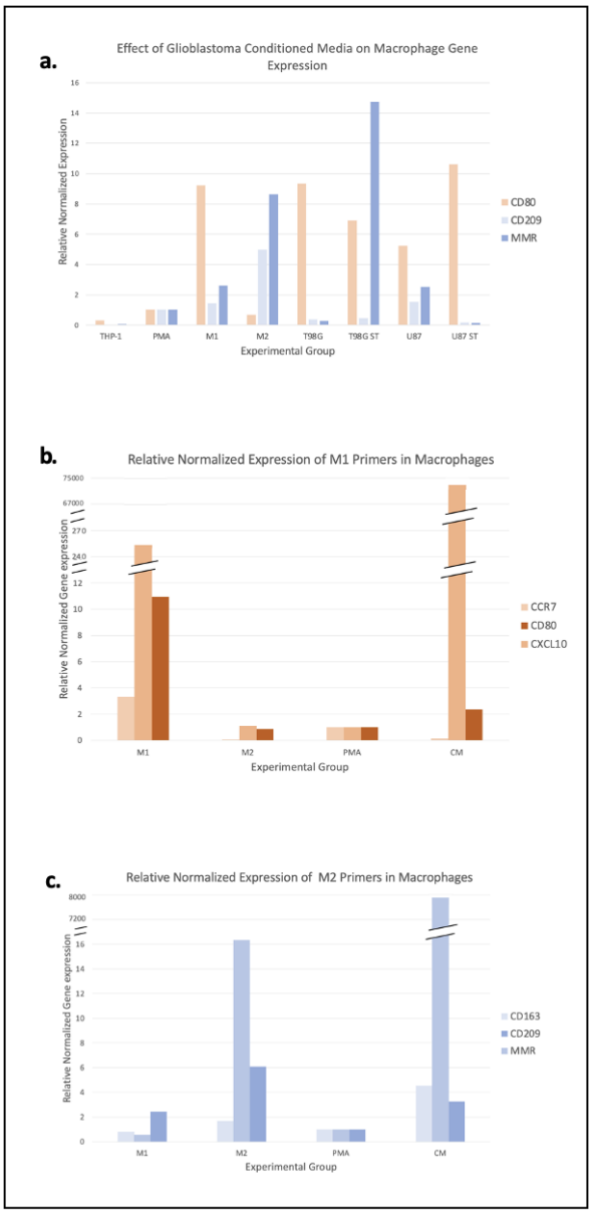

Figure 4. Conditioned Media This figure contains graphical representations of THP-1 gene expression following conditioned media exposure: **(a)** effect of various types of conditioned media on THP-1 expression levels, **(b)** M1 gene expression, and **(c)** M2 gene expression.

Co-culture

To characterize the interaction between glioblastoma cells and macrophages, we decided to optimize the co-culture protocol. Beforehand, T98G conditioned media exposure caused CD80 upregulation and CCR7 and MMR down- regulation compared to the PMA control (**Figure 5a**). Because the macrophages and glioblastoma cells are grown in different medias, we decided to test whether there is an optimal media which allows for adequate growth of both cell types. THP-1 cells are non-adherent and typically grow in RPMi media (3.9×10^4 cells, **Figure 5b**). When THP-1 cells are grown in EMEM, confluency significantly decreases (2.3×10^4 cells, **Figure 5c**); however, a 1:1 mixture of RPMi and EMEM results in growth similar to that of the RMPi only growth conditions (5.1×10^4 cells, **Figure 5d**). T98G cells are adherent and typically grow in EMEM

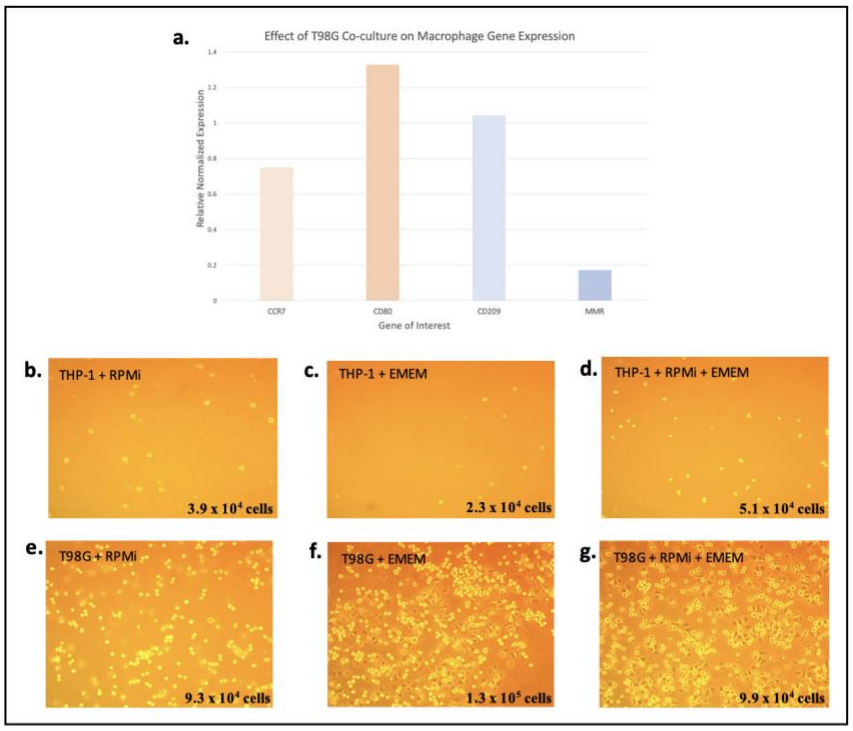

Figure 5. Co-culture This figure contains a graphical representation of THP-1 gene expression following co-culture with T98G cells and visual representations of the cells grown throughout these experiments. **(a)** M1 and M2 gene expression following T98G co-culture. **(b-d)** THP-1 monocytes grown in RPMi, EMEM, and mixed media, respectively. **(e-g)** T98G glioblastoma cells grown in RPMi, EMEM, and mixed media, respectively.

media (1.3×10^5 cells, **Figure 5f**). Growth in RPMi results in a decrease in confluency (9.3×10^4 cells, **Figure 5e**). Similar to the THP-1 cells, a 1:1 mixture of RPMi and EMEM results in growth similar to that of the EMEM only conditions (9.9×10^4 cells, **Figure 5g**). This indicates that use of a 1:1 media mixture will result in THP-1 and T98G growth comparable to that of their respective normal growth conditions.

Cytokine Array

Figure 6 shows the first two membranes from the cytokine array: (a.) EMEM with FBS (b.) conditioned media. **Figure 7** shows the last two membranes: (c.) EMEM without FBS and (d.) stressed conditioned media. Eighty cytokines were tested on each membrane, and the ones that were secreted more as indicated by darker, larger blots are identified in the color keys. The integrated optical density of each blot was determined using ImageJ. If every pixel within the circle was black, the optical density was recorded as 0. For this reason, the formula was inverted and values of zero were assigned the value $10^{-10}$to allow calculations. This yielded percent increase in the amount of each cytokine relative to the corresponding control, and the data is recorded in **Tables 1 and 2**.

For the conditioned media, greatly increased secretion was found in 16 cytokines: GRO, IL-6, IL-7, IL-8, MCP-1, MCSF, SDF-1, Angiogenin, VEGF, BDNF, IGFBP-2, IGFBP-3, LIF, Osteoprotegerin, TIMP-1, and TIMP-2. In the stressed conditioned media, only IL-6, MCP-1, SDF-1, BDNF, Osteoprotegerin, TIMP-1, and TIMP-2 secretion was noticeably increased. The difference in blot size between the conditioned media and stressed conditioned media groups was analyzed in **Table 3** to determine the effect of stress on glioblastoma cytokine secretion. All four cytokines exhibited decreased diameters in the stressed group.

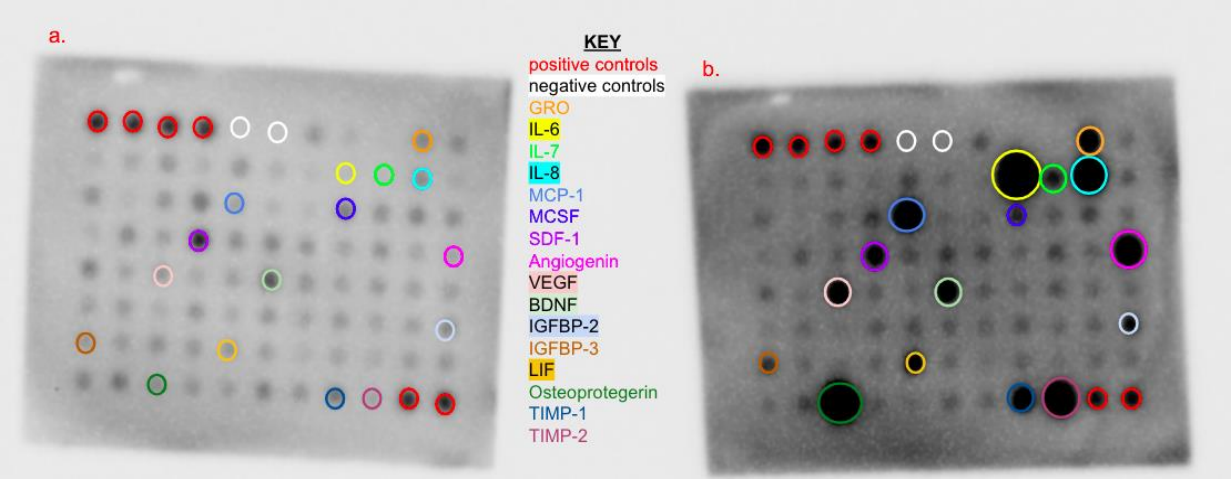

Figure 6: Chemiluminescent Imaging for Cytokine Array. (a.) The EMEM with FBS, which served as a control for b. (b.) Conditioned media. Positive and negative controls as well as significant cytokine blots are color coded according to the key. Darker dots correspond to secreted cytokines in the media. These are quantified in Table 1.

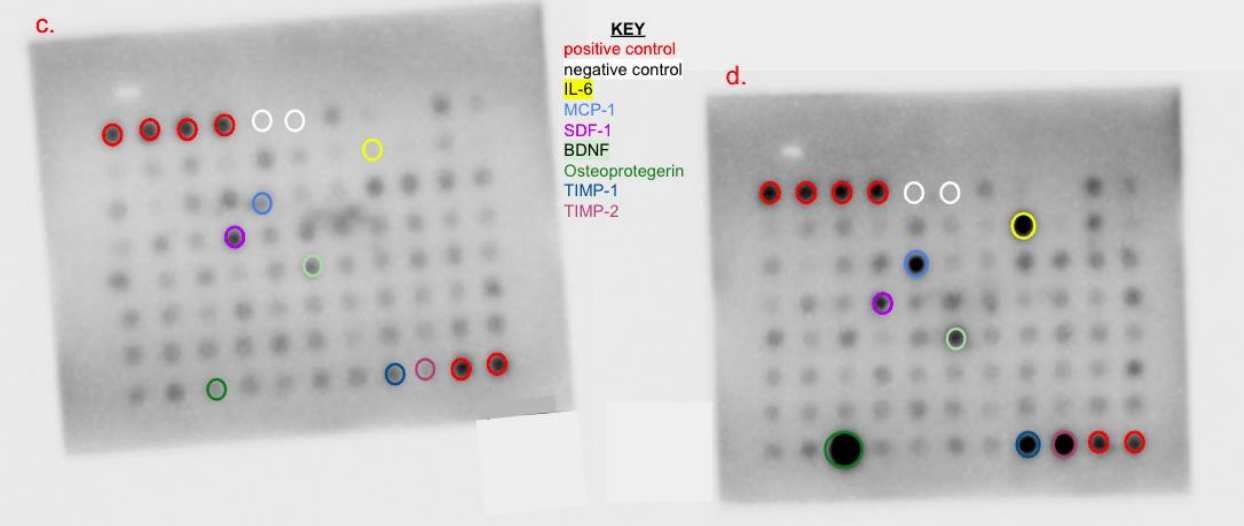

Figure 7: (c.) EMEM without FBS, which served as a control for d. (d.) Stressed conditioned media. Positive and negative controls as well as significant cytokine blots are color coded according to the key. Darker dots correspond to secreted cytokines in the media. These are quantified in Table 2.

CYTOKINE	xFOLD INCREASE
GRO	3.43E+02
IL-6	4.01E+08
IL-7	1.58E+00

IL-8	3.21E+08
MCP-1	3.63E+08
MCSF	8.17E-01
SDF-1	5.00E-01
Angiogenin	3.65E+08
VEGF	3.46E+08
BDNF	2.34E+08
IGFBP-2	4.84E-01
IGFBP-3	1.52E+00
LIF	5.52E+01
Osteoprotegerin	3.16E+08
TIMP-1	2.88E+08
TIMP-2	3.65E+08

Table 1: Cytokine Array Data for Conditioned Media. The integrated optical densities of the blots for conditioned media were compared to the control, EMEM with FBS. The percent increases correspond to secretion of the cytokines by the glioblastoma cells. Though 80 cytokines were tested, only the ones with the darkest blots are shown. The largest increases were for IL-6, IL-8, MCP-1, angiogenin, VEGF, osteoprotegerin, TIMP-1, and TIMP-2, which were all to the eighth power. Values were normalized to the positive control. Pink boxes indicate largely increased secretion, while yellow boxes indicate somewhat increased secretion.

CYTOKINE	xFOLD INCREASE
IL-6	4.59E+01
MCP-1	5.41E+00
SDF-1	6.45E-01
BDNF	1.15E+00
Osteoprotegerin	6.87E+08
TIMP-1	1.05E+03
TIMP-2	1.72E+00

Table 2: Cytokine Array Data for Stressed Conditioned Media. The integrated densities for each blot on the stressed conditioned media membrane was compared to the control of EMEM without FBS. Osteoprotegerin secretion was upregulated the most, with a factor of 10^{11}%. Values were normalized to the positive control. Red boxes indicate largely increased secretion, while yellow boxes indicate somewhat increased secretion.

CYTOKINE	CM to ST CM xFold Decrease
IL-6	1.06E-07
MCP-1	1.52E-08
SDF-1	1.14E+00
BDNF	3.72E-09
Osteoprotegerin	2.12E+00
TIMP-1	3.82E-06
TIMP-2	4.94E-09

Table 3: Cytokine Array Data Comparing Conditioned Media and Stressed Conditioned Media. Values are normalized to the positive control and the 3.586x multiplier from Table 4 was used to account for difference in cell counts.

BCA Assay

A BCA assay was performed to quantify the amount of protein in the conditioned media and stressed conditioned media. Because the overall amount of proteins secreted into the media should not vary widely between groups, differences can be attributed to the different cell counts from which the media was collected. The BCA assay can be used to normalize the cytokine array data and determine if secretion was truly decreased, or more likely increased, from the conditioned to the stressed groups. It was found that the conditioned media group had about 3.586 times more cells than the stressed group, so this multiplier was used for the cytokine array data.

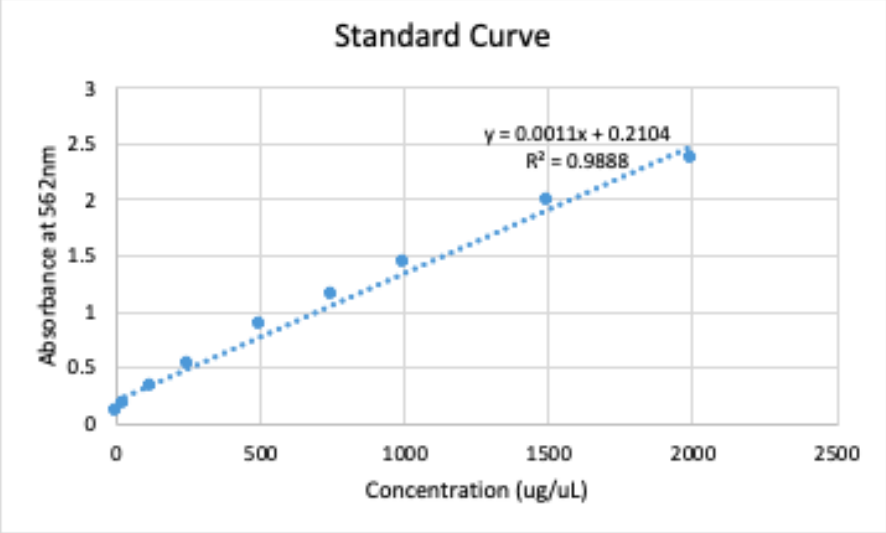

Figure 8: BCA Assay Standard Curve. This was used to determine the concentration of protein in the conditioned media and stressed media.

	Absorbance	Concentration
CM	3.9145	3367.364
Stressed CM	1.24325	938.9545

Table 4: Protein Concentrations of Conditioned Media and Stressed Conditioned Media. Conditioned Media has 3367.365 ug/uL of protein, and the stressed conditioned media has 938.955 ug/uL of protein. The multiplier for SCM to CM is 3.586, indicating there were 3.586x more cells in the conditioned media group.

Discussion

qPCR Primers

To analyze macrophage gene expression in future experiments, we decided to test the reliability of various genes of interest. In this experiment, primers were considered reliable if they exhibited upregulation in their respective experimental groups. Because CCR7, CD80, and CXCL10 were all expressed at a higher level in the THP-1 cells exposed to M1 cytokines than in those exposed to M2 cytokines, they are considered reliable and will be used as M1 genes of interest in subsequent experiments (**Figure 2a**). Conversely, since iNOS was expressed at a higher level in the M2 cytokine group, it is considered unreliable (**Figure 2b**). CD163, CD209, and MMR were all expressed at a higher level in THP-1 cells exposed to M2 cytokines than in those exposed to M1 cytokines (**Figure2c**). Interestingly, MMR was expressed at a lower level in the M1 cytokine group than in the PMA control, suggesting MMR is downregulated in M1 macrophages. Because CD209 and MMR exhibit the largest differences in expression levels between the two cytokine groups, they will be the primary M2 markers in future experiments. Since Arg-1, CCL18, and PD-L1 were expressed at lower levels in the M2 cytokine group than in the M1 cytokine group, they are considered unreliable and will not be used in the future (**Figure 2d**). The small variation in β-actin and GAPDH expression when normalized to cyclophilin (the reference gene used in all our experiments) indicates that they are comparable to cyclophilin as reference genes and could be used in future experiments (**Figure 2e**).

Polarization

To characterize the induction of the M2 phenotype in THP-1 cells by glioblastoma cells, it was necessary to optimize the protocol through which we obtain M2 macrophages. For this experiment, successful macrophage polarization occurs when the phenotypic markers are upregulated in their respective experimental groups. M-CSF exposure resulted in increased expression of CCR7, CD209, and CXCL10 compared to the PMA control; however, only CXCL10 was upregulated when the THP-1 cells were exposed to both M-CSF and M2 cytokines

(**Figure 3b**). Because downregulation was observed when both M-CSF and M2 cytokines were added, this experiment indicates that M-CSF priming is not a necessity in optimizing macrophage polarization.

Conditioned Media

In order to study how conditioned media from glioblastoma cells affects macrophage polarization, we decided to test various types to determine the optimal conditions. Due to the M2 phenotypic nature of TAMs, conditioned media exposure is expected to result in M2-like gene expression. Addition of U87 conditioned media (both normal and stressed) resulted in gene expression levels most similar to those of the M1 cytokine group; however, addition of T98G stressed conditioned media yielded significant upregulation of MMR, just as in the M2 cytokine group (**Figure 4a**). Since the gene expression levels following T98G conditioned media more closely resemble the M2 cytokine group, compared the levels following U87 conditioned media exposure, only T98G conditioned media will be used in future experiments.

To determine the effect of glioblastoma cells on macrophage polarization, we analyzed differences in gene expression that occurred because of exposure to normal T98G conditioned media. While CCR7, CD80, CD163, and MMR expression levels in the conditioned media group were similar to the expression levels in the M2 cytokine group, CXCL10 and CD209 expression was more similar to the M1 cytokine group (**Figure 4b-c**). The presence of similarities to expression levels of both cytokine groups indicates that the conditioned media is not completely polarizing the macrophages toward one phenotype. One possible explanation for the discrepancies is that the conditioned media used was EMEM which the T98G cells were cultured in for several days. Since THP-1 cells are not grown in EMEM, this likely resulted in cell death, affecting relative levels of gene expression.

Co-culture

To characterize the interaction between glioblastoma cells and macrophages, we decided to optimize the co-culture protocol, enabling us to observe the effects of the monocytes' direct exposure to glioblastoma cells. Analysis of THP-1 gene expression following co-culture demonstrated higher overall expression of M1 markers than M2 markers, compared to the PMA control (**Figure 5a**). A possible explanation for these results could be the nature of our culturing and harvesting protocols. Without a way to effectively isolate only THP-1 cells after culture in the same dish as T98G cells, we used a transwell insert and a 6-well plate – THP-1 cells in the plate and T98G cells in the transwell. Upon PMA priming, THP-1 cells adhere to the plate and are difficult to remove. Even after trypsinization and scraping, there were still adherent cells remaining. This in addition to the rigorous scraping necessary to remove the cells we did likely resulted in cell loss, affecting the relative expression levels observed.

Additionally, because the macrophages and glioblastoma cells are grown in different medias, we decided to test whether there is an optimal media which allows for adequate growth of both cell types. Compared to normal growth conditions in RPMi, THP-1 cells exhibited

decreased growth in EMEM (**Figure 5b-c**). Conversely, compared to normal growth conditions in EMEM, T98G cells exhibited decreased growth in RPMi (**Figure 5e-f**). Both THP-1 cell and T98G cells demonstrated similar confluencies in a 1:1 EMEM and RPMi mixture to that of their respective normal growth conditions (**Figure 5d** and **5g**). This indicates that the use of a 1:1 media mixture will result in adequate growth of both monocytes and glioblastoma cells when used in future experiments.

Cytokine Array

A cytokine array was performed to detect secreted factors in the media that may influence macrophage polarization. For the conditioned media, sixteen cytokines exhibited increased secretion: GRO, IL-6, IL-7, IL-8, MCP-1, MCSF, SDF-1, Angiogenin, VEGF, BDNF, IGFBP-2, IGFBP-3, LIF, Osteoprotegerin, TIMP-1, and TIMP-2 **(Figure 6)**. Of these, IL-6, IL-8, MCP-1, and Osteoprotegerin were present in particularly large amounts as seen in the increased diameter of their blots. Each of these cytokines' densities had percent increases to the eleventh power **(Table 1)**. In the stressed conditioned media, only IL-6, MCP-1, SDF-1, BDNF, Osteoprotegerin, TIMP-1, and TIMP-2 secretion was increased **(Figure 7)**. Of these, Osteoprotegerin exhibited the largest increased density, with 6.87E+08 fold increase **(Table 2)**. Osteoprotegerin is typically known to aid bone remodeling, but in the tumor microenvironment it plays a role in tumorigenesis of healthy cells and metastasis[6].

Prior research suggested that glioblastoma cells secrete IL-1, IL-6, IL-8, SDF-1, TNF, GDNF, HGF, VEGF, MCSF, and GM-CSF and that these play a role in macrophage polarization[2]. IL-6 in particular leads to alternative macrophage activation[4]. However, IL-1, GDNF, HGF, and GM-CSF were not amongst the most secreted as expected. This may be due to the isolated cell culture environment; the tumor cells were not in communication with surrounding tissues the way they would be in a body. Another unexpected finding was that GRO, MCP-1, BDNF, IGFBPs, LIF, and TIMPs were secreted at high levels. GRO is known to be secreted by tumor cells because it aids metastasis, angiogenesis, and invasion of tissues[3]. MCP-1 is a monocyte chemoattractant that likely helps the glioblastoma recruit monocytes that are then matured by glioblastoma-secreted MCSF and polarized[3]. BDNF, or brain-derived neurotrophic factor, helps neural cells survive, so it may aid glioblastoma growth[18]. TIMP 1 and 2 activity is mainly associated with pain attenuation and inflammation[9]. The increased secretion of these cytokines by glioblastoma cells is reasonable.

The stressed conditioned media showed that without FBS, T98Gs seem to reduce secretion of the cytokines tested in this assay. Even the more-secreted factors were still decreased relative to their conditioned media counterparts. This may suggest that when the cancer cells are forced into survival mode, they pause nonessential activities. The cells in the stressed media flask were sparse and visibly stressed. To account for this, a BCA assay was performed which revealed there was about 3.586 times more protein, and likely more cells, in the conditioned media compared to the stressed media (**Table 4**). This means the amounts of cytokine secreted from the stressed cells should be multiplied by 3.586 to be comparable to the

conditioned media data. In the future, it may be beneficial to only culture the T98Gs in EMEM without FBS for the two or three days it takes to collect the conditioned media, rather than for one or two weeks at a time. Alternatively, the cells could be counted once the media is collected and then the cytokine amounts can be normalized to cell count. It would also be highly beneficial to study normal glial cell conditioned media, so the differences in cytokines of cancerous cells can be identified. These factors may yield clues into the signaling pathways that alter macrophage function in the tumor microenvironment.

Conclusion

This project sought to understand the interactions between glioblastoma cells and tumor associated macrophages in the tumor microenvironment. Through numerous qPCR experiments, reliable M1 and M2 primers were identified. For observing M1 polarization, CCR7, CD80, and CXCL10 were determined to be reliable. For M2 polarization, CD163, CD209, and MMR exhibited the expected trends and were considered adequate biomarkers. Progress was made in optimizing the protocol to produce tumor-associated macrophages. MCSF was deemed unnecessary for polarization. It was found that the co-culture couldn't yield enough genetic material in the six-well plate setup, so in the future efforts can focus on using six centimeter petri dishes to grow T98Gs and THP-1s on the same surface. Separating the macrophages for qPCR analysis will require more experimentation. The conditioned media experiments suggested incomplete polarization, so alternative means of confirming macrophage polarization should be explored. Finally, a cytokine array was performed to analyze the signaling factors in the conditioned media secreted by glioblastoma cells under stressed and unstressed conditions. This suggests MCP-1, angiogenin, VEGF, and osteoprotegerin (amongst others) exhibit increased secretion, and that IL-6 may be one of the key players in the alternative activation of tumor-associated macrophages.

In addition to exploring co-culture options, the cytokine array should be repeated with a normal glial cell control as well as normalization to the cell count that secretes the cytokines in the media for better comparison between groups. In the future, flow cytometry may be a useful tool for confirming polarization because it can identify surface proteins implicated in the signaling pathways activating the alternative functions of TAMs. Finally, more experimentation with M2 inhibitors could help determine if the alternative activation of TAMs can be reversed back to the classical M1 phenotype. Successful inhibition could lead to harnessing the body's natural immune system to eliminate glioblastoma alongside chemotherapeutic treatments.

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Addendum

ARRAY MAP											
	a	b	c	d	e	f	g	h	i	j	k
1	Pos	Pos	Pos	Pos	Neg	Neg	ENA-78	GCSF	GM-CSF	GRO	GRO-alpha
2	I-309	IL-1alpha	IL-1beta	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	IL-8	IL-10
3	IL-12 p40/p70	IL-13	IL-15	IFN-γ	MCP-1	MCP-2	MCP-3	MCSF	MDC	MIG	MIP-1b
4	MIP-idelta	RANTES	SCF	SDF-1	TARC	TGF-beta1	TNF-alpha	TNF-beta	EGF	IGF-1	Angiogenin
5	Oncostatin M	Thrombopoietin	VEGF	PDGF-BB	Leptin	BDNF	BLC	Ck beta 8-1	Eotaxin	Eotaxin-2	Eotaxin-3
6	FGF-4	FGF-6	FGF-7	FGF-9	Flt-3 Ligand	GCP-2	GDNF	HGF	IGFBP-1	IGFBP-2	IGFBP-2
7	IGFBP-3	IGFBP-4	IL-16	IP-10	LIF	LIGHT	MCP-4	MIF	MIP-3alpha	NAP-2	NT-3
8	NT-4	Osteopontin	Osteoprotegerin	PARC	PlGF	TGF-beta2	TGF-beta3	TIMP-1	TIMP-2	Pos	Pos

Figure 23: Cytokine Array Map. This is provided as reference for the blots in Figures 6 and 7.