IDENTIFICATION OF MICROGLIAL CELLS DURING CHICK EMBRYO BRAIN DEVELOPMENT AND HUMAN XENOGRAFT TUMOR FORMATION

by

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TABLE OF	CONTENTS
----------	----------

LIST (OF TA	BLESvi
LIST (OF FI	GURES
ABST	RACT	viii
1	INTI	RODUCTION1
	1.1 1.2 1.3	Glioblastoma
		1.3.1Microglia and Glioma
		1.3.2.1 Primary Chick Studies
	1.4	Specific Aims and Objective
2	MAT	TERIALS AND METHODS
	 2.1 2.2 2.3 2.4 2.5 2.6 	Glioblastoma Cell Line
3	RES	ULTS
	3.1	Antibodies Screening Using the Normal Chick Embryo Brain18
		3.1.1 LT40 Antibody to Visualize Microglia Changed Across Development
		3.1.2 Tomato Lectin and Isolectin B4
		3.1.3 Ricinus Agglutins Lectin
		3.1.4 Staining for Endothelia Using Antibodies: Anti-Laminin and Anti- Integrin
		3.1.5 Differentiation of RCA-Positive Microglia from RCA-Positive Endothelia Using Double Staining
	3.2	Glioma and Microglia in E14 and E15 Brains
4	DISC	CUSSION

4.1	Optimal Antibodies	
	4.1.1 Anti-Chick CD45: LT40	
	4.1.2 RCA-I Lectin Specifically Labels Microglia	
	4.1.3 Use 3H11 and V2E9 to Differentiate RCA-I Positive	
	Endothelial from Microglia	
4.2	Tumor and Microglia	
4.3	Implications for Future Research	
4.4	Conclusions	
REFERENC	CES	
Appendix A	٠	
А ОРТ	TIMIZATION IN STAINING PROTOCOLS	
A.1	Autofluorescence (AF) Reduction Attempts	
A.2	Additional Images	
B.2	Additional Antibodies	

LIST OF TABLES

Table 1. Primary antibodies reported to be specific to microglia only.	.12
Table 2. Antibodies reported to be specific to endothelial cells only	. 13
Table 3. Lectins reported to stain both microglial and endothelial cells	. 14
Table 4. Summary of immunostaining results in normal brain versus U-118 glioma injected brain.	.36
Table 5. Results seen using different solutions for immunostaining cryosections usin lectin primary	ng . 52
Table 6. List of secondary and tertiary antibodies used throughout the study	. 56
Table 7. Antibodies that was tested during the study but did not show any positive staining in the chick brain cryosections.	. 58

LIST OF FIGURES

Figure 1. Amoeboid shaped HIS C7 positive cells (B) and primitive ramified microglia (C) in chick optic tectum (Cuadros et al., 2006)7
Figure 2. Widefield microphotographs of chick embryo cryosections at different ages showing changes in quantity and morphology of microglia stained with LT40
Figure 3. Isolectin B4 staining a microglial like cell in the E7 chick brain cryosection
Figure 4. RCA-I staining in the E19 Optic tectum
Figure 5. RCA-I staining of microglia and blood vessel in a normal E19 Optic Tectum
Figure 6. Endothelial staining in chick optic tectum sections
Figure 7. Double stained E15 Optic Tectum for RCA-I (green) and 3H11 (magenta)
Figure 8. Double staining of RCA-I and V2E9 to tell apart microglia from blood vessels in the E15 Optic Tectum
Figure 9. Close proximity of U-118/L1LE tumor traveling along RCA-I positive blood vessels in E14 optic tectum
Figure 10. Amoeboid shaped microglia at periphery of U-118/1879 (red) glioma cells in E15 optic tectum
Figure 11. Triple staining of an E14 U-118/L1LE optic tectum with RCA-I (green) and V2E9 (purple) showing extensive microglia and blood vessel 34
Figure 12. Triple staining of an E14 U-118/1879 optic tectum showing cross section through the peripheral tumor mass in an E14 optic tectum 35
Figure 13. Autofluorescent images taken at the 488 channel and 594 channel
Figure 14. Double staining of blood vessel using CSAT-s and RCA-I in E14 optic tectum injected with U-118/1879 (not shown here)
Figure 15. LT40 staining (magenta) of E14 optic tectum injected with U-118/L1LE cells (red)

ABSTRACT

Glioblastoma (GBM) is the most aggressive and invasive human brain tumor, which despite advancement in surgery and immunology, only 10% of patients survive 18 months after diagnosis. The Galileo lab studies GBM, focusing on the role of the protein L1CAM on increasing the motility, proliferation, and invasiveness of GBM cells using in vitro cell tracking and a novel xenograft chick embryo brain tumor model. My work focused on microglial cells, which are resident brain immune system cells that are not well understood. Previous research in mice showed that microglial cells concentrated in areas with greatest cancer cell invasion. It was hypothesized that a difference in microglia will be seen in the chick embryo brain that corresponds with areas of human xenograft tumor growth and invasion of glioma cells. The first aim for my project was to find biomarkers that specifically identify microglial cells in normal chick embryo brain during development. The second aim was to use those markers to examine any potential interaction microglial cells have with xenograft tumors in the chick embryo brain.

Chick embryo brains were fixed, frozen, and cut into serial cryostat sections or fixed and vibratome sectioned, followed by immunofluorescent staining using antibodies or lectins that identified microglial cells in other model organisms. The optimal staining protocol required overnight primary antibody/lectin with 0.1% Triton X-100 detergent and 5% normal goat serum in phosphate buffered saline. The antibodies and lectins that I investigated included Isolectin B4, *Ricinus Communis* Agglutinin I (RCA-I), 3H11, V2E9 and anti-CD45. Several of the antibodies and lectins tested either did not stain cells specifically or exhibited a high background staining. Immunofluorescence analysis revealed RCA-I to stain microglial cells with

viii

the most specificity. Anti-CD45 staining reveal microglial cell morphology to progressively change from a compact amoeboid shape (E5) into ramified branched shape (E12 and E15). Instead of locating throughout the brain, microglial cells appeared to be located as clusters in certain areas. In the chick brains with xenograft human tumors, there appeared to be a cluster of amoeboid shaped microglia in the tumor periphery. Further investigations are needed to verify the increased presence of reactive microglial cells and their interaction with tumor cells.

Chapter 1

INTRODUCTION

1.1 Glioblastoma

Glioblastoma (GBM), an astrocytic glioma grade IV, is a highly invasive primary brain cancer that is resistant to all current treatment methods with recurrence rate extremely high in patients. Currently, GBM is also the most common primary malignant glioma in adults (Alves et al., 2011). Characteristics of malignant glioma include angiogenesis, necrosis, diffuse growth pattern and heterogeneity. Amongst the most vascularized tumor in human, GBM invades the brain along blood vessels, leaving behind hypoxic conditions (Chen & Hambardzumyan, 2018). Glioma cells exhibit a great extent of inter- and intra- tumor diversity, allowing for resistance to current therapeutics. The standard of care treatment for GBM is surgical resection followed by Temozolomide chemotherapy and radiation (Johnson & O'Neill, 2012). The median survival time of GBM patients following diagnosis is 14.6 months which, despite advancement in scientific research, has not shown improvement in the past decade.

1.2 Brain Tumor Microenvironment (TME)

GBM's resistance to surgical resection is owed to its aggressive infiltration of the physical barriers within brain such as extracellular matrix and a functional immunosuppression in the microenvironment. The tumor microenvironment includes all non-cancerous cells such as glial cells, immune cells, endothelial cells and

biomolecules produced by those cell types within the environment. Glioma cells produce cytokines and chemokines that lead to tumor promotion, immunosuppression and chemo attraction. There is widespread hypoxia at the center of the tumor population caused by inappropriate neovascularization and excessive oxygen consumption in proliferating glioma cells. Specifically, hypoxia-inducible factor- 1α (HIF-1 α) induces vascular endothelial growth factor (VEGF) production which leads to endothelial cell proliferation and suppression of immune cells (Razavi et al., 2016). The immunosuppressive cytokines, such as interleukin (IL)-10, IL-6 and TGF- β are found throughout the TME, aiding the irregular angiogenesis (Matias et al., 2018). One strongly immunosuppressive cytokine is expressed in high concentration in microglial cells when in close proximity to GBM cells thereby inducing T-cells apoptosis (Magnus et al., 2005). Glioma cells produce chemoattractants such as VEGF, SDF-1 and CX3CL1 to attract macrophages (Carvalho Da Fonseca & Badie, 2013). The majority of the cells in the TME are tumor associated macrophages. Of increasing interest in recent therapeutic intervention is the finding that up to 30% of the tumor mass consists of microglial cells (Charles et al., 2012).

1.3 Microglia

Microglia are specialized resident macrophages that are found in the central nervous system and functions to maintain overall brain immunity and homeostasis. These resident macrophage cells, distinct from blood marrow derived macrophages, invade the brain very early in development and differentiate into ramified microglial cells (Hanisch & Kettenmann, 2007). Myeloid progenitor cells that entered the central nervous system (CNS) have longevity and a limited self-renewal (Razavi et al., 2016). Microglia harbor a unique phenotype distinct from peripheral macrophages located extrinsic to the blood brain barrier. As immune cells, microglia have long processes to scan the central nervous system, removing plaques and damaged or unnecessary neurons and synapses in the presence of injury and pathologic conditions.

The morphology of microglia closely aligns with development and its function. During development, microglia resemble a compact amoeboid shape, round with short processes, and gradually extend processes to acquire a ramified shape, a small soma with fine cellular processes (Helmut et al., 2011; Kaur et al., 2017). In the healthy mature CNS, "resting microglia" are highly motile cells, constantly surveilling for any altered neuronal activity that causes a loss of brain homeostasis. When a response is detected, microglia becomes activated, undergo migration and releases cytokine factors to restore homeostasis. An "activated microglia" acquires an amoeboid shape and changes in receptor expression patterns to carry out phagocytic and inflammatory role (Nakamura et al., 1999).

1.3.1 Microglia and Glioma

However, in the presence of malignant cancer, microglia seem to acquire a unique phenotype unlike that of an activated type. In the native CNS research have shown that the maximal concentration of microglial cells coexists in areas with the greatest cancerous cell number (Bryukhovetskiy et al., 2016; Charles et al., 2012). Glioma cells release chemoattractants in the TME, which bind to the receptors on microglia. Such microglia are termed tumor associated microglia or macrophages (TAM) which has been shown to undergo a complex relationship with glioma cells benefitting glioma migration while suppressing the normal "activated" phenotype in the resident brain immune cell. Activated microglia have a functional phenotype similar to M1 phenotype of hematopoietic macrophages. M1 activated microglia are

amoeboid shaped and stimulate anti-tumor immune responses through proinflammatory cytokines and chemokines such as tumor necrosis factor (TNF) α . In contrast, M2 microglia are ramified shaped and secrete anti-inflammatory factors such as Arginase-I and interleukin-10 which are essential to tissue repair and wound healing. TAM have a morphological amoeboid appearance typical of M1, but a M2 functional phenotype, contributing to immunosuppression. Malignant glioma has a greater proportion of M2 phenotype microglia compared to more benign glioma (Charles et al., 2012). In presence of glioma, microglia cells behave in a way that is distinct from

TAM have been shown to promote glioma proliferation and migration by engaging in a glioma cross talk. Glioma produces soluble factors which attracts microglia and leads to pro-invasion outcomes. In response to soluble factors released by glioma cells, microglia produce type 1 metalloproteases (MT1-MMP) which in turn helps to activate metalloprotease 2 (MMP-2) released subsequently by glioma cells (Markovic et al., 2009). In addition, glioma cells release CX3CL1 which binds to CX3CR1 on microglia to also cause a cascade of MMP production in glioma cells. MMP breaks down extracellular matrix to provide for extensive angiogenesis and outgrowth of glioma. In addition to aiding invasion, glioma leads microglia to a M2 phenotype by pathways such as inhibition of (IL)-1 β and TNF and an upregulation of transforming growth factor (TGF)- β 1. IL-1 β is a pro-inflammatory cytokine that is typically released by activated macrophages (Yin et al., 2017). Glioma secretes prostaglandin 2 which inhibits tumor necrosis factor (TNF) production in microglia (Matias et al., 2018). TGF- β act by promoting angiogenesis through vascular

endothelial growth factor expression and immune suppression by reducing the ability of microglia to acquire a pro-inflammatory functional phenotype.

1.3.2 Relevant Primary Microglia/Glioma Study Models

Identification of microglia through staining procedures was made possible by cell surface-associated molecules. In vivo studies were primarily performed in the rodent brain using antibody-based and lectin-histochemical procedures that yielded pivotal outcomes. Markovic et al. (2008) injected glioma cells into embryonic mouse brain and stained for MMP and microglia marker, Iba-1 and Griffonia simplicifolia isolectin-B₄ (ILB4). The density of microglial cells was much higher in the glioma region than in normal brain while the staining of MMP is expressed in microglia associated with glioma. Furthermore, transgenic mice expressing a CD11b mutation causing reduced microglia population also led to reduced glioma growth. Iba1 is a microglia-specific protein important in calcium homeostasis that has proven most helpful in visualizing microglia in experimental rodent models (Ito et al., 1998). ILB4 is a plant lectin that in addition to identifying microglia, also stains endothelial cells. Imaging for endothelial cells was often done along with gliomas studies because of the close proximity between glioma invasion and blood vessels. Other lectins such as Lycopersicon esculentum (tomato) lectin and Ricinus agglutins lectins have also been shown to stain both microglia and endothelial cells. Another study by Kostianovsky et al. (2008) formed cocultures of primary human microglia cells isolated from GBM specimens with immune cells prevented subsequent activation of freshly isolated monocytes. Therefore, malignant glioma cells rendered microglia cell unable to activate normal brain inflammatory processes. Antibodies against CD11b, unique to microglia was used to positively select for microglia cultures.

Both of the above studies have shown that microglia play an important role in glioma progression using both in vivo rodent model system and in vitro human coculture experiments. However, there remains so much that is unknown in the resident CNS immune cell role in glioma. To further understand the relationship of microglia in glioma progression, these experiments must be repeated and the process of the glioma invasion must be studied in greater depth.

1.3.2.1 Primary Chick Studies

Experiments performed using the chick brain have shown that it can be a promising model for studying microglia. Cuadros et al. (2006) used a chick embryo model to examine the characteristic of microglia development in response to pathological death of motoneurons. In the chick spinal cord, microglial cells marked with anti-chicken CD45, carried out the normal immune functions as described within human CNS. Chick microglial cells were present at an early developmental stage and performed phagocytic functions to remove dying cells. Both the number and shape of chick microglia transformed during development. In another chick study, Calderó et al. (2009) used anti-CD45 antibody (HIS-C7) and Ricinus communis (RCA I) lectin to stain for microglial cells. Positive immunostaining occurred as early as embryonic day three (E3), which appeared with an amoeboid shape in the neuroepithelium, thereby matching the results from rodent studies. On the twelfth embryonic day, both amoeboid and ramified shaped cells were stained in the optic tectum of the midbrain. An addition study performed by Ignácio et al. (2005) also identified and localized microglial cells within the cerebral hemispheres during the normal development of chicken. The morphology of chick microglial were divided into an amoeboid, primitive ramified or ramified cell group as shown in Figure 1. Chick microglia were

labeled using tomato lectin, which also stained rodent microglial cells. Although studies are limited, the results obtained have revealed the chick brain to be a promising model system to study changes in morphology and quantity of microglia.



Figure 1. Amoeboid shaped HIS C7 positive cells (B) and primitive ramified microglia (C) in chick optic tectum (Cuadros et al., 2006).

The Galileo lab has shown the chick embryo to be an efficient model system to study GBM invasion (Cretu et al., 2005). Glioma cell line injected into the chick embryo on E5 have been visualized using confocal and wide field microscopy. Therefore, it would be a great model to examine the combined effect of the glioma cell and microglia in the chick brain.

1.4 Specific Aims and Objective

It is proposed that differences in microglia will be seen in the chick embryo brain that corresponds with areas of human xenograft tumor growth and invasion of glioblastoma cells. The two aims for this project are:

1. To find biomarkers that specifically identify microglial cells in normal chick embryo brain during development.

2. To use those markers to examine any potential interaction microglial cells have with xenograft tumors in the chick embryo brain.

Chapter 2

MATERIALS AND METHODS

2.1 Glioblastoma Cell Line

The cells line used for injection was U-118 MG, a human glioblastoma cell line obtained from American Type Culture Collection (ATCC, Manassas, VA). Two variants of U-118 cell lines, U-118/1879 and U-118/L1LE, were used for injections. U-118 cells transduced with an empty K1879 lentiviral vector created a L1-negative U-118/1879 cell line. U-118 cells transduced with K1879 vector containing the L1 long ectodomain (L1LE) produced U-118/L1LE cells. These cell lines then were infected with another lentiviral vector encoding the mCherry fluorescent protein. The cells were cultured in Dulbecco's Modified Eagle Medium (Thermo Fischer Scientific), 10% fetal bovine serum (Atlanta Biologicals), 2mM L-Glutamine (Sigma) and 100µg/mL Penicillin/Streptomycin (Cellgro). The cells were cultured in a humidified incubator at 37°C and 5% CO₂ and passaged with 0.25% Trypsin/EDTA solution (Cellgro).

2.2 In vivo model: Chick Brain

Fertilized White Leghorn chicken eggs were obtained from the University of Delaware Department of Animal and Food Sciences and incubated at 37.5°C in a humidified force draft incubator. To analyze microglia development, the chick embryos were sacrificed at multiple embryonic (E) ages, E5, E9, E12, E15 and E19. E0 referred to the day that the fertile eggs were placed into the incubator. E5 chick brain was too small for dissection of the brain, hence the entire embryo was used for analysis. E9 and E12 brains were dissected and fixed as whole brains. E15 and E19 brains were further dissected into their cerebellum and optic tecta. The respective tissues were fixed in 2% paraformaldehyde in 0.1M cacodylate buffer (Electrion Microscopy Sciences) overnight. On the second day, the tissue was rinsed in phosphate buffer saline (PBS) for three times at one hour each. After the fixative was rinsed, the tissues were immersed in 30% sucrose and allowed to fully sink. Afterwards, the tissues were embedded in Tissue-Tek OCT compound, frozen at -80°C and sectioned on Leica CM3050 S Cryostat at -20°C. Sections were collected on Superfrost plus slides (Fisher), air dried and stored in a desiccated airtight container at -20°C until immunostaining. Cryostat sections were cut at thicknesses of 16µm and 10µm. The thinner sections were cut to allow for better antibody penetration during immunostaining.

2.3 Injection of Established Glioma Cells into Chick Embryo Brain

On E5, the chick optic tectum was injected with U-118 cell lines to examine the interaction of GBM cells with the brain endothelial and microglial cells. Preparation were the same as previously described in Cretu et al. (2005). The cells used for injection were virally infected with fluorescent marker, mCherry, and injected on E5. The eggs were sterilized with 70% ethanol and a small hole was cut in the blunt end of the egg. After exposing the membrane, saline was dropped to coat the space and approximately 5μ L of 10,000cells/ μ L glioma cells were injected into the optic tectum with a PV830 pneumatic picopump (World Precision Instruments). Before injection, the cells were trypsinized and counted using a hemocytometer. Following injection, a few drops of ampicillin was added into the egg and the opening sealed with tape. The eggs were returned to the incubator until their dissection on E14. On E14, the chick embryos were sacrificed and the same fixation and sectioning

procedure were performed. Only the optic tectum in injected chick brains was sectioned for later analysis.

2.4 Search for Biomarkers

Since microglia research in chick brain is so minimal, much of this study was spent on finding adequate antibodies and lectins to specifically bind onto antigens specific to microglia. Additional antibodies binding to endothelial cells were sought to better analyze the interaction between microglia, vasculature and glioma. All of the primary antibodies and lectins used in this study to identify microglia, endothelial and both cell types are listed respectively in Tables 1-3.

		- ·		
Antigen	Antibody	Company/	Dilution/Meth	Reference
		Product Code	od	
Chicken CD45	LT40	Southern	1:50 overnight	N/A
	IgM	Biotech	3 Steps	
	C	8270-01	-	
Chicken CD45	Um16-6	Bio Rad	1:100	N/A
	IgG2a	MCA2413GA	overnight	
		Lot: 150839	3 Steps and 2	
			Steps	
C-terminal of	Iba-1*	Wako	overnight	Ito et al., 1998
Iba1 (rodents)		019-19741,	2 Steps	
		Lot PTE0555	-	
Ferritin	Ferritin heavy	Bioss	overnight	Kaneko et al.,
	chain	Bs-5907R	1:100	1989
	Polyclonal			
	Antibody			
	(Rabbit IgG)			

Table 1. Primary antibodies reported to be specific to microglia only.

^{*} Borrowed from Dr. Jaclyn Schwarz lab.

Antigen	Antibody	Company/	Dilution/Type of
		Product Code	Protocol
human von	Polyclonal Rabbit	Agilent Dako	1:100
Willebrand	Anti-Human Von	A0082	2 steps overnight
factor/factor VIII	Willebrand Factor		
complex	(VWF)		
Laminin-1, avian	3H11-s (IgG1)	DSHB*	1:20
			2 steps or 3 steps
			overnight
Laminin 111	Rabbit Polyclonal	Encore Bio	1:500
	Antibody to	Lot 197-013019	2 steps overnight
	Laminin (RPCA-		
	Laminin) (IgG)		
β_1 integrin	CSAT-s (IgG2b)	DSHB*	1:10
	_		2 steps overnight
β_1 integrin	V2E9-s (IgG1)	DSHB*	1:20
			2 steps overnight

Table 2. Antibodies reported to be specific to endothelial cells only.

^{*} Developmental Studies Hybridoma Bank, University of Iowa, Department of Biology, 028 Biology Building East, Iowa City, Iowa 52242-1324

Antigen	Lectin	Company/ Product Code	Dilution/Type of Protocol	Reference
[GlcNAc]1-3, N- Acetylglucosa mine	Lycopersicon Esculentum (tomato) lectin, Biotinylated	Vector Labs B-1175 Lot: ZF0412	1:200, 2 Steps Streptavidin Secondary	Ignácio et al., 2005 Kaur et al., 2017 Villacampa et al., 2013
Galactose, Lactose	Ricinus Communis Agglutinin I (RCA I, RCA120), Biotinylated	Vector Labs B-1085-5	1:500 2 Steps Streptavidin Secondary	Cuadros et al., 2006; J. Y. Kim et al., 2014; Mannoji et al., 1986;Shin et al., 2003
Galactose	Griffonia Simplicifolia Lectin I (GSL I) isolectin B4, Biotinylated	Vector Labs B-1205 Lot: ZB1017	1:50, 2 Steps Streptavidin Secondary	N/A
α-D-galactosyl	Griffonia simplicifolia (BS-I) Isolectin B4 Biotin Conjugate	Sigma Aldrich L2140 Lot: 039M4066	1:100	Dailey et al., 2013; Ito et al., 1998

Table 3. Lectins reported to stain both microglial and endothelial cells.

2.5 Immunostaining

Multiple procedures were used to stain for different antibodies and lectins which is described in more details in the Appendix. The optimal staining procedure will be discussed here. Since all of the biomarkers were new to the lab, optimal staining conditions had to be determined empirically. The initial dilution of primaries was determined based off of distributors' and past researchers' recommendations. New primary concentrations were increased until a positive signal could be determined. A two-step protocol using a fluorescent conjugated streptavidin secondary was used for all biotinylated lectins. A three-step protocol for antibodies was performed in two ways. The first consisted of a biotinylated secondary directed against the host species of the primary and a fluorescent conjugated-streptavidin tertiary. The second way was a secondary antibody directed against the host species of the primary and a fluorescent-conjugated tertiary antibody directed against the host species of the primary

Frozen sections were allowed to thaw until the case containing the sections were at room temperature. Using Super Peroxidase-anti-peroxidase (PAP) Pen, a hydrophobic barrier was drawn surrounding each brain section to allow for a well to contain the staining solution. For all staining, sections were blocked for 30 minutes in "PBSTG" which consisted of 5% Normal Goat Serum (NGS), 0.1% Triton X-100 (Sigma) and PBS. Hereafter, "PBSTG" will refer to the same concentration of NGS and detergent. All primary antibodies or lectin (except for RCA-I lectin) were left overnight in a dark humid chamber at 4 °C. RCA-I lectin was applied for one hour at room temperature. All primary antibodies in Table 1 and Table 2, with the exception of Iba-1 and Ferritin, were stained in both 2-step and 3-step antibody incubation protocol to determine the best staining condition. Since there was no adequate

secondary antibody against the host of Iba-1 and Ferritin, rabbit, only a 2 step protocol was performed using Alexa Flour 647-conjugated Goat anti-Rabbit IgG (H+L) (Jackson Immunoresearch catalog #111-605-144) secondary. Following the primary incubation, the solution was aspirated off of the section using a Pasteur pipet and vacuum then rinsed 3x at 5 minute intervals with PBSTG. After the final incubation step, nuclei were counterstained with Bisbenzimide (Hoechst 33258) diluted to 2μ g/mL for 5 minutes in PBS. Following nuclei staining, the sections were rinsed in PBSTG for 5 minutes. The slides were mounted using a glycerol based mounting media: 80% glycerol, 0.625% n-propyl gallate and 20% PBS. Immunostainings for each tissue section were performed using up to five markers. The sections containing five markers consisted of U-118/mCherry cells, Alexa flour 488, Alexa flour 546, Alexa flour 647 and bisbenzimide. Multiple variation of fluorescent secondary and tertiary were used to analyze the separate antibodies and lectins. For negative controls, sections were stained with secondary and tertiary without the primary. The edges of the coverslip were sealed with nail polish before storing slides at 4 °C.

2.6 Microscopy and Image Analysis

Sections were analyzed using both wide field fluorescence using a Nikon E800 microscope and a new confocal microscope (Nikon C2+si). The wide field microscope was an upright microscope with Plan Apo objectives and an exterior camera that could capture images. The new Nikon C2+ confocal microscope was used to take the majority of the later images and then analyzed using companion Nis Elements AR software. It used four lasers with wavelengths at 405nm, 488nm, 561nm, and 640nm. Due to the thinness of the cryostat sections, a single z-plane image was acquired at different x-y locations. For a few sections containing tumor, acquisition of "z-stacks"

were taken at thickness of 2.5 microns to acquire a thin 3D image of the brain. Most of those 3D images were converted into 2D by utilizing the "maximum intensity projections" function the Nis software which consists of the straight-on maximum brightness value in the z-stack for each x-y pixel.

Chapter 3

RESULTS

3.1 Antibodies Screening Using the Normal Chick Embryo Brain

Since the microglia research performed within the chick brain is so limited, all antibodies and lectins that I used had to be optimized for staining in thin frozen sections. In my literature search, there were two groups of biomarkers, protein based antibodies and carbohydrate based lectins, that seemed to be staining microglia and endothelia cells.

3.1.1 LT40 Antibody to Visualize Microglia Changed Across Development

LT40, an anti-chick CD45 antibody, was used to identify microglial cell. Bright LT40 positive cells were seen at all ages when viewed on the E800 widefield microscope as shown in Figure 2. E5 embryos were sectioned as a whole embryo because the brain was too small to fully dissect. While I was unable to identify the exact location of the section within the embryo, I saw small compact round amoeboid shaped cells that stained. This identical small shape was not seen at the older ages. E7 was the earliest in which the brain was dissected but not into its separate lobes. A section of the entire brain at E7, showed larger shape LT40 positive cells with a more ramified morphology distinct from E5. Beginning at E12, the chick brain was large enough to be dissected into optic tectum, hindbrain and cerebellum. Microglia with longer processes were seen in the E12 hindbrain. In the older brain, E15 optic tectum, both amoeboid and ramified shapes were seen. Thus, during the chick development, a difference in the morphology of brain microglia was observed.



Figure 2. Widefield microphotographs of chick embryo cryosections at different ages showing changes in quantity and morphology of microglia stained with LT40. Microglia were stained with LT40 (in green) three steps and nuclei counterstained with bisbenzimide (in blue). (A-C) Presence of compact amoeboid shaped LT40-immunolabeled cells in an E5 embryo. (D-F) In an E9 brain, microglia appeared larger and greater in quantity. (G-I) In the E12 hindbrain, branched ramified shapes were seen in the circled area. (J-L) On E15, many LT40 labeled cells had both ramified and amoeboid shapes. Scale bars = 50 μm, taken with 40x objective on E800 wide field microscope. Section thickness = 16 μm.

Another anti chick CD45 antibody, UM16-6, did not show any positive staining in all frozen sections. Both the 2-steps and 3-steps methods at different concentrations did not reveal any positive staining.

3.1.2 Tomato Lectin and Isolectin B4

Tomato lectin (TL) is a protein lectin obtained from *Lycopersicon Esculentum* which binds to poly-N-acetyl lactosamine sugar residues found on the plasma membrane and in the cytoplasm of microglia as well as in endothelial cells. Studies have shown a protocol using TL to stain microglia cells and endothelial cells in the chick (Ignácio et al., 2005) and rodent brain (Kaur et al., 2017; Villacampa et al., 2013). Another lectin, *Griffonia (Bandeiraea)* isolectin B₄ (ILB4), binds to α -galactose sugar residues on microglia and endothelial cells in rodents (Helmut et al., 2011; Pukrop et al., 2010). In my immunostaining, despite changing the staining protocol multiple times, no specific staining was shown on widefield or confocal microscopes. One ILB4 (1:200 in PBSTG overnight) positive microglia-like cell was seen in an E7 brain section shown in Figure 3. The cell appears to have distinct long processes typical to that of ramified microglia. No additional staining was able to replicate this finding.



Figure 3. Isolectin B4 staining a microglial like cell in the E7 chick brain cryosection. Shown in the circled area is an ILB4 (1:200 dilution in PBSTG overnight) positive cell resembling the morphology of a ramified microglia with multiple short processes extending from the soma. A red secondary was used for detection (Streptavidin Alexa Flour 594 Molecular Probes S-11227, Lot 99C1-1) Scale bars = 50 μ m, taken with 40x objective on E800 wide field microscope. Section thickness = 16 μ m.

Additional protocols used to improve TL and ILB4 immunostaining included altering the dilutions, incubation time, and fixation method. TL was tested at dilutions ranging from 2μ g/mL (1:200) to 10μ g/mL (1:1000) and ILB₄, 2.5μ g/mL (1:200) to 5μ g/mL (1:100). Length of primary incubation varied from 1 hour to overnight. Greater concentration and incubation times also increased the background fluorescence of the section. No distinct cells were observed. To improve lectin penetration and binding, different fixatives and dilution media recipes were tested. Some of the fixatives tested include: 15 minute methanol followed by dilutions in only PBS, PBSTG with greater detergent (0.2% Triton-X) and PBST (without addition of normal goat serum). Additional details for reducing the background fluorescence can be found in the appendix.

3.1.3 Ricinus Agglutins Lectin

Ricinus Communis Agglutinin I (RCA-I, RCA120) was found to specifically stain both endothelial and microglia in the chick brain previously (Mannoji et al., 1986; Shin et al., 2003). In my hands, the normal chick brain at ages, E15 and E19, RCA-I was shown to clearly stain cells with the morphology of microglia and vasculature. When stained for just RCA-I, positive cells were found throughout the optic tectum as shown in Figures 4 and 5. Optic tecta have distinct tecta layers with varying degree of nuclei concentration. By comparing the bisbenzimide nuclei staining density, I observed the various layers of the optic tectum that were well developed in an E19 chick as shown in Figure 4. Numerous blood vessels but few microglia were found throughout the layers. The microglia in the chick optic tectum shown in Figure 4C and 4E had long extended processes. The E19 optic tectum section shown in Figure 5 was from a different chick brain. Similar spacing of nuclei, blood vessels and microglia were also observed. A ramified microglia with four long processes is seen in close proximity to a blood vessel as shown in Figure 5 (bottom right). RCA-I stained the entire membrane outlines of blood vessel and microglia.



Figure 4. RCA-I staining in the E19 Optic tectum. All micrograph images were taken with RCA-I (magenta) using different objectives. (A) Superficial to deep Layers of the optic tectum were seen by following the nuclei (blue) patterns. A lot of the visible blood vessels and microglia which were further magnified in B-E. (B, B') A ramified microglial cell was visible with extended process near blood vessels. (D) A large cross section of RCA-I positive blood vessel is clearly stained on its membrane. (E) Circle is a clearly identified microglial cell with extended processes branching outward from the soma. Arrows indicate blood vessels that are encasing nuclei. Scale bar shown in image. Image taken with C2+. Cryosection Thickness = 10μm.



Figure 5. RCA-I staining of microglia and blood vessel in a normal E19 Optic Tectum. Images taken with RCA-I primary (1:250 one hour). (Top) Numerous cross section through tectum showing distinct nuclei (blue) layers and a lot of blood vessels and microglia cells (magenta) taken at 20x objective. (Bottom Left) A zoomed in image taken at higher objective (60x) showing clear blood vessel and microglia. (Bottom Right) cropped image displaying a ramified microglia near a blood vessel. Scale bar shown in image. Image taken with C2+. Cryosection Thickness = 10µm.

3.1.4 Staining for Endothelia Using Antibodies: Anti-Laminin and Anti-Integrin

Additional antibodies were found to stain specifically for blood vessels. Out of the list of antibodies in Table 2, 3H11 was shown to be most specific in staining endothelial cells. 3H11 is a polyclonal antibody binding laminin that is distributed by Developmental Studies Hybridoma Bank (DSHB). 3H11 distinctly stained the outer membrane of blood vessels leaving the lumen interior negative. Another successful but less strong antibody from DSHB is V2E9. V2E9 binds β 1 integrin on the blood vessels' membrane. Compared to 3H11, I had to raise the gain level of C2+ confocal NIS analyzing software to a greater intensity to better see signal. Moreover, V2E9 seems to stain the entire blood vessel including the lumen surface. The lumen interior is less distinct in V2E9 positive blood vessel than that of 3H11. I diluted both 3H11 and V2E9 to a greater concentration to achieve greater signal intensity. Due to time constraints from the university shutdown, I obtained most immunostaining following a two-step visualization. Distinct staining of thick blood vessels at ages, E15 and E19, are shown in Figure 6.



Figure 6. Endothelial staining in chick optic tectum sections. (A,B) Anti-integrin V2E9s (magenta) positive blood vessels. (A) E15 optic tectum section contains sagittal and cross section through long thick blood vessels. (C) In a different E15 chick brain, 3H11 (magenta) positive blood vessel is stained on the vessel membrane periphery under a 20x objective cropped. (B) E19 optic tectum section under a 20x objective cropped, V2E9s marks blood vessel. Nuclei are shown in blue. Confocal images taken with C2+. Cryosection thickness (A, C) = 16µm and (B) = 10µm.

3.1.5 Differentiation of RCA-Positive Microglia from RCA-Positive Endothelia Using Double Staining

Double staining with additional anti-endothelia were necessary to distinguish RCA-I positive microglia from blood vessel. Cells that were negative for 3H11 or V2E9 but positive RCA-I were identified as microglial cells. As shown in Figures 7 and 8, long and thick blood vessels were positive for RCA-I and either 3H11 or V2E9. Lumen of blood vessels also contained nuclei (blue) which helped to confirm the identification of cells. Microglia in E15 and E19 optic tectum and cerebellum were found through the layers. However, there was an area of dense amoeboid shaped and primitive microglia in the E15 tectum as shown in Figure 8. This interesting finding was also found in a different E15 tectum stained with LT40 (Figure 2K). Due to the nonspecific pattern of nuclei (not shown), I am unable to determine the exact location of the section within the tectum. Other tectum staining show microglia distribute throughout the superficial tectum layers with no specific location as seen with Figure 7.



Figure 7. Double stained E15 Optic Tectum for RCA-I (green) and 3H11

(magenta). Arrows show the RCA-I positive microglia which RCA-I positive but 3H11 negative. (Top row): E19 cerebellum with the red arrow drawn at same location in the three images. Merged imaged shows the close overlapping of RCA-I and 3H11 positive endothelial cells. (Bottom 2 rows): images are all taken from the same E15 optic tectum section but zoomed in showing the separated channels of RCA-I and 3H11. More superficial layers are designated as layers to the right. Circled region in (Last row) are the RCA-I positively stained microglia but no staining in the 3H11. Scale bar shown in image. Confocal image taken with C2+: Top and Bottom row = 60x objective; Middle row = 20x objective. Cryosection Thickness = 10µm for E19 cerebellum and 16µm for E15 optic tectum.



Figure 8. Double staining of RCA-I and V2E9 to tell apart microglia from blood vessels in the E15 Optic Tectum. Both images are of the same section. RCA-I (Left circle) positive microglia shown in circle are not stained in V2E9 (Right circle). Numerous RCA-I positive microglia are amoeboid shape densely packed in the tectum. Long RCA-I positive endothelia are also positive for V2E9. Confocal images are taken with C2+ at 20x objective. Cryosection thickness = 16 μm.

3.2 Glioma and Microglia in E14 and E15 Brains

I was able to visualize tumor invasion in the chick brains on the dissected ages, E14 and E15. RCA-I immunostaining revealed blood vessels and microglia scattered throughout non-tumorous region of the optic tectum at both ages. Multiple large irregular blood vessels are located near large and smaller diffused tumor masses (Figure 9). Tumors seem to prefer perivascular region more than non-vascular region. Smaller tumor mass that may have migrated away from the large tumor nodule were seen along with RCA-I positive blood vessels at the tumor border. Under a greater magnification, round punctate RCA-I positive cells were also found near those perivascular region. The morphologies of those cells, distinct from circular or elongated endothelia, were granule-like and a few had short branches, hence I identified the cells as amoeboid microglia. A concentrated band of amoeboid and primitive ramified microglia was also found in a separate E15 tectum injected with U-118/1879 cells (Figure 10). Based on the pattern of the nuclei, the section was most likely from the inner layers of the tectum. Outside of this small region surrounding the tumor, RCA-I positive microglia were more scattered in farther non-tumorous regions. It was interesting to not find any positive RCA-I stained microglia nor vasculatures within the tumor center in the majority of the staining. However, I was able to identify string of nuclei taking the form of blood vessels within the tumor center in Figure 10. Those blood vessels seem to be lacking the antigen recognized by RCA-I.

To better identify immune cells, double staining with specific endothelia markers were also performed. Selective endothelial antibodies, targeting integrin (V2E9 in Figure 11) and laminin (3H11 in Figure 12), were associated with vasculature within and outside of the tumor. A maximum intensity projection through the entire cryosection, did not reveal significant RCA-I staining within the tumor

center (Figure 11) but V2E9 stained blood vessels were seen. In a separate chick optic tectum injected with U-118/1879 cells, endothelia labeled with 3H11 was also seen within the tumor center as shown in Figure 12. Outside of the tumor center, RCA-I stained blood vessels and microglia were scattered around the tumor periphery. The microglia surrounding tumor mass spanned from extremely amoeboid to branched ramified shape.



Figure 9. Close proximity of U-118/L1LE tumor traveling along RCA-I positive blood vessels in E14 optic tectum. (Top images) are different channels of the same image. RCA-I (magenta) positive endothelial referred to by arrows are near tumor. Additional smaller tumor masses were seen throughout the section. Circled region contains amoeboid shape microglia that is also in close distance to a large blood vessel and tumor zoomed in on the (Bottom image). Confocal image taken with C2+ at 20x objective. Cryosection thickness = 16 μ m.



Figure 10. Amoeboid shaped microglia at periphery of U-118/1879 (red) glioma cells in E15 optic tectum. All images were taken of the same section stained with RCA-I (magenta) and bisbenzimide (blue). (Top) Shown in circled region are round punctate shaped microglia at 10x objective. RCA-I positive microglia were located outside of the U-118 tumor core (red). At a higher 20x objective (Bottom), some of the microglia appear ramified shape (white circle) whereas others were round amoeboid shape (yellow circle). Neither blood vessels nor microglia were stained within the tumor center. White arrows within the tumor center highlights the outline of blood vessels. The nuclei within tumor center took the form of the blood vessels but those were not RCA-I positive. Confocal image taken with C2+ at 20x objective. Cryosection thickness = 16 μm.



Figure 11. Triple staining of an E14 U-118/L1LE optic tectum with RCA-I (green) and V2E9 (purple) showing extensive microglia and blood vessel. All three images are different channels of the same section. RCA-I positive and V2E9 negative cells are located at the periphery of the U-118/L1LE tumor mass and have an amoeboid shape. Blood vessels positive for V2E9 show a diffuse staining within the tumor mass. Maximum intensity projection image acquired with C2+ at 20x objective. 12 z stacks at thickness of 2.5 μm. Cryosection thickness = 16 μm.





Figure 12. Triple staining of an E14 U-118/1879 optic tectum showing cross section through the peripheral tumor mass in an E14 optic tectum. Images stained with 3H11 (magenta), RCA-I (green) and bisbenzimide (blue). The U-118/1879 (red) tumor mass was located near the lateral ventricle of the tectum. RCA-I negative and 3H11 positive blood vessels were seen within and surrounding the main tumor region (Top). Nuclei within the tumor region were more elongated then those in the surrounding region. Ramified microglia (circled area) positive for RCA-I but negative for 3H11, were found only in the peripheral region and not within the tumor mass. To get a better view, the image was cropped (rectangle region) and zoomed in (Bottom) to show the great extent of processes protruding from the soma. When shown in only the green channel, parts of a blood vessel and microglial cell's process identified with RCA-I were seen near the tumor exterior but not within the interior region. Confocal image taken with C2+ at 20x objective. Cryosection thickness = $16 \mu m$.

Antibody	Microglia in	Endothelia in	Microglia and
	Normal Brain	Normal Brain	Endothelia in
			Tumor Brain
LT40	Ramified and amoeboid shape seen at all stained ages throughout the brain but was not able to stain full extent of processes. ¹	N/A	Blurry positive staining of microglia seen in tumor peripheral region (Figure 15 in Appendix)
RCA-I	Clear amoeboid and ramified shape seen with all images scattered throughout the brain. ² Higher signal intensity and lower background when using a far red secondary.	Clear positive staining of membrane of blood vessels leaving the lumen negative	Using 488 secondary: few positive staining within large tumor mass but mostly located outside outside of the tumor. Using 647 secondary: no positive staining within large tumor mass

Table 4. Summary of immunostaining results in normal brain versus U-118glioma injected brain.

¹ With the exception of Figure 2K where a higher concentration of LT40 positive microglia seen in one tectum region.

² With the exception of Figure 11 where a higher concentration of RCA-I positive microglia seen one tectum region.

Table 4 Continued.

V2E9	N/A	Stains blood vessel	Blurry staining
		surface throughout	within the large
		tectum ³	tumor mass.
			Clear staining of
			vessels outside of
			the tumor mass.
3H11	N/A	Stains blood	Clear vasculature
		vessels throughout	staining both
		tectum and	within and outside
		cerebellum	the large tumor
			mass

³ No staining was performed in cerebellum or hindbrain so results were limited to chick tecta.

Chapter 4

DISCUSSION

4.1 **Optimal Antibodies**

4.1.1 Anti-Chick CD45: LT40

I am unable to fully replicate the findings of Cuadros et al. (2006) and Caldero et al. (2009). Both studies utilized an anti-CD45 antibody, HISC7, to immunostain the chick brain and spinal cord. Since the HISC7 antibody is no longer in production, an alternative anti-chick CD45 antibody, LT40, was used. CD45, also called leukocyte common antigen, is a transmembrane protein tyrosine phosphatase. The monoclonal antibody LT40 appeared to be staining microglial cells with the most specificity when the primary was left overnight followed by a secondary and tertiary antibody (Alexa Flour 488). However, the same staining could not be replicated in identical sections imaged with the C2+ confocal when a far red tertiary (Alexa Flour 647) was used for detection. An explanation for the difference in staining could be due to a nuisance that I endured throughout staining: autofluorescence (AF). AF is the fluorescence of endogenous fluorophores in the tissue which consists of light emission in the UVvisible, near-IR spectral region. Throughout the staining, most prominent AF was observed in the 488 channel and least in 647 channel. Cause of AF in the brain include lipofuscin, collagen and elastin (Croce & Bottiroli, 2014; Schnell et al., 1999). Lipofuscin is a pigment that accumulates in the cytoplasm of cells due to age and oxidative damage. Collagen and elastin are extracellular structural components present respectively in the basal lamina and neural interstitial matrix (Y. Kim et al., 2018). Lipofuscin, collagen and elastin have excitation in the UV-green spectrum and emission in the green-yellow spectrum. The excitation wavelength closely correlates

with the UV channel and 488 nm channel which may explain the additional positive signal in the LT40 stained sections detected with green fluorophore than those detected with far red fluorophore. An additional reason for the lack of anti CD45 staining, is the immunological composition of microglia. Studies often utilize CD45 to distinguish CD45 low resident microglia from CD45 high infiltrating macrophages. This suggests that the chick microglia may express a low level of CD45 that cannot be strongly detected. Therefore, the applicability of LT40 as a microglia biomarker in the chick brain remains to be suspicious.

4.1.2 RCA-I Lectin Specifically Labels Microglia

RCA-I was observed to specifically stain the cell bodies and processes of microglia in all frozen brain section. Although endothelial cells also reacted with RCA-I, they were distinguished morphologically from microglia. In contrast to microglia, endothelia have thick and long branch-like structure. Microglia morphology were observed to be spanning from a round amoeboid shape to an elongated branched ramified shape. I was not able to identify a qualitative difference between the shapes of microglia between ages E15 and E19 using RCA-I staining. At both ages, ramified and amoeboid shaped microglia were found.

For the majority of the brain sections, RCA-I positive microglia cell were found dispersed throughout the brain sections. However, in a few E15 optic tectum section, amoeboid shaped microglia were found in close proximity. This finding may be explained by previous study done in the chick brain. Shin et al. (2003) immunostained glial cells in the adult Leghorn chick optic tectum and found specific distribution of microglia. They found that although the resident immune cells were scattered throughout the tectum, most were prominently located in the stratum album

centrale (SAC) layer of the tectum. In the sections stained containing a large concentration of microglia cells, that region may have been a cross section through the SAC. No concentrated group of RCA-I positive cells were identified in the cerebellum and hindbrain.

4.1.3 Use 3H11 and V2E9 to Differentiate RCA-I Positive Endothelial from Microglia

It was difficult to tell apart smaller RCA-I positive cells as microglia versus endothelia. Hence, double staining with an endothelia specific antibody were performed. 3H11 is a monoclonal mouse antibody that binds onto the avian laminin-1 which has shown to specifically immunostain the outer membrane of endothelia in the chick brain. Laminin, expressed throughout the basement membrane surrounding chick's endothelia appeared as early as E6, was an indication of vasculature maturity (Risau & Lemmon, 1988). Embryonic chick brain accumulates fibronectin followed by laminin during embryonic angiogenesis. Therefore, my findings of extensive 3H11 positive vasculature at ages, E15 and E19, matches the literature. Based off of the age and morphological similarity to blood vessels, it was concluded that 3H11 specifically stains blood vessels. When 3H11 positive endothelia were cross referenced with RCA-I positive vasculature in double stain sections, microglia were distinguished. All 3H11 positive cells were also positive for RCA-I. Hence 3H11 negative cells and RCA-I positive cells were concluded to be microglia cells.

Another successful antibody used to identify endothelia was V2E9 which binds the avian β_1 integrin. When compared to 3H11 staining, a lower signal intensity image was obtained using the V2E9 antibody. Problems with background noise and lower signal intensity were more common in V2E9 staining. This may be due to the lower density of integrin distribution in the endothelia membrane than that of laminin in the basement membrane.

4.2 Tumor and Microglia

U-118 glioblastoma cells, microglia and endothelia were successfully identified in the chick embryo brain. Stained primarily with RCA-I, ramified and amoeboid microglia, were found throughout the optic tectum. Generally, tumor associated microglia are concentrated near glioma and have an amoeboid morphology (Markovic et al., 2009). When compared to the normal brains, a significance difference in microglia distribution or morphology was not qualitatively observed in brains injected with tumor. This finding may be explained by the relatively young age. I was limited to using only embryonic aged chicks with the oldest age being E15. Previous research studying microglia activation in glioma were performed using adult rodents (Bryukhovetskiy et al., 2016; Markovic et al., 2009). The RCA-I stained microglia that I imaged may not have been matured enough to react to soluble factors released by glioma cells. Or, glioma invasion may not have occurred to as great of an extent in an E15 brain.

However, I was able to observe distinct locations of vasculature and microglia in glioma sections. First, an irregular vasculature was seen in close proximity to U-118 cells. Multiple images showed the RCA-I positive vasculature near diffused tumor nodules. These findings were justified because glioma relies on rich vasculature for invasion. Second, vasculatures were seen both within and outside of the glioma mass when stained with anti-laminin and anti-integrin antibodies. Overall, the blood vessels that were positive for 3H11 and V2E9 within the main tumor nodule were negative for RCA-I. This indicated that the blood vessels within the tumor center had laminin and

integrin but not antigen recognized by RCA-I. Lastly, microglia stained with RCA-I and LT40 were predominantly observed in the perivascular spaces and in tumor periphery. Tumor and microglia cells were found near large blood vessels, alike that of a tumor perivascular niche, which may serve to support the tumor. Glioma are highly vascular tumor that relies upon nearby noncancerous cell such as endothelia and immune cells to support its invasion. The reasons for the preferential binding of RCA-I to tumor border instead of tumor center are not clear. Although this finding may be due to the location where tumor cell was injected do not usually inhabit cells. The tumor, injected into the midbrain, was often found within the lateral ventricle of optic tectum. The ventricle contains CNS fluid and not cells. Possible reason for RCA-I stained microglia surrounding the tumors periphery is that those cells were recruited tumor associated microglia. In human glioma biopsy specimens, Markovic et al. (2009) found MT1-MMP expression was much stronger in the tumor border compared with the center. MT1-MMP expression is upregulated in tumor associated microglia due to manipulation by glioma via toll like receptor signaling. Furthermore, the microglia found at the periphery of glioma had an amoeboid morphology which aligns with previous studies findings of an activated tumor associated microglia phenotype.

4.3 Implications for Future Research

Due to the pandemic, many essential experiments were cut short. Experiments included immunostaining thicker vibratome sections of 100 μ m and analyzing an E19 injected chick brain. Thicker sections would allow me to better analyze the full extent of microglia processes. Thin 16 μ m sections may have been a cross section through only the soma or branched region of microglia cells. Therefore, my morphological observations would be more accurate if a thicker section was analyzed. I have

attempted to obtain an older E19 chick brain with injected tumor but most of the injected chicks did not survive until older ages. Another restriction was the lack of three steps staining done with 3H11 and V2E9 antibodies. Future studies should perform staining using three step amplification to increase signal intensity.

Other essential studies to analyze the interaction between microglia and glioblastoma is using microglia cultures. Microglia cultures allows for a quantitative analysis of cytokines and chemokines. Microglia exposed to tumor would be expected to express greater receptors that help to stimulate glioma invasion. The culture could be used to analyze the secreted cytokines and chemokines in the extracellular environment. In addition, a polymerase chain reaction (PCR) experiment could also be performed to definitively analyze the functional phenotype of wild type microglia to that of a glioma exposed microglia. Microglia, similar to macrophages, are classified under M1 (activated) or M2 (alternative activation or deactivation) state. In gliomas, microglial cells are associated with the M2-like phenotype due to expression of anti-inflammatory factors. PCR can be used to analyze genetic expression of M1 or M2 markers such as ARG-1 and IL-10 (Matias et al., 2018). A large limitation of my study was the inability to determine if there was a difference in activation state of amoeboid versus ramified microglia. Results from such quantitative study would definitively define the functional phenotypes of microglia.

4.4 Conclusions

This study shows that microglia can be effectively identified in the embryonic brain model. Although I was unable to find an antibody specific to only chick microglia, I found a lectin that target both microglia and endothelia. Anti-CD45 immunostaining yielded inconsistent success across sections. Based on morphological

differences and double staining with anti-laminin and anti-integrin antibodies, I was able to identify microglia using the RCA-I lectin. Similar to human and rodent immune cells, chick microglia also display a difference in morphology from amoeboid to ramified branch state. In this study, the effectiveness of using the chick embryo brain model to study glioblastoma was restricted to qualitative immunostaining analysis. I could not see a significant difference in brain with tumor which may be explained by the young age of chick specimens. However, there seem to be a greater population of microglia of varying morphology surrounding tumor rather than within the tumor. Previous studies have revealed the increasing importance of tumor associated microglia in facilitating glioma invasion. Further studies are needed to understand the functional phenotype of those microglia surrounding the tumor and whether those are recruited into the region.

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Appendix A

OPTIMIZATION IN STAINING PROTOCOLS

My project is a pilot study to look into the feasibility of examining microglia interaction with glioblastoma cell in the chick xenograft tumor model. Here I want to describe additional methodologies that I have attempted so future students in the Galileo lab may improve upon my findings.

A.1 Autofluorescence (AF) Reduction Attempts

AF was a large nuisance throughout my staining. One of the ways that I knew a staining was not actual primary staining was by comparing the signal to a different channel. Both green and red channel showed a lot of background noise on the E800 wide field and new C2 plus confocal. Figure 14 shows one example of my finding. Although I double stained for LT40 (microglia marker) and ILB₄ (microglia and blood vessel marker) on E15 forebrain section using different secondaries. Identical images were seen in both green and red channels. The positive signals that looks like blood vessels were often seen throughout the sections of all ages. This finding suggests the presence of fluorochrome within the chick brain endothelium. Most likely origin of the AF are collagen and elastin.



Figure 13. Autofluorescent images taken at the 488 channel and 594 channel. Images taken on the E15 forebrain when stained LT40 (top) and ILB₄ (bottom). Top: LT40 primary and Streptavidin Alexa Flour 488 (Jackson Immunoresearch 016-540-084, Lot 131489). Bottom: ILB₄ primary and Streptavidin Alexa Flour 594 (Molecular Probes S-11227, Lot 99C1-1). There is high resemblance between the two images. Taken with 40x objective on E800.

To remove AF, commercial test kits were trialed. Here, I will explain the methodology that I attempted.

Lipofuscin ® TrueBlack Lipofuscin Autofluorescence Quencher (Biotium Catalog #23007): The protocol stated that the quencher could be added either before or after the immunostaining steps. When the quencher was added during the last step with Bisbenzimide, there were a lot of background covering up the positive staining. Therefore, the quencher was added at the beginning. I diluted the quencher 1:50 in 70% ethanol and applied the quencher prior to primary for 30 seconds followed by PBS rinse three times for five minutes each. Subsequent staining and rinsing solutions were diluted in 5% normal goat serum in PBS without detergent.

TrueBlack® IF Background Suppressor System (Biotium catalog # 23012) was another background suppressor used to reduce the background. The application consisted of blocking with PBSTG for 30 minute followed by blocking with the TrueBlack® IF Background Suppressor (Cat.# 23012A) for 15 minute. Primaries, secondary and tertiary were diluted in TrueBlack® IF Blocking Buffer (Cat.# 23012B) and rinse with PBS only.

Table 5. Results seen using different solutions for immunostaining cryosections using lectin primary.

Initial Treatment	Griffonia Simplicifolia Lectin	Lycopersicon Esculentum
	I, Isolectin B4 Results	(Tomato) Lectin Results
15 minute Methanol	No specific fluorescence	No specific fluorescence
Fixation	seen.	seen.
PBSTG (5%NGS,	High background.	High background.
0.1% Triton X-100,		
PBS)		
PBSTG (5%NGS,	Partial microglial and blood	No specific staining.
0.1% Triton X-100,	vessel staining on some	Lower background.
PBS)	sections.	
PBST (0.1% Triton	Very high background.	Very high background.
X-100, PBS)		
PBS	Very high background and	Very high background and
	much nonspecific staining.	much nonspecific staining.
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A.2 Additional Images

CSAT-s was another anti integrin antibody that I tested to identify blood vessels. CSAT-s is an IgG2b mouse antibody against avian integrin beta-1 subunit. I had a really difficult time finding positive staining for CSAT-s. I often had to turn the gain to really high value to see positive staining. Since I saw more blood vessels staining from 3H11 and V2E9 antibody, I only acquired few staining using CSAT-s. Figure 15 shows one of the very few successful immunostaining results from CSAT-s in an injected chick brain.

Further staining using the anti CD45 marker, LT40, did not yield staining of high intensity. LT40 stained microglia were of inferior quality when compared to RCA-I. Distinct branches were only seen in very few images (Figure 16). When viewed under higher objectives, the same positive objects seen at lower objective became blurrier. Further studies are needed to understand the nature of LT40 immunostaining.



Figure 14. Double staining of blood vessel using CSAT-s and RCA-I in E14 optic tectum injected with U-118/1879 (not shown here). Figure CSAT-s (red) which binds integrin, are shown in small quantity on the bottom of the image. Red arrow refers blood vessels positive for both CSAT-s and RCA-I. White arrows refers to RCA-I positive cells identified as microglia due to its branched morphology. Multiple blood vessel like cells were positive for RCA-I but really faintly positive for CSAT-s antibody. Confocal image taken with C2+ at 20x objective. Cryosection thickness = 16 μ m.



Figure 15. LT40 staining (magenta) of E14 optic tectum injected with U-

118/L1LE cells (red). Top image is a 20x objective acquired on C2+ confocal microscope. LT40 positive cells were located surrounding the perimeter of the tumor and scattered in nonspecific locations within non tumor regions. When the same field was acquired using a 60x objective, there were no improvement in image quality. Although blurry, LT40 stained cells were still localized to the peripheral region. Cryosection thickness = 16 μ m.

Antibody	Company/ Product Code	Notes ⁴	
Streptavidin Alexa Flour 488	Molecular Probes S-11223, Lot 84E3-1	Old	
Streptavidin Alexa Flour 488	Jackson Immunoresearch 016-540-084, - Lot 131489		
Streptavidin Alexa Flour 594	Molecular Probes S-11227, Lot 99C1-1	-	
Streptavidin Alexa Flour 647	Jackson Immunoresearch 016-600-084, Lot 124695	-	
Alexa Flour 488- conjugated Goat anti Mouse IgG	Jackson Immunoresearch 115-545-146, Lot 129708	-	
Alexa Flour 488- conjugated Goat anti Mouse IgM, μ chainJackson Immunoresearch 115-545-020, Lot 143364		New	
Alexa Flour 647- conjugated Goat anti Mouse IgG	Jackson Immunoresearch 115-605-146, Lot 124290	New	
Alexa Flour 647- conjugated Goat anti Mouse IgM	Jackson Immunoresearch 115-605-075, Lot 131576	New	

Table 6. List of secondary and tertiary antibodies used throughout the study.

⁴ The notes refer to approximate ages of the antibodies. Antibodies that I was unsure was left blank. "New" antibodies refer to those that were obtained between 2019 summer and 2020 spring.

Table 6 Continued.

Alexa Flour 488- thconjugated Goat anti Rabbit IgG (H+L)	Jackson Immunoresearch 111-545-144, Lot 108520	New	
Alexa Flour 546- conjugated Goat anti Rabbit IgG	New New		
Alexa Flour 647- conjugated Goat anti Rabbit IgG (H+L)	Jackson Immunoresearch 111-605-144, Lot 144078 New		
Biotin conjugated Goat Anti Rabbit	Jackson Immunoresearch 111-065-144	Very old	
Biotin conjugated Goat Anti Mouse IgG (H+L)	Jackson Immunoresearch 115-065-146 Lot 121635	-	
Biotin conjugated Goat Anti Mouse IgG	Jackson Immunoresearch 115-065-205, Lot 118650	-	
Biotin conjugated Goat Anti Mouse IgM	Jackson Immunoresearch 115-065-044	-	
Biotin conjugated Goat Anti Mouse IgM, μ chain specific	Jackson Immunoresearch 115-065-075, Lot: 139011	New	
Rabbit anti Mouse IgG (H+L)	Jackson Immunoresearch 315-005-003, Lot: 137825	; New	
Rabbit anti Mouse IgM (H+L)	Jackson Immunoresearch 315-005-049, Lot: 144896 New		

B.2 Additional Antibodies

Antigen	Antibody	Company/ Product Code	Dilution/Type of Protocol on Cryosection
Vimentin	MCA-2A52* mouse monoclonal IgG1	Encore Lot: 022217	1:500 2 steps overnight primary and 1 hour secondary
GFAP	MCA-5C10* mouse monoclonal IgG1	Encore Lot: 090817	1:500 2 steps overnight primary and 1 hour secondary
GFAP	MCA-2A5* mouse monoclonal	Encore Lot: 060717	1:500 and 1:200 2 steps overnight primary and 3 steps overnight primary
Vimentin	Rabbit polyclonal to (RPCA) Vimentin* IgG (serum)	Encore Lot: 77-012213	1:500 2 steps overnight primary and 1 hour secondary
Vimentin	MCA-2D1* mouse monoclonal IgG2a	Encore Lot: 080416	1:500 2 steps overnight primary and 1 hour secondary
Nuclei acid	SYTO 40 Blue Fluorescent	Thermo Fisher S11351	50nM-50mM in either TBS or PBS for 15 minutes or overnight
CD11c	mAb 8F2	Gift from Dr. Mark Parcells lab	1:50 3 steps primary overnight

Table 7. Antibodies that was tested during the study but did not show any
positive staining in the chick brain cryosections.

*Samples provided by Encore for me to trial in the chick brain.