

Multiplex Immunofluorescence Studies to Characterize Early Cellular Dynamics in Laser-Ablated Non-Tumor Mouse Brain

By

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Abstract

Introduction: Laser Interstitial Thermal Therapy (LITT) is a minimally invasive, hyperthermia-based laser cytoreductive method used in the treatment of several severe conditions including inoperable brain tumors, treatment-resistant epilepsy, and radiation necrosis. Laser-induced heat causes damage to the tissue and alters the brain-tissue microenvironment (TME) at the local treatment site and beyond. The regional effects include activation of resident brain cells, release of mediators of inflammation, immune cell infiltration, vascular leakage, and the appearance of heat-induced neo-antigens that may activate immune responses. Immunomodulatory responses to LITT in the brain microenvironment are largely unknown. One hurdle is the lack of affordable and efficient imaging protocols that enable the simultaneous spatial visualization of multiple immunoreactive cellular and molecular targets in the brain. Here, we describe a flexible and affordable multiplex immunofluorescence (mIF) staining method in our established LITT mouse model. This mIF protocol can be applied to the brain as well as other tissue types. In the post-LITT mouse brain, mIF revealed laser ablation-induced complex spatial responses by the resident brain and innate immune cells.

Methods: We developed a customizable and affordable mIF detection protocol that uses standard histological and microscopy equipment to assess TME changes on single sections of formalin-fixed paraffin-embedded (FFPE) mouse brain tissues at day 6 post-LITT. This mIF imaging procedure provided a comprehensive spatial analysis of multiple cellular and molecular immunotargets in resident brain cell responses and immune cell profiles in individual tissue sections, as exemplified with a mouse brain microenvironment on day 6 post-LITT.

Results: We optimized mIF imaging methodology on LITT-treated mouse brain FFPE serial sections to visualize the spatial distribution of resident brain cell cellular activation states and

the emergence and distribution of immune cell populations at the LITT site. Our mIF imaging protocol of mouse brain regions surrounding the LITT site revealed the activation of resident astrocytes and microglial cells and the presence of a diverse innate immune cell population in this region.

Conclusion: The combined use of LITT ablation treatment with multiplexed versus regular immunofluorescence offers a significant advancement and aids in our understanding of post-LITT repair and immune responses in the brain. We characterized the thermal ablation-induced modulation of resident brain cells and innate immune cell infiltrations.

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Declaration of Software Usage

In the preparation of this thesis, I have used the following software tools:

- Biorender – Used to create figures.
- Mendeley Reference Manager – Used to manage all the references
- Grammarly, Chatgpt, and Perplexity AI – Used to check grammar. AI software was not used to generate thesis content.
- Microsoft Office: MS tools were used to write the thesis.
- Zeiss Zen: Zeiss imaging software was used to image and quantify the thesis images.
- iThenticate: Used to check plagiarism. Report: 4%.

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List of Abbreviations

BiP	Binding immunoglobulin protein
CgA	Chromogranin A
DAMPs	Damage-Associated Molecular Patterns
DBS	Deep brain stimulation
DRFE	Drug-resistant focal epilepsy
ER	Endoplasmic Reticulum
FFPE	Formalin-fixed paraffin-embedded
GFAP	Glial Fibrillary Acidic Protein
H&E	Hematoxylin and Eosin staining
Iba1	Ionized calcium-binding adaptor molecule 1
IF	Immunofluorescence
LITT	Laser Interstitial Thermal Therapy
MDMs	Monocyte-derived macrophages
mIF	Multiplex immunofluorescence
Nes	Nestin
NGS	Normal Goat Serum
PBST	phosphate-buffered saline with Tween
Pdi	Protein disulfide isomerase
PRRs	Pattern Recognition Receptors
RAGE	Receptor for advanced glycation end products
RBN	Radiation-induced brain necrosis
ROI	Region of Interest
SMA	Smooth Muscle Actin
STAT3	Signal transducer and activator of transcription 3

TLRs	Toll-like receptors
TME	Tissue microenvironment
UPR	Unfolded Protein Response
Vim	Vimentin
VNS	Vagus Nerve Stimulation

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1. INTRODUCTION

1.1 Stereotactic Laser Ablation

In the late 19th century, thermal ablation was first described by alternating radiofrequency to induce thermal energy in biological tissues (d'Arsonval, 1891). The thermal ablation evolved with the discovery of the stereotactic frame. In the year 1908, the stereotaxic frame was first described, which paved the path for the first stereotactic neurosurgery (Rahman et al., 2009). A stereotaxic frame is a specialized instrument used to precisely target specific regions within the brain (Gildenberg, 1990). The device utilizes three-dimensional coordinates to locate and target the region of interest in the brain (Al-Rodhan & Kelly, 1992). Stereotactic Laser Ablation (SLA) is a minimally invasive neuro-surgical technique that uses laser energy to precisely destroy the targeted brain region (Buscarini et al., 2005; Goldberg et al., 2000; J. Wu et al., 2024). Over the years, different thermal ablation methods like cryoablation, microwave ablation, and laser ablation were developed (Knavel & Brace, 2013). Laser ablation, commonly known as Laser Interstitial Thermal Therapy (LITT), has become the preferred choice for patients with inaccessible brain tumors or drug-resistant epilepsy. Clinical LITT instruments integrate a real-time MRI monitoring system with laser functions (Desclides et al., 2023; Salem et al., 2019).

1.2 The origin and application of LITT

1.2.1 LITT in humans

The most desperate patients include those with brain tumors (primary and metastasis) located in regions of the brain (e.g., brainstem) that cannot be accessed surgically and do not respond to conventional therapies. These patients face a grim prognosis. Minimally invasive Laser Interstitial Thermal Therapy (LITT) is one of very few currently FDA-approved effective

treatment options with low complication rates for patients with inoperable brain tumors and drug-resistant epilepsy (Figure 1). Upon complete tumor ablation, LITT achieves a median survival rate between 5.8 to 19.8 months in brain metastasis recurred after stereotactic radiosurgery (C. Chen, Lee, et al., 2021) compared to the untreated patient survival rates of about 7-24 weeks (according to the survival statistics by the [Canadian Cancer Society](#)). LITT converts precisely focused laser light energy into heat. The operating temperature of LITT ($>43^{\circ}\text{C}$) is well above the physiological body temperature and cytotoxic to cells. The initial restricted clinical applicability of LITT has been broadened with the development of real-time MRI, which is now combined with LITT. Real-time MRI helps to monitor the actual temperature changes in the treatment regions during LITT (McNichols et al., 2004). Currently, the two FDA and Health Canada-approved LITT devices include the Visualase™ Thermal Therapy system (Medtronic Inc) and the NeuroBlate® system (Monteris Medical). The two LITT devices are known to produce similar effects but differ in the cooling mechanism and the type of laser used. However, the surgical procedure would be more precise with NeuroBlate as Monteris has a robotic control for directional ablation. This study involved the use of a laser device from Monteris Medical (customized for animal studies in laboratories) (Frain et al., 2024). The LITT's refined compatibility as a combinational treatment option with radiation and chemotherapy enabled LITT as a promising tool for a wide range of intracranial pathologies (Remick et al., 2020; Schwabe et al., 1997).

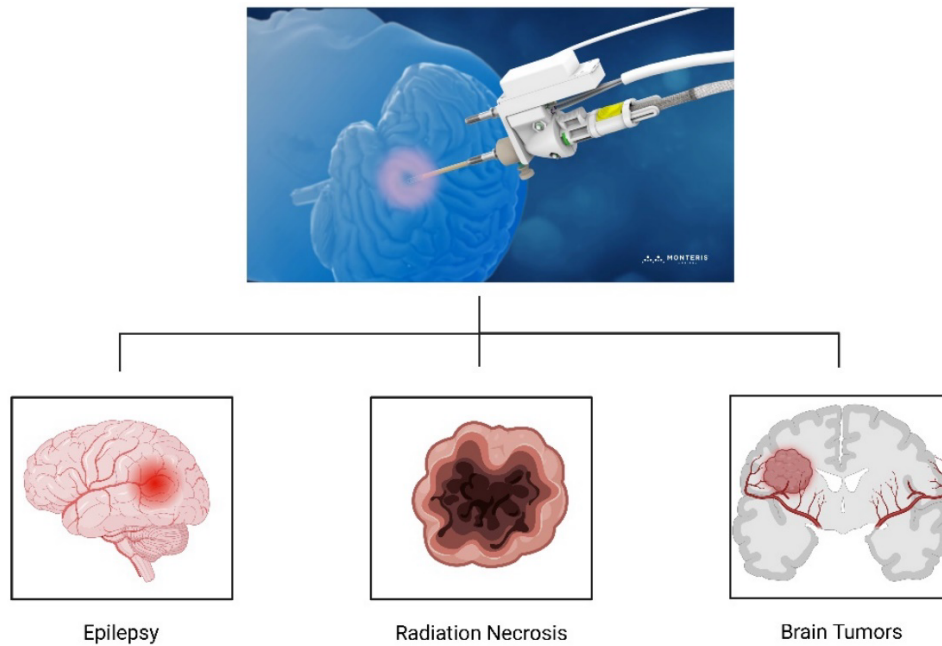


Figure. 1.1 Clinical applications of LITT: LITT is widely used to treat patients with medically refractory epilepsy, radiation necrosis, and brain tumors (both primary and metastatic brain tumors). This figure shows a LITT device representative image from Monteris Medical. *Created with BioRender.com.*

1.2.2 LITT to treat drug-resistant epilepsy

Epilepsy is a neurological disorder delineated by recurrent seizures, diverse etiology, and varying prognosis, and predominantly developed due to genetic disorders and brain abnormalities (Karlander et al., 2021; Oyrer et al., 2018; Sødal et al., 2024; Stevelink et al., 2023; Whelan et al., 2018). Treatment options include anti-epileptic drugs, surgeries for focal seizures, brain stimulations, and diet (Figure 1.2).

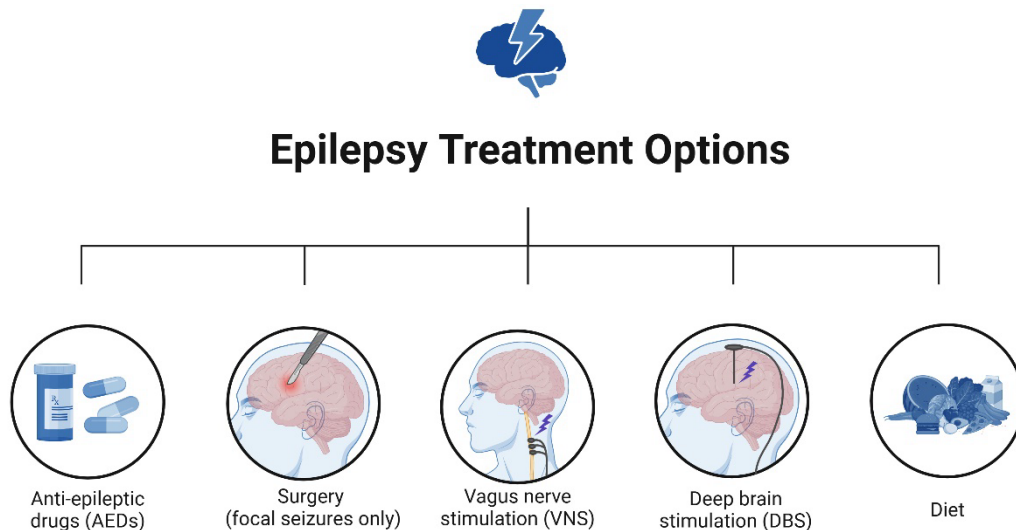


Figure. 1.2 Current Treatment Options for Epilepsy. The image illustrates the primary care options for Epilepsy, including anti-epileptic drug surgery for deep brain stimulation (DBS), focal seizures, vagus nerve stimulation (VNS), and dietary therapies like the ketogenic diet. These approaches aim to reduce seizure frequency and improve quality of life based on individual patient needs. *Created with BioRender.com.*

The minimally invasive LITT procedure is being used to treat drug-resistant epilepsy, hypothalamic hamartoma, mesial temporal lobe epilepsy, and other types of epilepsy due to structural lesions and damage. Over 30% of the diagnosed epileptic patients are under drug-resistant focal epilepsy (DRFE). DRFE is a type of seizure that originates from a specific region of the brain and is non-responsive to anti-seizure medications (Consales et al., 2021; Shan et al., 2021). Patients with identifiable focal seizure-generating regions and accessible to LITT probes are considered to be ideal candidates for LITT epileptogenic region ablation (Kang et al., 2016; Waseem et al., 2015; Willie et al., 2014). Clinical studies conducted with LITT on DRFE patients showed effective outcomes with high success rates for seizure control (C. Wu et al., 2019; Xue et al., 2018). LITT represents a promising, effective, and minimally invasive

treatment modality for selective DRFE patients (In et al., 2023). Optimizations of LITT parameters and clinical study data could make LITT a standard treatment option along with other conventional treatment options for epilepsy.

1.2.3 LITT to Treat Radiation Necrosis

Radiation necrosis in the brain, also known as radiation-induced brain necrosis (RBN), is a serious complication from radiotherapy for brain tumors. RBN occurs when the brain tissues become necrotic due to the ionizing radiation from the stereotactic radiosurgery or whole-brain radiation to treat brain tumors (Patel et al., 2014; X. Yang et al., 2021). The rate of incidence varies between 3% and 68%, determined by the radiosurgery conditions and patient-dependent factors (Miyatake et al., 2014). Clinically, RBN has been considered an irreversible condition. With the recent therapeutic advancements, RBN is being treated with corticosteroids to reduce cerebral edema and Bevacizumab to restore the function of destroyed blood vessels.

LITT has been applied to the RBN to ablate the radiation-induced necrotic regions with minimal damage to the neighboring healthy tissues. Studies have shown that the RBN-diagnosed patients treated with LITT demonstrated a high success with 75.7% to 83.1% of the patients with a complete progression-free RBN (Chan et al., 2023; Hernandez et al., 2019; Holste & Orringer, 2020a).

1.2.4 LITT to Treat Brain Tumors

LITT has emerged as a promising treatment option for patients with a variety of brain tumors. The minimally invasive treatment combined with real-time MRI monitoring assists surgeons in ablating the tumor tissue (A. H. Kim et al., 2020). LITT is particularly effective for patients with deep-seated focal tumors of size less than 3cm in diameter. LITT is also

recommended for recurrent tumor patients who have already undergone surgical resection. The unclear residual/recurrence margins and proximity to eloquent brain regions make the open surgical intervention risky as it may cause new neurological deficits. The scar tissue formation makes the second surgical resection complicated. Several clinical studies and patient outcomes have demonstrated LITT's efficacy in treating both low-grade and high-grade gliomas (C. Chen, Lee, et al., 2021). Patients who received LITT showed an increased median survival rate and improved Karnofsky Performance Status score (brain impairment test) compared to the patients who had undergone stereotactic radiosurgery (A. H. Kim et al., 2020). Clinically, studies are focusing on LITT as a combinational therapy along with chemo-radiation and surgical resection. Successful application of LITT on patients with complex tumor types collectively demonstrates the growing interest and the need for more studies to unlock the potential of LITT in brain tumors and other LITT neurological therapeutics.

Most of the studies on LITT are being conducted on patients as clinical studies. The survival outcome data from the clinical study participants is being used to assess the efficiency and effectiveness of the LITT in neurological disorders and as a combinational therapy. Due to the unavailability of post-LITT patient brain samples, there is no study conducted to understand the LITT-induced biological changes. Animal models are vital for understanding the biological mechanisms underlying diseases and treatments. However, limitations in animal models for LITT impede our understanding the post-LITT-induced biological change.

1.3 Development of an in-house spatial characterization technique for post-LITT brains

The growing interest and promising LITT clinical study outcomes required further understanding of the LITT-induced biological changes to enhance LITT therapeutic potential. Mouse LITT models are being developed to characterize the post-LITT ablation effects in mice

brains (Frain et al., 2024). The availability and ability of the normal mouse brains and tumor cells implanted in mouse brains to recover from the laser ablation made them suitable models for studying post-LITT effects. A study from our lab (Spence et al., 2024), developed an optimized LITT mouse model using C57BL/6 immunocompetent mice to study laser effects on tumor and non-tumor brains (Figure 1.3). In partnership with Monteris Medical, a laser device was designed for mice models (Frain et al., 2024; Spence et al., 2024).

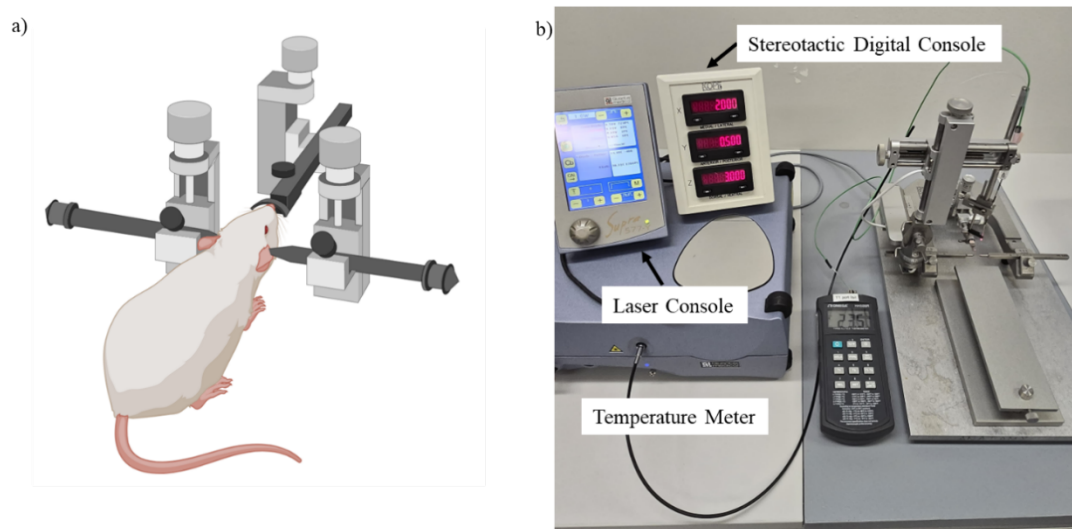


Figure 1.3 a) Stereotactic set-up for mouse. The stereotactic frame helps in enabling precise targeting of specific brain regions for experimental interventions. **b)** Laser console, temperature meter, and stereotactic digital console were connected to the stereotactic frame. *Created with BioRender.com.*

A thermocouple was used to monitor the temperature during the mouse LITT surgery replacing the real-time MR thermometry in clinical LITT devices. A clinical observation from a cohort of post-LITT patient MRI and the comprehensive review by (Lerner et al., 2022) described concentric tissue destruction at the brain region immediately next to the laser probe firing tip forming the necrotic core. MR thermometry showed a steep decrease in the treatment

temperature away from the necrotic core forming zones with varying degrees of damage. Thus, leading to the treatment temperature-dependent modulation of brain microenvironment post-LITT. The complex LITT mouse brain microenvironment requires a novel histological spatial staining method to characterize the post-LITT cellular modulations.

1.3.1 Multiplex-Immunofluorescence

Immunofluorescence (IF) is an extension of immunohistochemistry that facilitates the selective staining of antigens in tissues and cells by exploiting antigen-antibody interaction (Buchwalow et al., 2015; Ehrlich, 1877). IF can be performed either by fluorophore-conjugated primary antibodies (Direct IF - does not require secondary antibodies) or by utilizing primary-fluorophore-conjugated secondary antibody interaction (Indirect IF). Indirect IF involves the use of antibodies from a host species that exhibit a high degree of sensitivity and specificity for an antigen of the target species which makes it the most preferred IF method (Coons et al., 1942) (Figure 1.4). This is followed by the selective binding of fluorophore-conjugated secondary antibodies raised against the host species of the primary antibody to form an antigen-antibody complex. Upon excitation at an appropriate wavelength, the fluorophore emits light of a longer wavelength when returning to its resting state, which allows visualization of the antibody target. Although directly fluorophore-conjugated primary antibodies are an alternative, they are more expensive, and their use for other immunodetection methods may be limited. Concurrent utilization of multiple primary antibodies requires that these primary antibodies are from different host species. This may be a limiting factor as far as quality, specificity, availability, and cost are concerned (Tan et al., 2020).

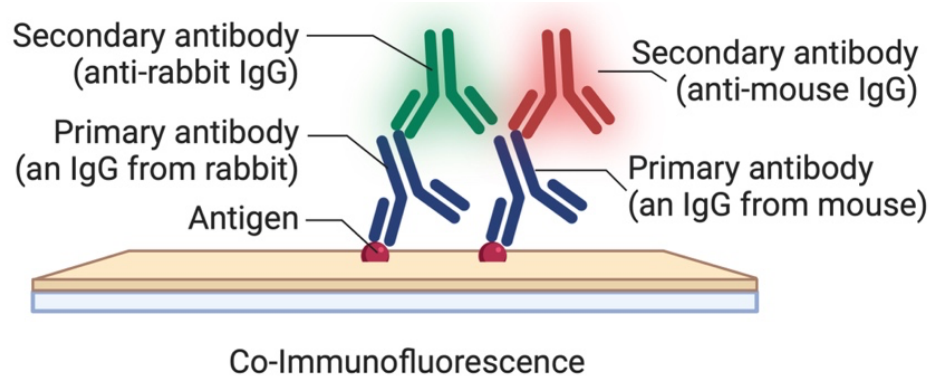


Figure 1.4 Co-immunofluorescence: The figure demonstrates the principle of Indirect co-immunofluorescence, where two primary antibodies from different host species (rabbit and mouse IgG) bind to specific antigens and are detected by species-specific fluorescently labeled secondary antibodies for visualization under fluorescent microscope. *Created with BioRender.com.*

There is an emerging need for multiplexed immunodetection of more than two different antigens to elucidate dynamic alterations of cellular and molecular markers and/or illustrate complex interactions occurring within the brain microenvironment. This is particularly evident in LITT mouse models investigating the complex post-LITT cellular and molecular modulations in healthy (non-tumor) brain and tumor brain tissues. Upon laser activation, thermal energy is released into brain tissue, and this heat dissipates into the surrounding brain regions, forming a thermal gradient cone. This requires an efficient serial tissue sectioning method to investigate the cellular modulation along the LITT-induced temperature gradients. LITT-treated cerebral tissues should be utilized most effectively to enable and expedite studies on dynamic cellular and molecular compositional changes in serial sections, thereby reducing the number of mice required for each experiment.

Spatial mIF facilitates the simultaneous examination of multiple biomarkers in the same tissue section and enables visualization of the topographic distributions. Recently, mIF has

undergone a remarkable development in the immune-oncology field (Tan et al., 2020). Numerous sophisticated instruments and microscopes have been engineered to study up to 100 markers concurrently. However, the results obtained with extensively multiplexed staining procedures are often difficult to interpret. These multiplexed immunodetection assays frequently utilize primary antibodies that have not been optimized for the particular tissue under investigation. Moreover, the substantial costs of acquiring a spectral microscope and employing these assays are prohibitive for many laboratories with limited financial resources. Developing an in-house multiplex immunofluorescence protocol for serial FFPE sections with individually optimized antibodies would provide a cost-efficient opportunity to explore the spatial distribution of LITT-induced cellular modulations (Figure 1.5).

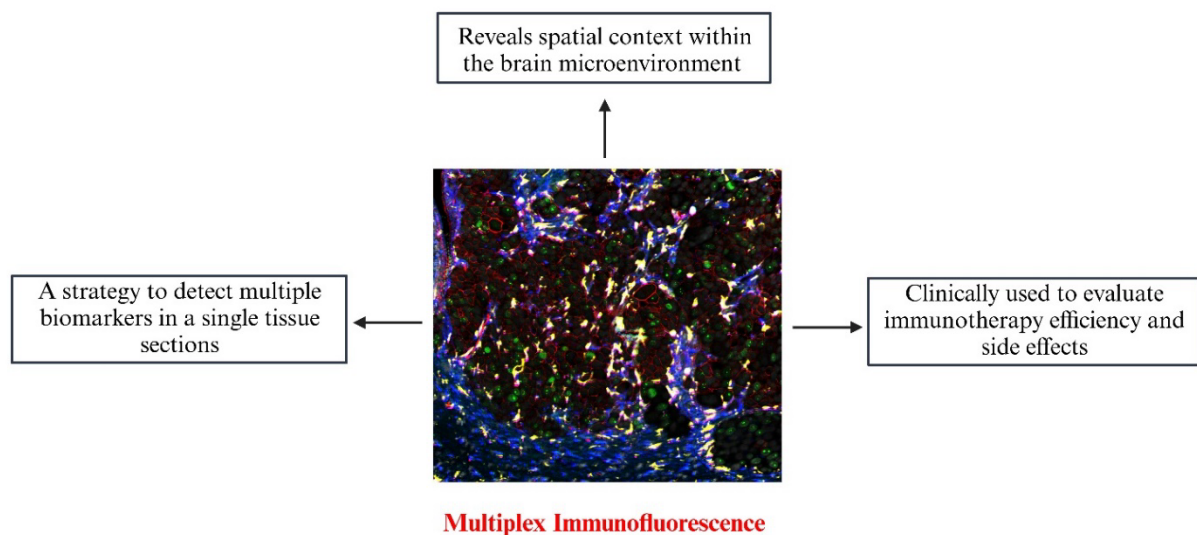


Figure 1.5 Multiplex Immunofluorescence is a powerful histological staining technique to detect multiple biomarkers within a single tissue section, providing a spatial context of the brain microenvironment and aiding in the clinical evaluation of immunotherapy efficacy and safety. The Image shown here is a representative image of multiplex staining with each color coding for an antigen.

1.4 LITT-induced resident brain cell damages

Mouse LITT requires the insertion of a laser probe and a thermocouple, which creates an insertional canal and disrupts the resident brain cells in their path. Along with the cellular damage from the laser probe insertion, the laser energy induces heat damage, leading to the activation of apoptosis and necrosis. Histological studies have shown significant damage to the mouse brain created by the laser probe insertion (Frain et al., 2024), which is yet to be characterized. Zonal-based damage induced by the LITT has been characterized based on MR thermometry and histological studies (Figure 1.6). The innermost necrotic zone, closer to the laser probe, was found with complete laser-ablated necrosis. Drastic cellular modulations were found in the regions next to the necrotic core. MR thermometry recorded the treatment temperature ranging from 41°C to 45°C, which induced severe reversible cell stress. Histological studies done on the patient biopsy revealed the complex brain microenvironment post-LITT treatment (Elder et al., 2019). This is a unique study conducted using a alive post-LITT patient biopsy making it the only preliminary data available on post-LITT-induced brain modulations. The study showed a central necrotic core (devoid of cells), a zone of granulation tissue with macrophages, followed by a zone of activated astrocytes (Elder et al., 2019; Lerner et al., 2022). However, the study lacks the spatiotemporal aspect for the characterization of LITT-induced cellular modulations.

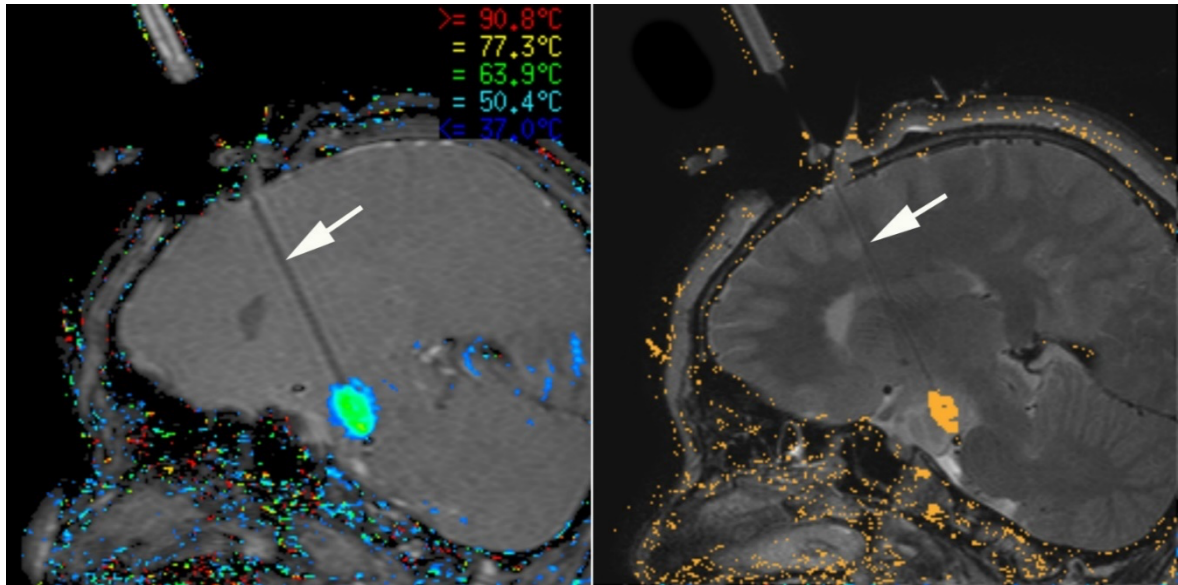


Figure. 1.6 MRI-guided LITT procedure: The left image shows a thermal map overlay indicating temperature changes during laser ablation, with the core therapeutic temperature of 50.4 °c and the arrow pointing to the laser probe. The right image displays post-ablation confirmation of the treatment zone (arrow) with minimal surrounding tissue damage. *Image source: [UCSF Brain Tumor Center newsletter](#).*

1.4.1 LITT-induced neuronal damage and astrocytic activation

Neurons are the fundamental units of the brain and the nervous system. The functionality of the brain neuronal network depends on resident oligodendrocytes forming myelin sheaths around neuronal axons to enable expedient action potential transmission. Post-LITT mouse brain histological staining performed by (Frain et al., 2024) showed the absence of Hematoxylin & Eosin (H&E) staining in the insertion canal and the ablation zone. As shown by (Elder et al., 2019), activation of astrocytes along the periphery of the necrotic core marks the beginning of regions with LITT live cells. Astrocytes are the most abundant glial cells in the central nervous system, and they are important for maintaining blood-brain barrier integrity,

nutrient support to neurons, and extracellular ions homeostasis (Escartin et al., 2021; Yuan & Wu, 2022).

Pathological conditions, such as glioma and traumatic brain injury, cause astrocytes to change their morphology and strongly increase Gfap expression, indicative of elevated astrocyte activation (Braun et al., 2017; Nespoli et al., 2024; Shinozaki et al., 2017; S. Wang et al., 2020). Astrocytes express five intermediate filaments: Glial Fibrillary Acidic Protein (Gfap), Vimentin (Vim), Nestin (Nes), Synemin, and Lamin. These cytoskeletal proteins form filaments of approximately 10 nm in diameter and play an important role in structural and mechanical support, cell migration, cell cycle, and cell proliferation (Coelho-Rato et al., 2024; Lowery et al., 2015; Potokar et al., 2020).

The neurons and astrocytes play an important role in the functioning and maintaining the homeostasis of the brain. Hence, it is crucial to evaluate the intensity of the ablation-induced resident brain cell response and recovery.

1.5 LITT-induced immune response

The laser ablation killed cells at the necrotic core cause the breakdown of the cell membrane, leading to the release of intracellular components like DNA, RNA, lipids, and cellular debris into the brain microenvironment (G. Y. Chen & Nuñez, 2010; Ma et al., 2024). These intracellular components released by the dying cells induce further cellular modulations (Brock et al., 2020). Signals from dying neurons and damaged myelin trigger the immediate local response characterized by the activation of resident brain cells (astrocytes and microglia), inflammasome formation, and promoting pro-inflammatory microenvironment (Kono & Rock, 2008; Laird et al., 2014; Rostron et al., 2022).

A major role of the microglia is the clearing of dead cells and orchestrating neurorestorative processes that promote neurological recovery. During microglial activation, they undergo drastic morphological changes from a ramified state to an amoeboid shape (Vidal-Itriago et al., 2022) (Figure 1.7). This makes it difficult to distinguish activated microglia from infiltrated monocyte-derived macrophages (MDMs). Despite microglia being the first injury responder, it has been described that MDMs are more phagocytic in function than activated microglia (Chang et al., 2021). The activated microglia recruit MDMs by releasing pro-inflammatory cytokines and chemokines. The activation of microglia/macrophage can polarize into two broad phenotypes – Classically activated M1 (pro-inflammatory) or alternatively activated M2 (anti-inflammatory) (Italiani & Boraschi, 2014; Kapellos et al., 2019). M1 phenotype macrophages induce the release of pro-inflammatory mediators to enhance the initial innate immune response. M2 phenotype macrophages play an important role in tissue repair and neuroprotection. The microglial/ macrophage transition between these two phenotypes is crucial to prevent neurotoxicity and promote brain damage recovery (Gottlieb et al., 2022; G. Wang et al., 2013).

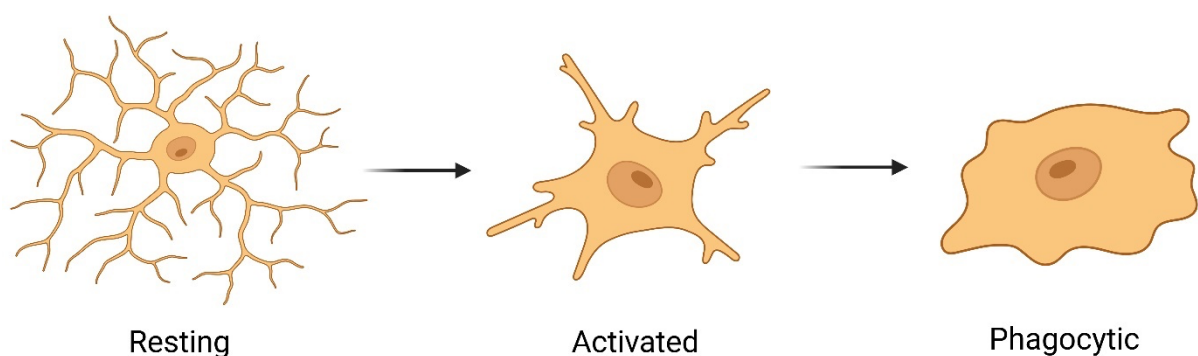


Figure. 1.7 Stages of microglial activation: Immuno-vigilant resting microglia with ramified processes to monitor pathogens, cellular debris, or cellular damage. Resting microglia

transition to an activated state with retracted processes, eventually adopting a phagocytic morphology for debris clearance. *Created with BioRender.com.*

The signal transducer and activator of transcription 3 (STAT3) play an important role in regulating the DAMPs-induced inflammatory response. STAT3 acts as a critical mediator linking DAMP recognition to downstream inflammatory signaling (Ma et al., 2024). DAMPs interact with receptors like Toll-like receptors (TLRs) or the receptor for advanced glycation end products (RAGE), which leads to the activation of several intracellular signaling pathways, including the JAK/STAT3 pathway. (Y. Wu et al., 2018) identified that STAT3 activation is crucial for the promotion of post-injury inflammation. Most importantly, STAT3 activation plays a dual role in mediating pro- and anti-inflammatory responses to promote tissue repair (Mühl, 2016).

This study will evaluate the spatial distribution of immune response response-associated biomarkers such as microglial activation, macrophage sub-populations, and inflammatory response-associated members.

1.6 Endoplasmic Reticulum (ER) stress in the brain

The endoplasmic reticulum (ER) is responsible for protein folding, lipid synthesis, and calcium storage. Any brain damage induces the accumulation of unfolded proteins and overwhelms ER functioning, leading to ER stress (Y. Yang et al., 2024). Unfolded Protein Response (UPR) gets activated to bring ER homeostasis. In brain injuries, ER stress is a major contributor to neuroinflammation and neuronal apoptosis (Truettner et al., 2007; Y. Yang et al., 2024). GRP78, or binding immunoglobulin protein (BiP), is a molecular chaperone that

controls the UPR. The function of BiP is to keep the UPR sensors inactive. BiP acts as a first-line defense by refolding damaged proteins and stopping them from aggregating together. During the acute stages of brain damage, BiP activates adaptive UPR pathways to support cell survival. BiP's protective function is diminished in situations when ER stress persists. Long-term UPR pathway activation causes downstream signaling that induces apoptosis (D. Y. Wang et al., 2021). Similar to BiP, PDI is a thiol oxidoreductase and ER-resident chaperone that catalyzes the creation, reduction, and isomerization of disulfide bonds in proteins to ensure appropriate protein folding (Parakh & Atkin, 2015). PDI either causes PERK-mediated apoptosis to eliminate cells with irreversible cellular damage or aids cells in adapting to temporary ER stress (Perri et al., 2016). Hence, BiP and PDI are used as potential biomarkers to detect ER stress activation, and their expression pattern in response to LITT will be investigated in this study.

1.7 Rationale

LITT is a fast-growing therapeutic option for patients with inoperable brain tumors, treatment-resistant epilepsy, and radiation necrosis. There is currently a knowledge gap on the post-LITT cellular responses and the unavailability of the LITT mouse model to investigate these responses. This knowledge gap is significant given the potential of the LITT to activate resident brain cells, release inflammatory mediators, and induce immune cell response. Preliminary experiments with our pilot LITT mouse showed significant changes in the brain microenvironment post-treatment. These findings highlighted the need for an efficient, cost-effective spatial imaging protocol to perform routine immune staining and study the cellular modulations while minimizing the tissue sample requirement.

This study addresses the need for an affordable and efficient imaging protocol for visualizing multiple immunoreactive targets in the brain simultaneously. By developing a flexible multiplex immunofluorescence (mIF) staining method, this research aims to provide a comprehensive spatial analysis of cellular and molecular changes in the post-LITT non-tumor brain microenvironment.

1.8 Hypothesis

LITT hyperthermic damage gradients induce the resident brain cell activation and an influx of innate immune cells into the acute non-tumor LITT tissue microenvironment.

1.9 Objectives

The objectives of this study are to (1) establish and optimize conditions for an in-house multiplex immunofluorescence protocol to (2) study the early post-LITT resident brain cell activations and characterize the non-tumor LITT-induced cellular infiltrations and modulations in mouse brain.

2. MATERIALS & METHODS

2.1 LITT procedure and tissue processing

LITT experiment procedures were approved by the University of Manitoba's Animal Care Committee and performed strictly following the ethical guidelines set by the Canadian Council for Animal Care (CCAC). The experiments utilized an immunocompetent model, C57BL/6 mice aged 6-10 weeks (n=8). Our lab-optimized LITT mouse model was used for the experiments (Spence et al., 2024).

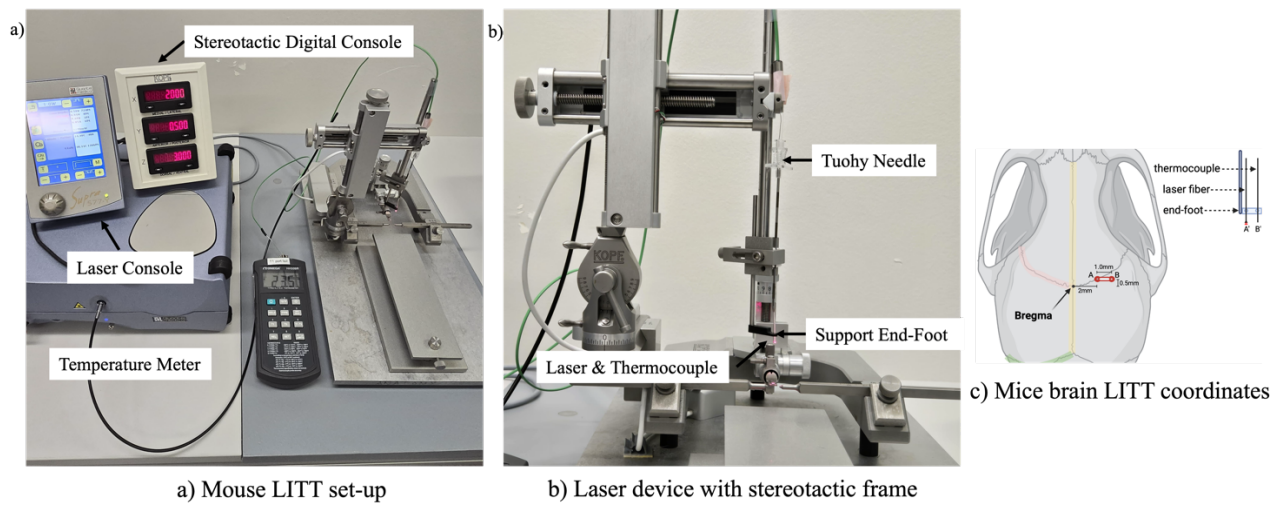


Figure 2.1: Mice LITT set-up and coordinates. a) Laser console, temperature meter, and stereotactic digital console were connected to the stereotactic frame. b) The stereotactic frame supports the laser probe and the thermocouple closer to each other. The support end-foot keeps the laser probe and thermocouple separated. c) Surgical coordinates using mice's brain anatomy. Point A for the laser probe is 2mm lateral and 0.5mm anterior to the bregma and point B indicates the thermocouple at 1mm away from point B.

The LITT parameters were optimized in proportion to human LITT parameters to access the brain microenvironment changes (Table 2.1). Similar to human LITT conditions, intermittent laser firing with regular intervals combined with a temperature-controlled thermometry method was employed. A normal, healthy mouse brain was used as a control. This study was completely done using a 6-day post-LITT non-tumor mouse model. LITT

surgery, MRI imaging to assess the ablation, and mice brain collection were performed by the members of the Klonisch lab (Spence et al., 2024).

Table 2.1 LITT treatment conditions for the study

Experiment	Duration/Target Temp	Intensity (W)	PW (ms)	IPI (ms)	Days	n
LITT	51 pulses/43C	2	100	200	6	8

Table 2.1: PW – Pulse width, IPI – Inter pulse interval.

Gross sectioned brain tissue and overnight fixed in a 10% buffered formalin solution of at least 20x tissue volume overnight at room temperature. To prepare the tissues for histological examination, the PBS-washed tissues were placed into tissue cassettes and placed in a tissue processor. Following tissue processing, the tissues were placed in the desired orientation on metal molds and embedded using double-filtered paraffin wax. The tissue processing procedure was performed by the members of Histology Services, Imaging Facility, Electron Microscopy Platform, University of Manitoba.

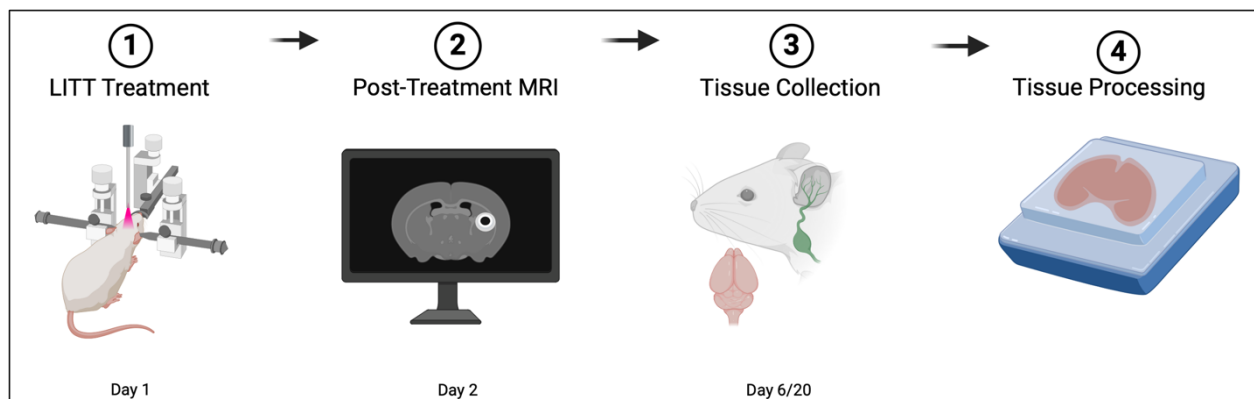


Figure 2.2 Mice treatment and tissue processing: Mice brains are MRI scanned 1-day post-LITT to assess the treatment damage and the brains are collected at different time points (6 days, 20 days). The mice's brains were grossly sectioned and fixed overnight in a 10% buffered formalin solution before tissue processing.

2.2 Serial sectioning optimization:

The paraffin blocks were cooled on ice for 2-3 hours before sectioning to ensure optimal cutting conditions. Using the Shandon finesse ME, the blocks were sectioned at 5µm thickness. The serial sections were obtained by continuous sectioning to form ribbons. Once 10 sections were cut, the ribbon was carefully placed onto a water bath at room temperature, allowing the sections to float and flatten. Slides were made as soon as the tissue started to appear. Up to three floating serial tissue sections were collected on Superfrost plus slides for enhanced tissue adherence. The slides were dried on a warming plate overnight to ensure proper adhesion and readiness for subsequent staining procedures.

Table 2.2 Materials and Equipment Tools

Name	Brand	Cat.no
Microtome – Shandon finesse ME	Fisher, Waltham, MA	77500102
Microtome Blade S22	Fisher, Waltham, MA	12-631P
Superfrost plus slides	Fisher, Waltham, MA	1255015
Xylene Histological grade	Fisher, Waltham, MA	X3P-1GAL
Ethanol	Greenfield Global	P016EAAN
Baking oven isotemp	Fisher, Waltham, MA	51030503
Liquid blocker super pap pen	Emsdiasum, Hatfield, PA	71310
Normal goat serum (NGS)	Sigma, Oakville, ON	G9023
Fluoromount-G	Fisher, Waltham, MA	00-4958-02
Phosphate Buffered Saline (PBS)	Fisher, Waltham, MA	BP399-4
Triton-X 100	Sigma, Oakville, ON	T8787
TrueVIEW Autofluorescence quencher	Vector, Newark, CA	VECTSP8400
Vectaplex antibody removal kit	Vector, Newark, CA	VECTVRK1000

Coverslips	Epredia, Kalamazoo, MI	152455
Permunt	Fisher, Waltham, MA	SP15-500
Tween 20	Sigma, Oakville, ON	P1379
Humidifying chamber		
Double distilled water		
Microwave oven		
Zeiss M2 Scan Microscope	Zeiss, Hebron, KY	

2.3 Hematoxylin and Eosin (H&E) staining:

H&E staining was performed to locate the LITT damaged sites and identify the treatment coordinates for subsequent immunofluorescence (IF) staining. Every 10th slide was stained with H&E by dewaxing the slides using xylene and rehydrating the sections through a descending ethanol series. Hematoxylin was then applied to stain the slides, and the stain was differentiated by briefly dipping the slides in acid ethanol. The blue color of the hematoxylin was enhanced by treating the slides with a lithium carbonate solution. Counterstaining was performed using eosin-phloxine B solution and the sections were dehydrated through an ascending ethanol series, cleared in xylene, and coverslips were mounted using Permunt. A Zeiss Imager M2 microscope was used with objective Zeiss 20X/0.08 ∞ /0.17/OFN25, Plan-Apochromat, Zeiss Filter Set 112, and AxioCam 305 camera for image acquisition.

2.4 Multiplex Immunofluorescence (mIF)

2.4.1 Slide preparation

H&E-stained sections were first examined to confirm LITT damage and appropriate slides were selected for the mIF. The slides were deparaffinized by placing them in an oven at 60°C overnight. Deparaffinization included three times 10 minutes of xylene washes, followed by rehydration through an ethanol gradient series (100%, 95%, 80%, 70%, 60%). Slides were rinsed in ddH₂O and washed in PBS with 0.1% Tween 20 (PBS-T). Antigen retrieval was performed using a citrate buffer solution made of 9 mL solution A [(21.01g C₆H₈O₇ + H₂O in 1L ddH₂O (citric acid) in 1L ddH₂O] and 41 mL solution B [(0.1M) 29.441g C₆H₅O₇Na₃ X H₂O (Sodium-citrate dibasic trihydrate) in 1L ddH₂O] and bring the final volume to 500 ml with ddH₂O.

The solution was prepared in a microwave-safe container and boiled with the slides for a few minutes until boiling bubbles were found and then transferred to a 90°C water bath for 20 minutes before cooling. After washing in PBS-T, slides were treated with 0.1% Triton X-100 for permeabilization. Sections were outlined with a PAP pen (EMS #71310, Hatfield, PA), and non-specific binding sites were blocked using 10% normal goat serum (NGS) in PBS-T for 30 minutes. F(ab) blocking reagent was applied for an hour to block endogenous immunoglobulins and reduce background when using antibodies from mouse species on a mouse FFPE section.

2.4.2 Antibodies incubation

A primary antibody cocktail and corresponding IgG controls were prepared in 10% NGS. The cocktail included all three primary antibodies at their recommended dilutions, with

the total volume adjusted to 100µl per tissue section in 10% NGS/PBST. Isotype-specific control non-immune IgG antibodies were prepared at the same concentrations as their corresponding specific antibodies. Each tissue section was then covered with 100µl of either the primary antibody cocktail or the IgG control solution. The slides were placed in a humidifying chamber protected from light and incubated overnight at 4°C to allow for optimal antibody binding to the targeted antigens. The next day, the slides were then washed and incubated with a cocktail of corresponding secondary antibodies.

2.4.3 Autofluorescence quenching and DAPI staining

Vector TrueVIEW Reagent (#VECTSP8400, Vector, Newark, CA) was prepared by mixing Reagents A, B, and C at a 1:1:1 ratio, with the order of mixing being crucial. First, equal volumes of Reagent A and B were combined in a test tube and mixed for 10 seconds. Then, Reagent C was added to the mixture, maintaining the 1:1:1 volume ratio, and mixed again for 10 seconds. To apply the reagent, the excess buffer was drained from the tissue section, and approximately 100µl of the prepared Vector TrueVIEW Reagent was added to completely cover the tissue. The section was then incubated for 25 minutes, followed by a 5-minute wash in PBS-T buffer using a magnetic stirrer. The excess buffer was drained from the section and incubated with DAPI (1:50,000) for 5 minutes. The slides were then cover-slipped and prepared for imaging. Slides were imaged prior to stripping for the next round of IF.

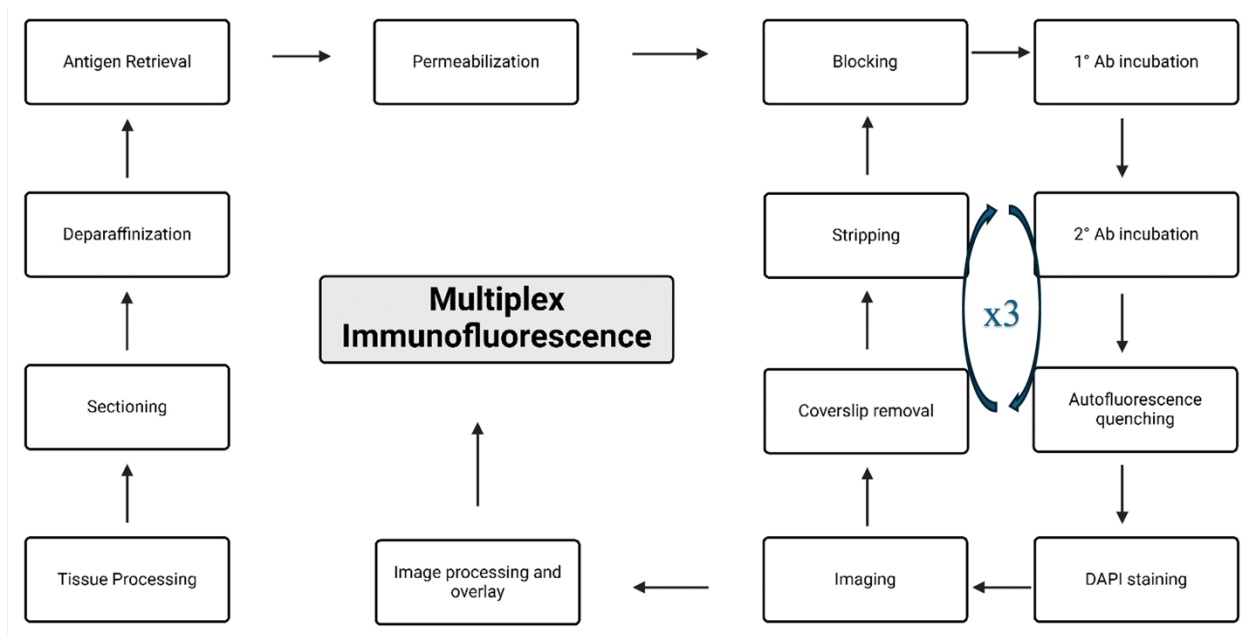


Figure 2.3 Flowchart of the multiplex immunofluorescence process

2.4.4 Second round of IF

The imaged slides were placed in PBS-T for 15-30 minutes to detach the coverslip from the slides. Thereafter, the coverslip was carefully removed from the tissue section. The section was then thoroughly washed with PBS-T (pH 7.4) to remove any residual mounting media. VectaPlex™ Reagent A was applied to completely cover the tissue section (70-100μL) and incubated for 15 minutes at room temperature. Following a brief PBS-T wash, VectaPlex™ Reagent B was similarly applied to cover the tissue section (70-100μL) and incubated for another 15 minutes at room temperature. Finally, the slides were washed with PBS-T for 5 minutes and the blocking step was repeated prior to incubation with a new set of antibodies and imaging. A total of three rounds of IF were done on the same sections.

Table 2.3 Multiplex Immunofluorescence Primary Antibodies

Antibody	Species	Source	Cat. No	RRID	Dilution
αSMA	Mouse monoclonal	sigma	A2547	AB_476701	1/150
AQP1 [EPR11588(B)]	Rabbit monoclonal	Abcam	ab168387	AB_2810992	1/100
ARG1 (D4E3M™) XP	Rabbit monoclonal	CST	93668	AB_2800207	1/100
Calreticulin (D3E6) XP	Rabbit monoclonal	CST	12238	AB_2688013	1/100
CD11b [EPR1344]	Rabbit monoclonal	Abcam	ab133357	AB_2650514	1/4000
CD11c (D1V9Y)	Rabbit monoclonal	CST	97585S	AB_2800282	1/500
CD163 [EPR19518]	Rabbit monoclonal	Abcam	ab182422	AB_2753196	1/200
CD20 (E3N7O) XP	Rabbit monoclonal	CST	70168S	AB_2943602	1/200
CD206/MRC1 (E6T5J) XP	Rabbit monoclonal	CST	24595s	AB_2892682	1/150
CD31	Rabbit polyclonal	Abcam	ab124432	AB_2802125	1/100
CD3ε (E4T1B) XP	Rabbit monoclonal	CST	78588S	AB_2889902	1/100
CD4 [EPR19514]	Rabbit monoclonal	Abcam	ab183685	AB_2686917	1/1000
CD68 (E3O7V)	Rabbit monoclonal	CST	97778	AB_2928056	1/150
CD8 alpha [EPR20305]	Rabbit monoclonal	Abcam	ab209775	AB_2860566	1/2000
CD86 (E5W6H)	Rabbit monoclonal	CST	19589	AB_2892094	1/150
Chromogranin A	Rabbit polyclonal	Abcam	ab15160	AB_301704	1/100
CRYGS	Rabbit polyclonal	Proteintech	PA5-57155	AB_2640182	1/100
F4/80 (D2S9R) XP	Rabbit monoclonal	CST	70076	AB_2799771	1/125
FoxP3 (D6O8R)	Rabbit monoclonal	CST	12653S	AB_2797979	1/200
GFAP	Chicken polyclonal	Aves	GFAP	AB_2313547	1/100
GFAP	Mouse monoclonal	Sigma	G3893	AB_477010	1/200
GRP78 BiP [EPR4040(2)]	Rabbit monoclonal	Abcam	ab108613	AB_10859806	1/100
IBA1	Chicken polyclonal	Aves	IBA1	AB_2910556	1/100
IBA1	Rabbit polyclonal	Wako	019-19741	AB_839504	1/250
Ki67	Chicken polyclonal	Aves	Ki67-1010	AB_2941673	1/100
MAP2 [EPR19691]	Rabbit monoclonal	Abcam	AB183830	AB_2895301	1/500
MBP	Chicken polyclonal	Aves	MBP	AB_2313550	1/100
MBP (2H9)	Mouse monoclonal	Novus	NBP2-22121SS	AB_3266940	1/200
NCAM1 (E7X9M) XP	Rabbit monoclonal	CST	99746S	AB_2868490	1/100
Nestin	Chicken polyclonal	Aves	NES	AB_2314882	1/100

NeuN	Rabbit polyclonal	Sigma	ABN78	AB_10807945	1/350
NFH	Chicken polyclonal	Aves	NFH	AB_2313552	1/100
NFL	Chicken polyclonal	Aves	NFL	AB_2313553	1/100
NFM	Chicken polyclonal	Aves	NFM	AB_2313554	1/100
NLRP3 (D4D8T)	Rabbit monoclonal	CST	15101	AB_2722591	1/100
OLIG2	Chicken polyclonal	Aves	OLIG2	AB_2924438	1/100
Olig2 (E6G6Q) XP	Rabbit monoclonal	CST	65915S	AB_2936997	1/100
PD1/CD279	Mouse monoclonal	Proteintech	66220-1-ig	AB_2881611	1/100
PDGF Receptor β (C82A3)	Rabbit monoclonal	CST	4564	AB_2236927	1/100
PDI (C81H6)	Rabbit monoclonal	CST	3501	AB_2156433	1/100
PDL1/CD274	Rabbit polyclonal	Proteintech	17952-1-AP	AB_10597552	1/100
pStat3 (Tyr705) (D3A7)XP	Rabbit monoclonal	CST	9145	AB_2491009	1/100
S100B (E7C3A)	Rabbit monoclonal	CST	90393		1/100
SQSTM1/p62	Rabbit monoclonal	CST	8025	AB_10859911	1/100
Stat3 (79D7)	Rabbit monoclonal	CST	4904	AB_331269	1/150
Sox2 (L1D6A2)	Mouse monoclonal	CST	4900	AB_10560516	1/100
Vimentin (D21H3) XP	Rabbit monoclonal	CST	5741	AB_10695459	1/150

Table 2.4 Multiplex Immunofluorescence Secondary Antibodies

Antibody	Species	Source	Cat. No	RRID	Dilution
Alexa Fluor 568	Goat anti Chicken	Invitrogen	A11041	AB_2534098	1/1000
Alexa fluor 647	Goat anti Rabbit	Invitrogen	A21245	AB_2535813	1/1000
Alexa Fluor 488	Goat anti Mouse	Invitrogen	A11029	AB_2534088	1/1000
DAPI	nuclear counterstain	Sigma	D9564		1/50000

2.5 mIF protocol optimisation

2.5.1 Antibody optimization

The antibodies were optimized individually before starting a mIF experiment. The concentration of the antibodies was adjusted to reduce the background and locate the staining region in the LITT mouse brains. The citrate buffer-based antigen retrieval method was used for all antibodies tested in the mIF experiments.

2.5.2 Autofluorescence quencher:

The Vector TrueVIEW Reagent, autofluorescence quencher optimization process was optimised for each tissue type. IF experiments were initiated with serial FFPE sections from a LITT-treated mice and processed until the blocking step when a Vector TrueView Autofluorescence quencher was added to all sections and incubated for a series of times, ranging from 5 minutes to 45 minutes. Following incubation, the sections were briefly washed in PBS-T for 5 minutes, DAPI stained, and cover-slipped. The sections were viewed under a fluorescence microscope using the filter for AF488 to reveal a green auto-fluorescence channel to optimize the duration of exposure with the quencher. This process determined that an incubation time of 20-25 minutes with TrueView Autofluorescence Quencher was optimal for LITT mouse brains.

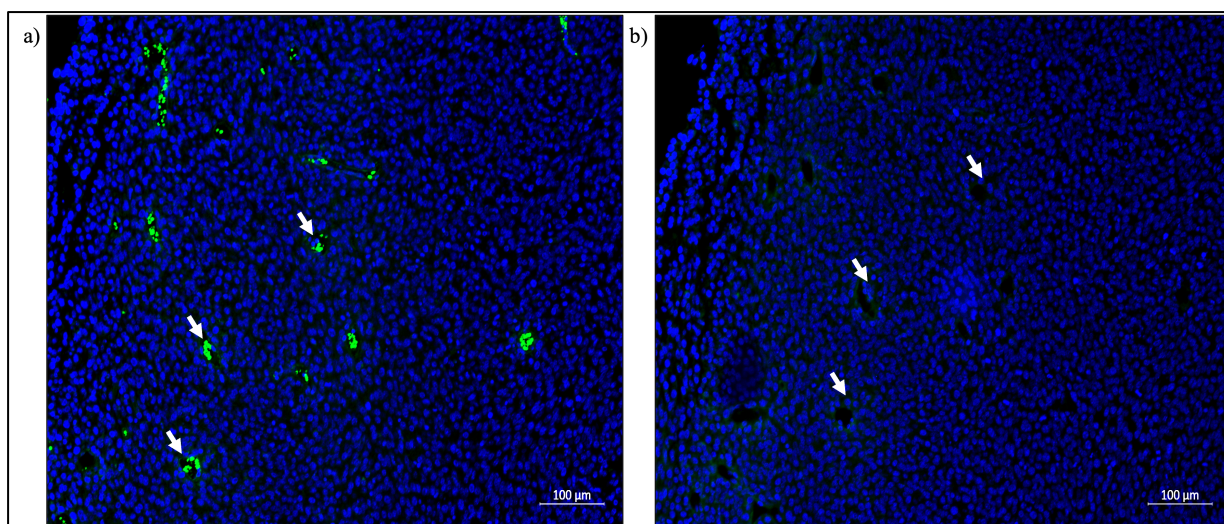


Figure 2.4 Autofluorescence quencher optimization: FFPE mice brain tumor sections with high autofluorescence (AF) were used for the Vector TrueVIEW Reagent optimization. a) shows the image without the AF quencher. The complete IF protocol was followed except for the AF quenching step. GAM AF488 secondary antibody was to view the autofluorescence. b) A serial section from the same tissue was treated with a Vector TrueVIEW autofluorescence quencher. Incubation times ranging from 5-25 minutes were tested and 20-25 minutes was the optimal AF quencher incubation time. Figure a and b show the same tissue region before and after autofluorescence quenching.

2.5.3 Stripping solution:

FFPE brain tumor tissues stained with the marker for resident microglia, Iba1, were used to optimize the Vectaplex antibody stripping solution. Upon completion of the IF staining protocol and imaging of specific immunoreactive Iba1 with an AF647 labeled goat anti-rabbit (GAR) secondary antibody, coverslips were removed, and the tissue sections were incubated with a stripping solution. Stripped tissue sections were again blocked with 10% NGS and incubated exclusively with the AF647 labeled GAR secondary antibody and DAPI. The datasheet suggested an incubation time of 15 minutes for both reagents A and B worked well.

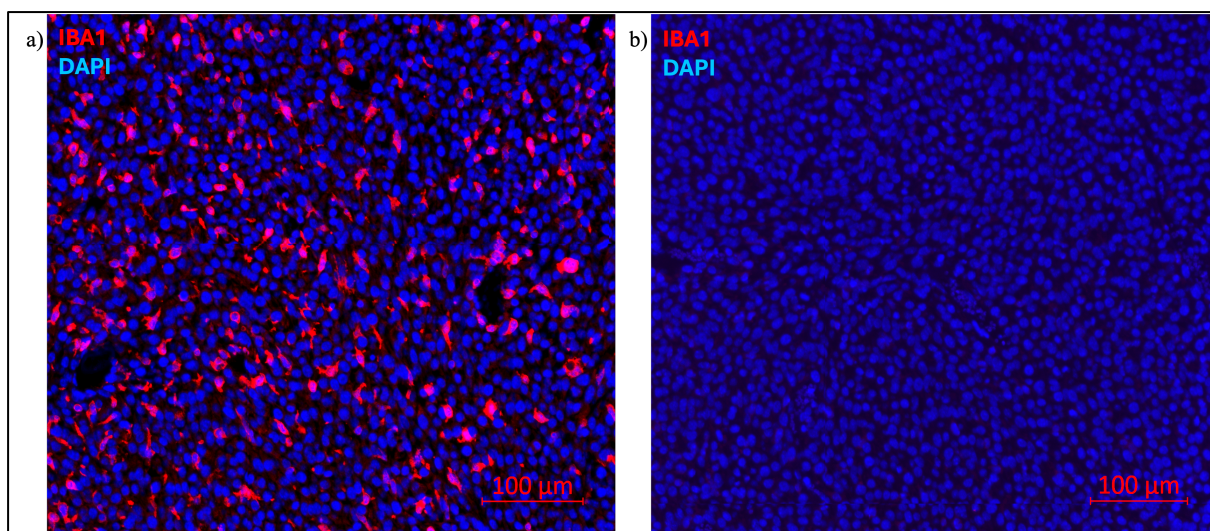


Figure 2.5: Antibody stripping solution optimization: Similar to AF quencher optimization experiments, mice tumor brain FFPE with dense Iba1 infiltration was chosen to optimize Vectaplex antibody stripping solution. After imaging the IBA1 staining using the respective GAR AF647 antibody, the section was treated with the Vectaplex antibody stripping reagents A and B following the manufacturer's protocol. The suggested incubation time for the quencher of 15 minutes was used and sections were incubated again with the GAR AF647 to check if the stripping solution removed the primary antibody Iba1 along with its secondary antibody GAR AF647. The manufacturer-recommended treatment time of 15 minutes worked well and was used from hereon in all quenching steps.

2.6 Image acquisition and processing:

A Zeiss Imager M2 microscope, equipped with a Zeiss 20X/0.08 ∞ /0.17/OFN25 Plan-Apochromat objective, Zeiss Filter Set 112, and an AxioCam 305 camera, was used for image acquisition. The Zeiss ZEN software (ZEN 3.8 Pro) was opened to calibrate the stage, enable the tile option, and calibrate the 4-slide holder. The region of interest was located, and the magnification was adjusted to 20X. The region to be tiled was marked, and focus points were

set. Multiple regions on the same slide could be marked simultaneously. A multi-slide holder and multiple regions of interest (ROIs) were used to calibrate and image several regions automatically for efficiency. Distributed focus points were ensured to cover the entire ROI before adjusting the focus and verifying each point. It was critical to maintain focus for each region of interest and to set appropriate exposure times for each marker based on single antibody optimization. Adjustments to focus points were made as needed during the process, as each tile of the image needed to remain in focus; out-of-focus images would compromise the quality of the final compiled mIF image. Imaging was repeated after each cycle of immunofluorescence staining.

After completing all cycles of immunofluorescence (IF) and image acquisition, the images were opened in ZEN Blue software. For cases where multiple tile regions were imaged within a single acquisition, the software outputted one image containing multiple scenes (ROIs). The "create image subset and split" option was used to extract individual scenes, and the stitching option under the processing tab was applied to integrate the individual tiles into a single image. Channel colors were then switched to suitable pseudo-colors for the targeted cellular markers from different IF cycles. Pseudo-colors were used to distinguish between various targeted cellular markers and to aid in visualizing their co-localization. Complementary colors were applied for co-localizing cellular targets; for example, red and green colors produced yellow when overlaid, indicating the co-localization of two targets from different IF cycles. To enhance visibility, a high-contrast color coding was selected for more relevant targets, while lighter colors were used for less significant markers. Tile-stitched .czi files were utilized for ZEN Connect overlay as the preferred method, and pseudo-colors were provided using merged multiplex images.

2.6.1 Image overlay using Zen Connect:

The images from multiple rounds of IF were opened in a Zen Blue Image Processing window. The Zeiss Zen Connect toolkit package enabled the image alignment option. A new project was created and all images from the different rounds were opened. The first image was used as the reference, and the opacity of the second image was adjusted while hiding all other images. The second image was selected and the align option was activated. Hovering over the first image and using a unique anatomical structure from the images as a reference for better alignment the exact region was selected. After applying the alignment, the second image was hidden, and the same process was repeated for all remaining images. The DAPI staining was aligned in the same location across all IF rounds and served as the reference channel for merging images into a single multiplex immunofluorescence image.

"Custom Carrier 1" was selected from the Zen Connect tab to include all images in the project, and the "Single Image Export" option was used to obtain a single merged image. In the export window, the merge option was changed to "Intensity-based," with the export format set to "CZI image multi-channel" and pixel size to "largest," while default settings were used for the remaining options before clicking "Export data" to initiate the merging process. Zen Connect merged the images tile by tile, resulting in a merged image in .czi format with all previously assigned colors removed. The channel tabs displayed details of the image along with its channel number, which could be used to identify staining and assign new pseudo-colors. Regions of interest (ROIs) were selected from the full-size merged multiplex image, and the .tiff file was exported using Zen Blue, allowing for specific channel combinations to be selected for export to create several panels of multiple images.

2.6.2 Image overlay using Adobe Photoshop:

The .tiff files of multiple IF rounds were opened in Adobe Photoshop. The .tiff file from the second round of IF was copied and pasted onto the first cycle IF .tiff file. The screen option was enabled to overlay only the colors from the second round onto the first round IF images. Similar to the Zeiss Zen Connect procedure, the images were moved over each other to match the DAPI signals, and the .tiff file was exported as a single multiplex immunofluorescence image.

2.6.3 Cell segmentation and quantification:

Zeiss Intellesis software (Zeiss, Jena, Germany) was used for segmentation analysis. In ZEN Blue, we switched to the analysis tab, created a new Intellesis Trainable Segmentation model, and initiated training. An immunofluorescence (IF) image was imported, and the “multi-channel” option was set to train multiple targets simultaneously. For segmentation training, tools in the “Labelling options” were used to label positive stains for each marker and assign a class to each marker, such as Class 1 for DAPI, Class 2 for Iba1, and Class 3 for the background. A background class was added to label all non-specific signals and empty spaces. Once all classes were labeled, the “train” option was clicked, generating a new segmentation channel with all annotations. The training preview was checked, and additional signals were labeled under each class where the software failed to accurately predict targets to improve machine learning efficiency. The model was re-trained repeatedly until the Intellesis segmentation model achieved accuracy. This trained model was then exported for use in the ZEN Blue Image Analysis workflow. A new Image Analysis program was created by clicking "Setup Image Analysis," which opened a wizard guiding through the workflow steps. The “model class” option was selected, and “fill holes” was enabled for the DAPI channel class to

prevent segmentation errors. Thresholds were optimized for all classes to avoid detecting false positives or cell debris. In the “features” step of the wizard, options such as “Image name,” “Count,” and other required features were added to include image details and the number of positive cells in the analysis output. The segmentation results were previewed, and the training wizard was finalized to save the analysis workflow. This analysis setting was then applied to all mIF images from the experiment, generating data tables and analysis masks for each image. Other Zeiss Zen Blue tools were used to measure the area, radius, and length of the positive signal cells were used to quantify the images.

3. RESULTS

3.1 LITT produces a heat damage gradient

The LITT-treated non-tumor mouse brains were extracted, gross sectioned, and processed for formalin fixed paraffin embedded sectioning. 5 μ m sections were made using a microtome. Serial sectioning protocol for the FFPE mouse brain blocks was optimized to study the heat-induced damages at different depths away from the ablation core (point 0, closer to the laser probe, which received the maximum heat damage). An initial assessment of the LITT-induced heat damage gradient was performed using the hematoxylin and eosin (H&E) staining. Every 10th slide was H&E stained to locate the LITT region in the brain. **Figure 3.1** shows the H&E staining images on Day 6 of post-LITT brains at different depths from 3 different mouse brains. Depending on the region at which the brain was gross sectioned after extraction, the LITT damage begins to appear on the FFPE tissue block. Significant damage was observed on the 1st slide of sections in mouse 1 and mouse 2, whereas, in mouse 3, the damage started to be visible only after the 11th slide of sections. Based on the gross sectioned location, either increasing or decreasing damage was observed in the H&E-stained images.

The dotted lines in **Figures 3.1** and **3.2** mark the zone of LITT damage. These images show the impact of laser probe insertion and its heat damage. The heat energy induced by the laser probe coagulates the tissue, and the insertion of the laser fiber and thermocouple device forms an insertion canal. From the H&E-stained images, the heat damage is characterized by reduced staining intensity, pycnotic or destroyed nuclei, and infused blood cells from ruptured blood vessels (**Figure 3.2**). These H&E-stained slides were used to pick the slides for the mIF experiments based on assessing the LITT-treated induced cellular changes in the mouse brains. **Figures 3.1** and **3.2** are representative images from 8 mice with the same LITT parameters. The FFPE sections from these eight mouse brains were used in all the experiments.

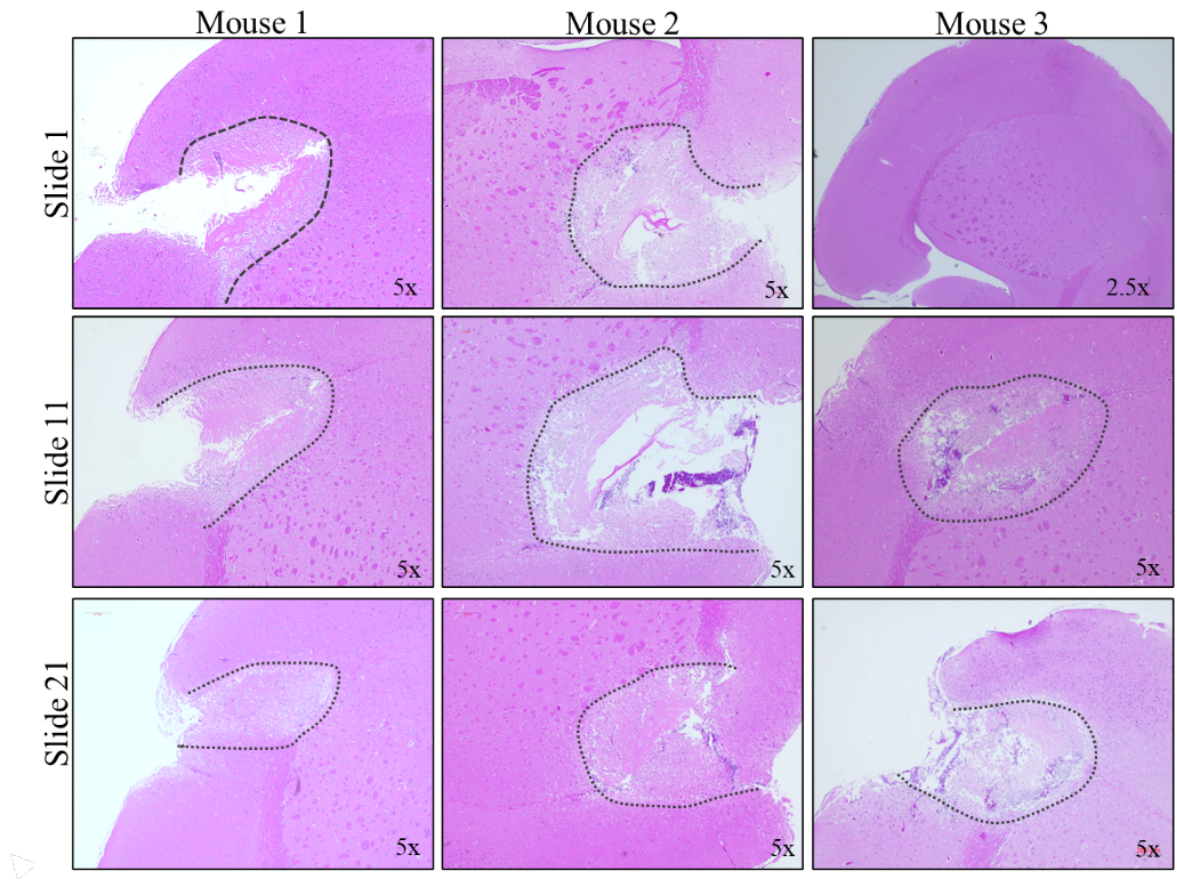


Figure 3.1 Tiled H&E images of serially sectioned FFPE mouse brains: H&E-stained images from serially sectioned slides 1, 11, and 21 of 3 different non-tumor mouse brains at Day 6 post-LITT showed a difference in the treatment-induced damage. The dotted line marks a histological LITT damage boundary to seemingly intact mouse brain tissue. All images were captured using the Zeiss Zen Tiled imaging option at 5x or 2.5x magnification. The images shown are the representative images of n=8.

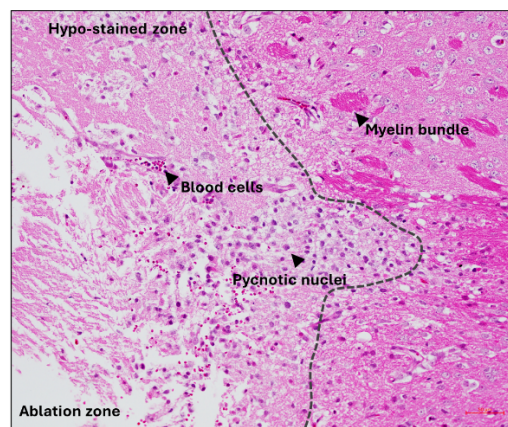


Figure 3.2: H&E image of the LITT zone 20x. The image showed the myelin bundles and normal tissue morphology away from the ablation zone. The dotted line marks the LITT damage boundary. Blood cells and the hypo-stained zone indicate LITT-induced tissue damage close to the ablation zone. Pycnotic nuclei (shrunk nuclei) were frequently observed close to and in the ablation zone.

3.2 Map2 and NeuN mark the extent of LITT-induced Neuronal Damage

Multiplex immunofluorescence (mIF) was used to assess the LITT-induced early neuronal and myelin damage in Day 6 post-LITT non-tumor mouse brains (**Figure. 3.3**). Neuronal nuclei marker NeuN (blue) was used to differentiate neuronal nuclei from nuclei of surrounding cells in the LITT brain. There was significant neuronal damage at the LITT site. A reduction in NeuN staining intensity and pycnotic neuronal nuclear morphology was typical at the ablation core (**Figure 3.3b**). Map2 (microtubule-associated protein 2) (red) was used to stain the neuronal cell bodies and dendrites, whereas Mbp (Myelin Basic Protein; green) stained myelin bundles and myelin sheaths in the brain. Similar to NeuN, a loss or reduction in the Map2 and Mbp staining was observed closer to the LITT damaged area (**Figures 3.3a, 3.3b**). The cortex showed more intense Map2 staining than the striatum due to the density and complexity of dendrites in the cortex. Neurons and myelin fiber tracts were destroyed at the thermal ablation core, with fragmented and reduced Mbp immunodetection along myelin bundles with altered morphology (**Figure 3.3b**). These results demonstrated substantial localized LITT-induced brain damage, which involved neuronal cells and myelin.

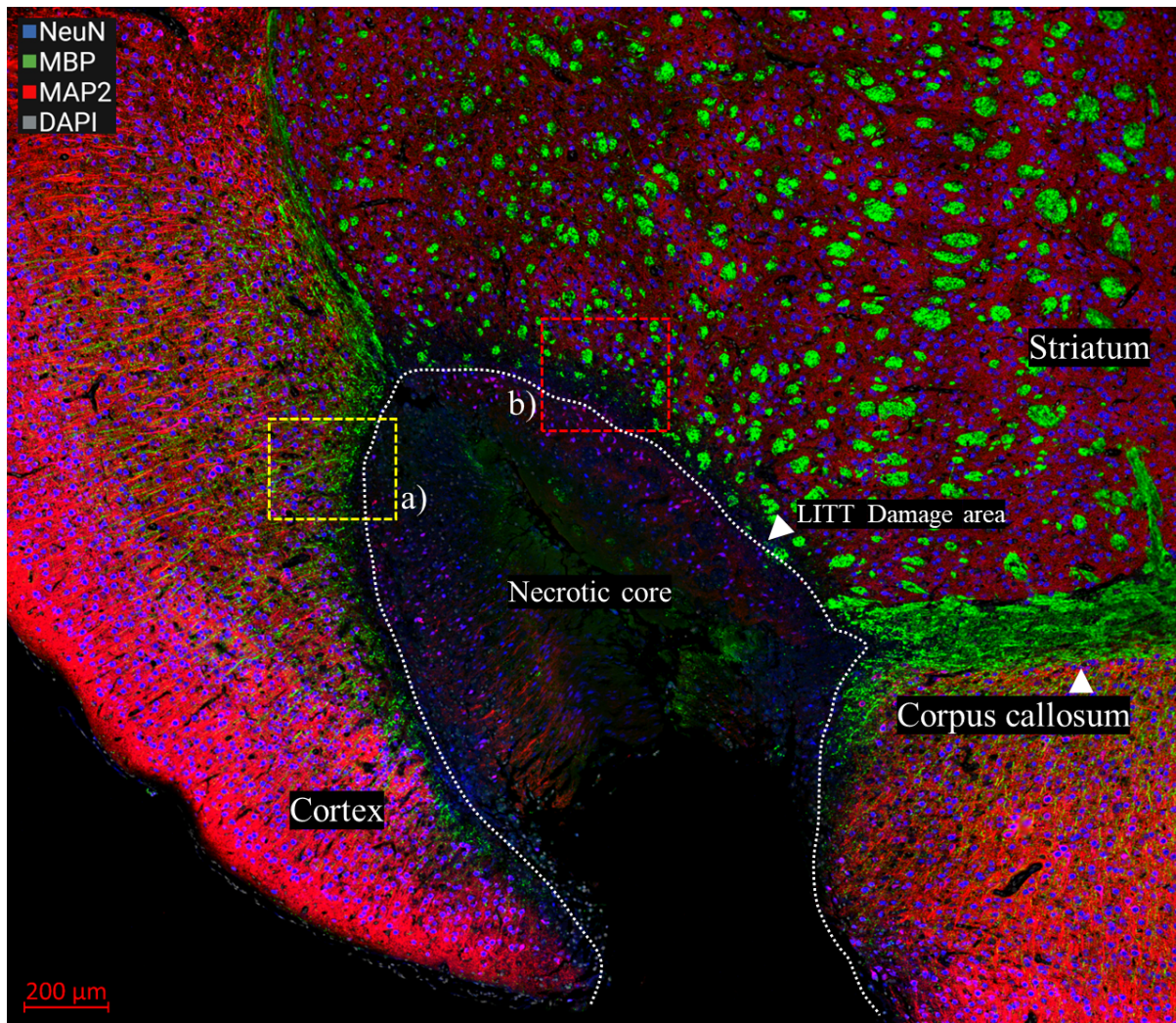


Figure 3.3 Map2, NeuN, and Mbp mIF: The overview image of the mIF shows the LITT site in the mouse brain cortex and striatum. The projection of dendrites and myelin sheaths stained by Map2 (red) and Mbp (green), respectively, clearly differed between the cortex and the striatum. Distinct strongly Mbp immunostained myelin bundles were observed in the corpus callosum and the striatum but absent in the cortex. DAPI staining was intentionally in a grey pseudo-color to minimize interference with NeuN (blue). The images shown are representative images of n=4.

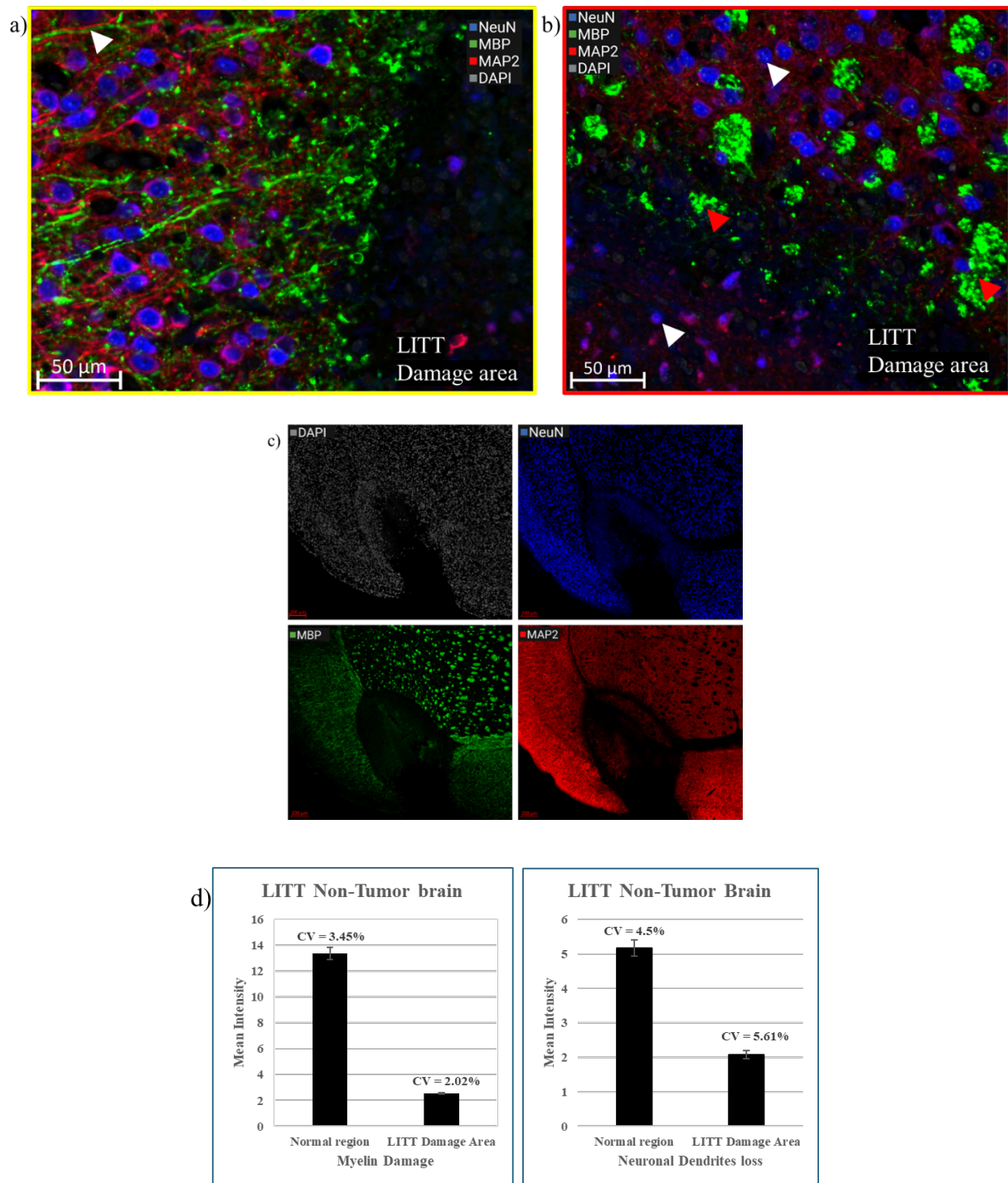


Figure 3.3 **a)** Myelin sheaths and dendrites were visible in the brain cortex. The white arrowhead shows separate Map2- and Mbp-immunostained dendrites and myelin sheaths. These structures did not co-stain. Close to the LITT core, Map2 (red) staining rarefied, and Mbp (green) immuno-stain became granular, suggesting extensive myelin damage. **b)** Differences in nuclear morphology were observed between NeuN⁺ immune-stained neurons

within the LITT damage area and away from the damage site (white arrowheads). Pycnotic neuronal nuclei, less intense and shrunken neuronal nuclei, were observed at the ablation area. Fragmentation observed within the Mbp immunostaining marked the LITT-induced myelin damage (red arrowheads). **c)** Single-channel images from the multiplexed image. **d)** Loss of Mbp and Map2 staining in the laser damage area was quantified using the Mean Intensity Estimation option in the Zeiss Zen Blue software. A coefficient of variation analysis was performed to calculate variation between technical replicates ($CV < 10\%$ =Low variation relative to mean). The images shown are representative images of $n=3$.

3.3 Zone of Regeneration as an early LITT event: Intermediate Filament Markers

Astrocytes and their intermediate filaments are major constituents of the brain. To assess the early LITT-induced cellular changes in astrocytes, we optimized a multiplex panel to study the effects of LITT on astrocytes (**Figure 3.4a**). Glial fibrillary acidic protein (Gfap) was used to stain activated matured astrocytes. Abundant Gfap⁺ cells were found in proximity to the LITT damage area. Gfap⁺ cells were completely absent in the ablation core. Gfap⁺ immunostaining gradually reduced away from the LITT damaged area, indicating spatially constrained laser effects on astrocyte activation (**Figure 3.4a**). Gfap was combined with other IF markers, including nestin (Nes), vimentin (Vim), and S100b, to identify their presence in astrocyte populations in post-LITT mouse brains. Co-stained Nes⁺ and Vim⁺ cellular extensions were identified closest to the necrotic core surrounded by a region of Gfap⁺ immunopositivity (**Figure 3.4b**). Nes⁺ immunostaining colocalized with both Vim⁺ and Gfap⁺ cellular structures. Nestin has been associated with astrocyte plasticity, suggesting different states of astrocyte regeneration close to the LITT ablation (Jin et al., 2004; Sahin Kaya et al., 1999; Yu et al., 2008).

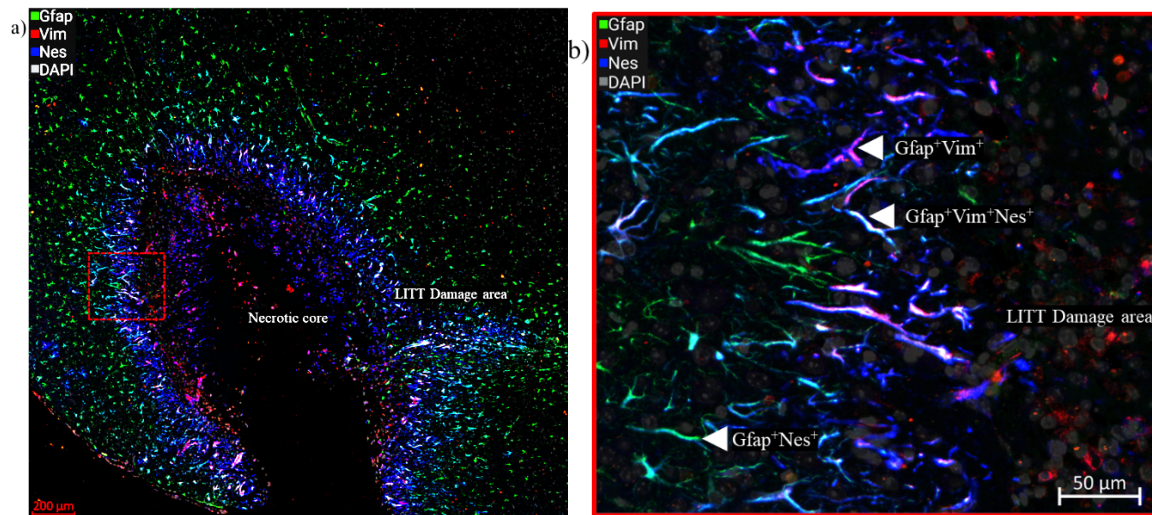


Figure 3.4 Intermediate filaments mIF panel 1: **a)** The overview image of Day 6 post-LITT mouse brain stained for intermediate filaments Gfap (green), Vim (red), and Nes (blue). Zonal expression of different astrocytic sub-populations was identified. **b)** Zoomed-in image of **a)** shows that the region adjacent to the necrotic core contained Gfap⁺, Vim⁺ and Gfap⁺, Vim⁺, Nes⁺ astrocytes. This zone had a high expression of Vim and Nes. Surrounding this region, away from the ablation site, were Gfap⁺ cells, some of which showed Gfap⁺ and Nes⁺ co-staining.

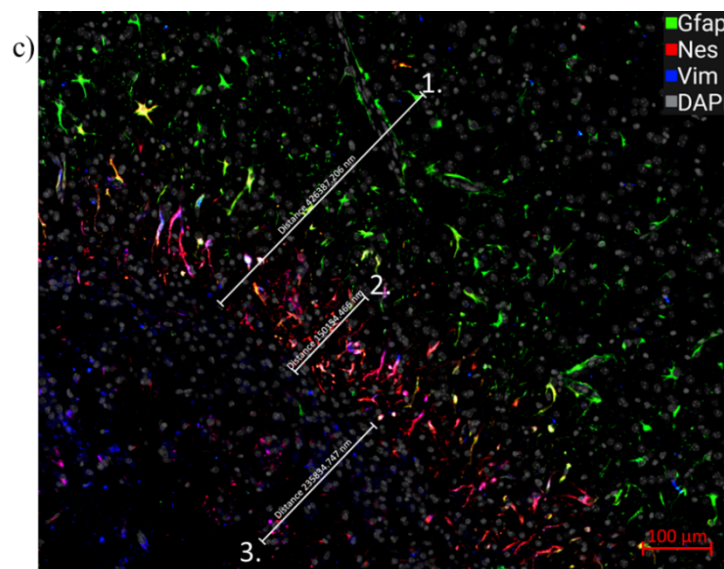


Figure 3.4: **c)** Zeiss Zen Blue was used to measure the expression pattern of different astrocytic sub-populations spanning from the necrotic LITT core to the intact brain. Three different zones

could be delineated. Zone 1 included activated astrocyte sub-populations with a total expression area of 426.38 μ m. Included in zone 1 was the smaller zone 2 adjacent to the LITT core (150.15 μ m) with Nes⁺ and Vim⁺ astrocytes of higher plasticity. Zone 3 included the necrotic core, which was devoid of activated astrocytes but did contain DAPI-positive nuclei.

To further characterize the changes in the astrocytic sub-populations, S100B was included in the mIF panel (**Figure 3.5a**). Similar to Map2, high background staining for S100B was found at the cortex due to the astrocytic density. S100b⁺, Vim⁺ cells resembling immature astrocytes (Dahl, 1981; Pixley & de Vellis, 1984), Gfap⁺, S100b⁺ mature astrocytes (Raponi et al., 2007a), and S100b⁺, Vim⁺, Gfap⁺ astrocyte sub-populations (J. Kim et al., 2024) were identified and reflected intricate differences in astrocytic injury response and developmental status (**Figure 3.5b**).

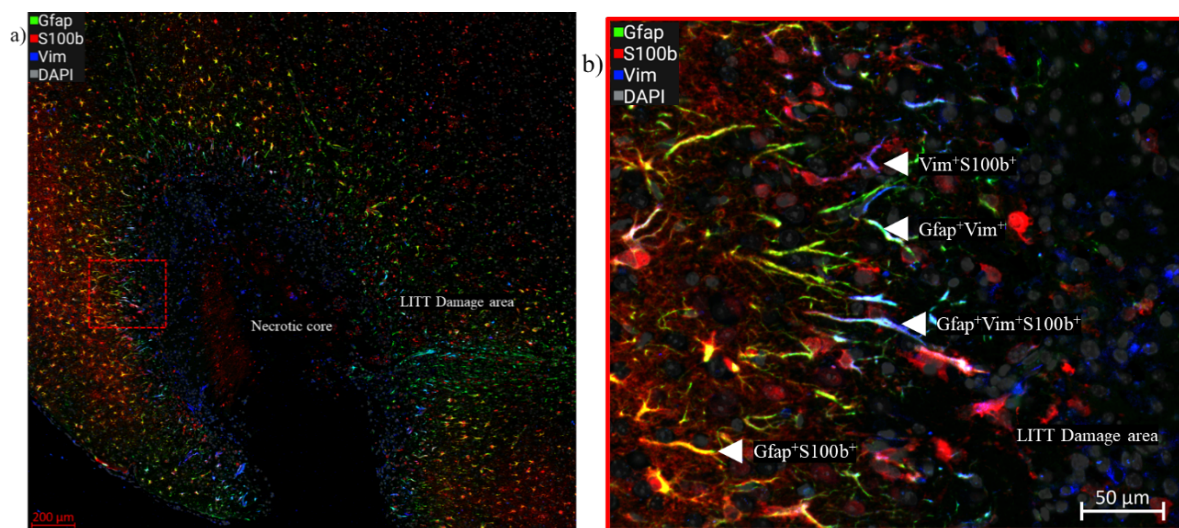


Figure 3.5 Intermediate filaments mIF panel 2: **a)** The overview image of Day 6 post-LITT mouse brain stained for intermediate filaments Gfap (green), S100b (red), and Vim (blue). **b)** Zoomed-in image showing the predominant S100b expression in mature astrocytes along with Gfap⁺ cells. Few S100⁺ Vim⁺ immature astrocytes were identified closer to the necrotic core.

Astrocytic activation was also studied at different tissue depths from the laser ablation core. Serial tissue sections closer to the laser probe and located farther away from the laser probe in the mouse brain were used (**Figure 3.6**). The comparative analysis showed a gradual decrease in Gfap⁺ activated astrocytic response farther away from the ablation core (**Figures 3.6b, d**), confirming our earlier findings in tissue sections showing maximal laser damage that Gfap positivity was highest closer to the ablation site and gradually declined away from the ablation core. This suggested that gradually reduced laser energy affected astrocyte activation accordingly in all directions in the brain. **The images shown were representatives of n=3.** The 8 mouse brains treated with the same LITT parameters were analysed using H&E staining. The post-LITT brains were confirmed to have similar histological changes and n=3 mouse brains were allocated for different types of cellular characterisation. This was done to look at the core LITT region for all the experiments.

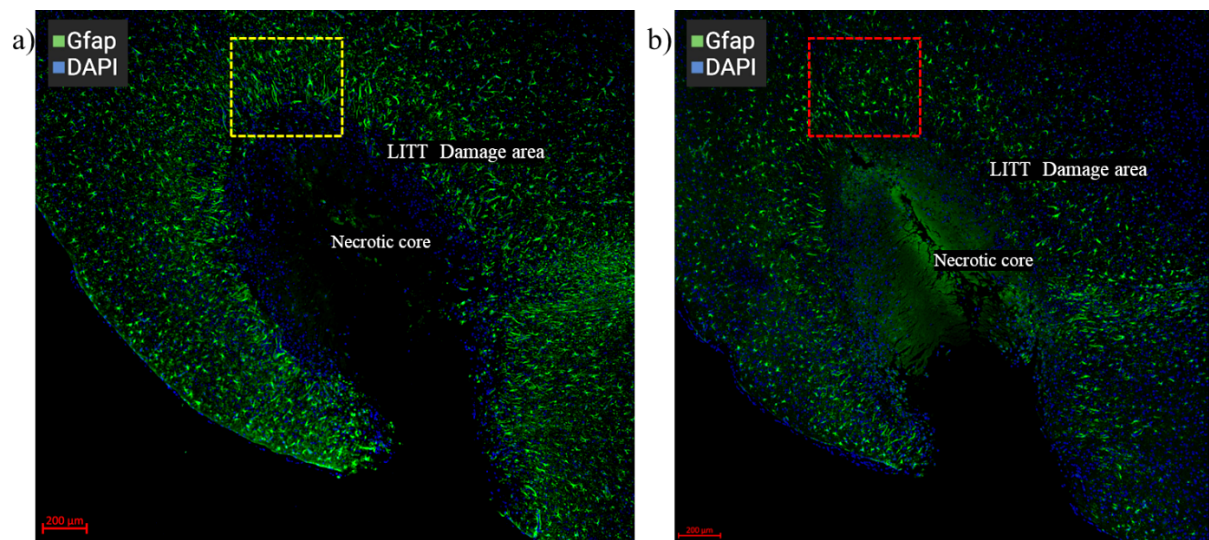


Figure 3.6 GFAP expression at different depths from the laser probe: Astrocytic activation marker Gfap (green) and nuclear (blue) stained overview images of a mouse brain from a section at a level **a)** closer to the laser probe (high Gfap⁺ cells) and **b)** farther away from the laser probe (weaker Gfap⁺ immuno-stained cells).

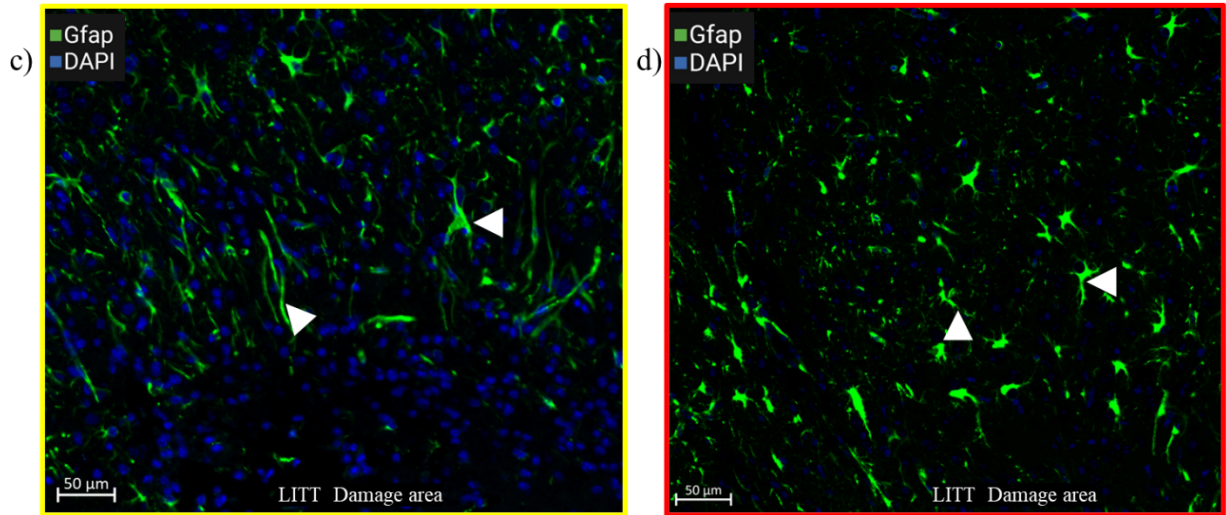


Figure 3.6: c) and d) were the zoomed-in images from a) and b) respectively. c) The region closer to the laser probe had higher Gfap immunostaining and longer astrocytic extensions (green) (DAPI-stained nuclei=blue). Away from the laser probe, there were fewer Gfap⁺ cells with shorter extensions.

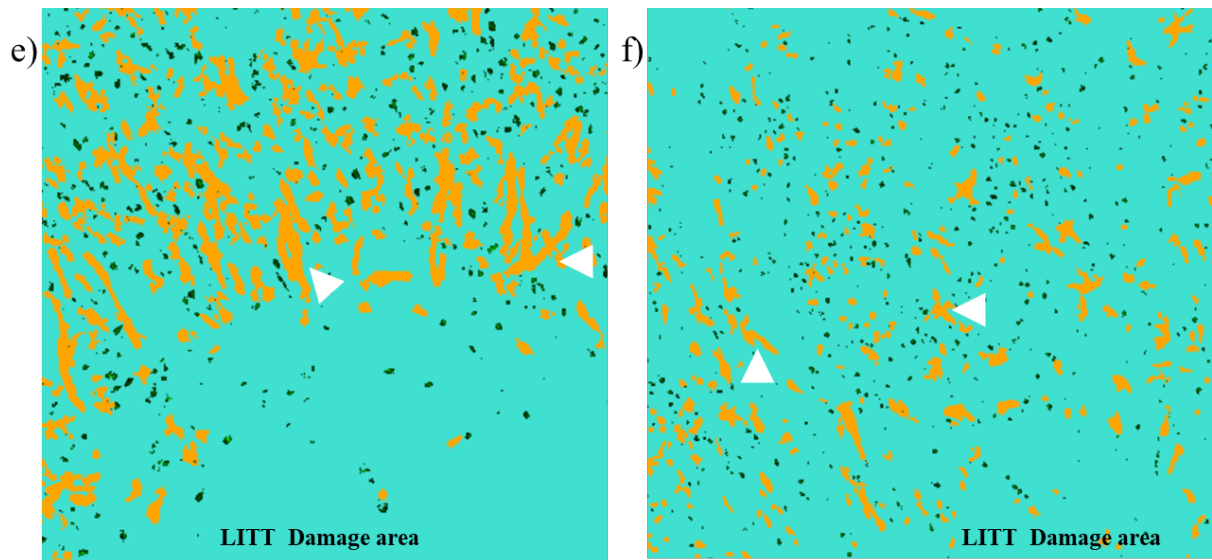


Figure 3.6: e) and f) show the representative images c) and d) segmented regions using Zeiss Zen Intellesis clearly show the difference in the length of each astrocyte. The region deeper f) from the laser probe shows less astrocytic activation than the region close e) to the laser probe. Gfap segmentation was performed using Zeiss Zen Intellesis software.

3.4 Spatial Distribution of Distinct Functional Microglia and Macrophage Populations

At the necrotic core, neuronal and glial cell staining was absent. However, DAPI-positive cell nuclei were identified. The region adjacent to the necrotic core received high laser energy that induced significant cellular damage. Innate immune cell markers were used to identify the type of cells present in this region, which was devoid of astrocytes and contained damaged neurons and fragmented myelin bundles. The resident brain microglia marker Iba1 identified the presence of activated microglia at the necrotic core (**Figure 3.7a**). Iba1⁺ microglia can be at different levels of microglial activation. Amoeboid-shaped phagocytic Iba1⁺ microglia were identified at the necrotic core. Away from the necrotic core, the amoeboid-shaped microglia gradually change to a ramified morphology with fewer extensions. Highly branched resting microglia with low Iba1⁺ immunoreactivity were identified farthest away from the LITT core (**Figure 3.7b**) (Donat et al., 2017a; G. Wang et al., 2013).

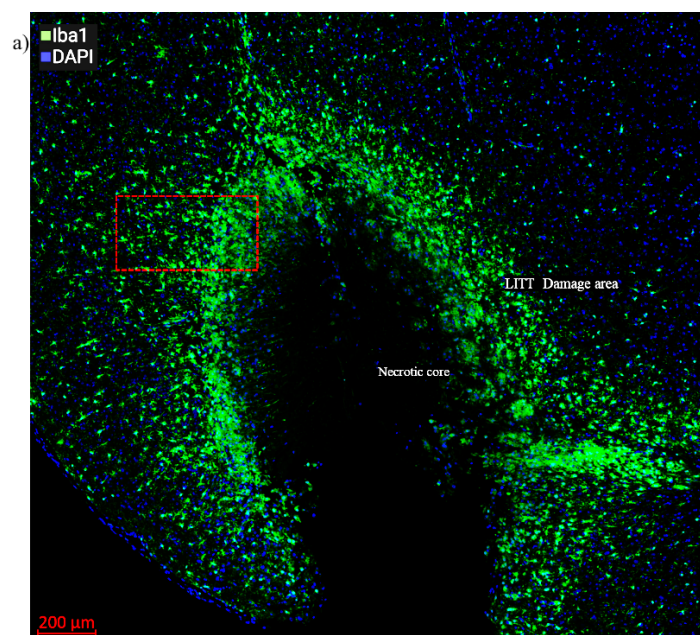


Figure 3.7 Resident brain microglia activation: a) overview image of activated resident brain macrophage marker Iba1⁺ cells (green) in a Day 6 post-LITT mouse brain. Most of the

Iba1⁺ cells were found to accumulate at the periphery of the necrotic core and some were identified within the LITT core region.

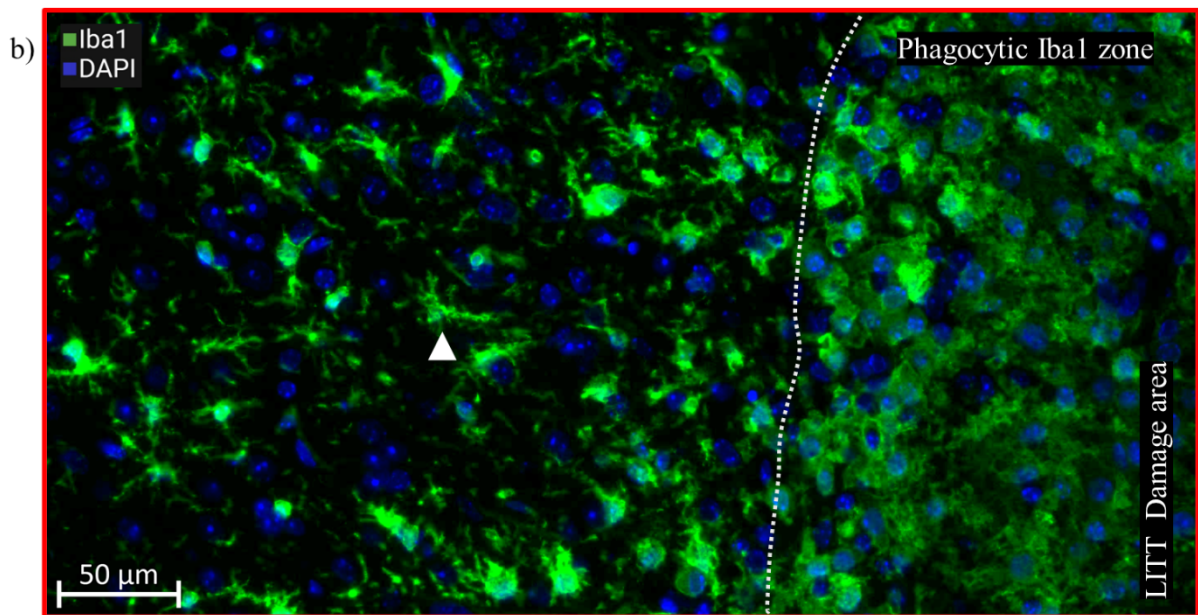


Figure 3.7: b) Zoom-in image shows plump amoeboid-shaped phagocytic Iba1⁺ microglial cells in the LITT damaged area on the right side of the dotted line and close to the LITT core. The white arrowhead shows the ramified activated microglia with frequent extensions located at a distance from the LITT damage area.

We co-immunostained for Iba1 and CD11b to confirm microglial cell activity in the LITT-damaged tissue area. CD11b is an integrin expressed predominantly on microglia and other immune cells and, along with Iba1, immuno-stains all microglia (Uff et al., 2022). **Figure 3.8a** shows the co-expression of CD11b and Iba1, thus, confirming the presence of activated microglia at the necrotic core. CD11b also stained other phagocytic Iba1 negative cells in the LITT ablation core.

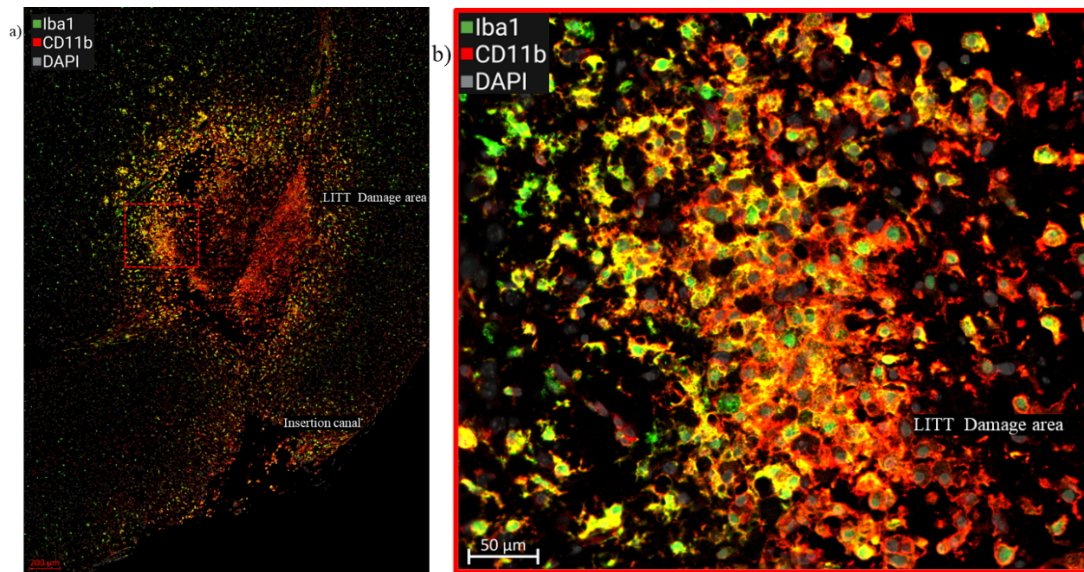


Figure 3.8 CD11b and Iba1 positive microglia: a) Overview image of a Day 6 post-LITT mouse brain co-stained with Iba1 (green) and CD11b (red) confirmed a high microglial activity status in the LITT damage area. Iba1⁺, CD11b⁺ (yellow) activated microglial cells were found in abundance close to the LITT damage area and the LITT probe insertion canal. b) Zoomed-in image from a) shows Iba1⁺, CD11b⁺ resident brain phagocytic cells in yellow.

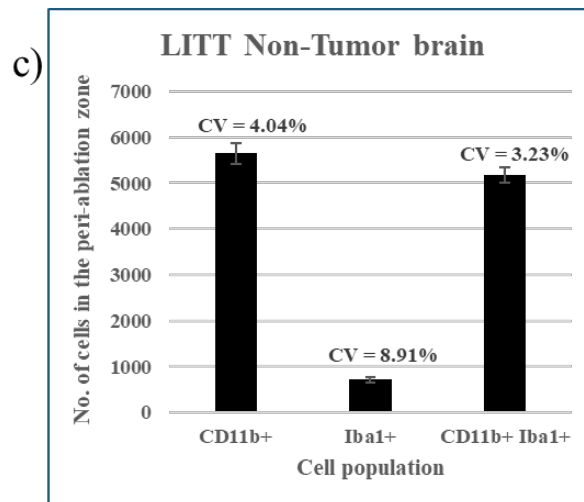
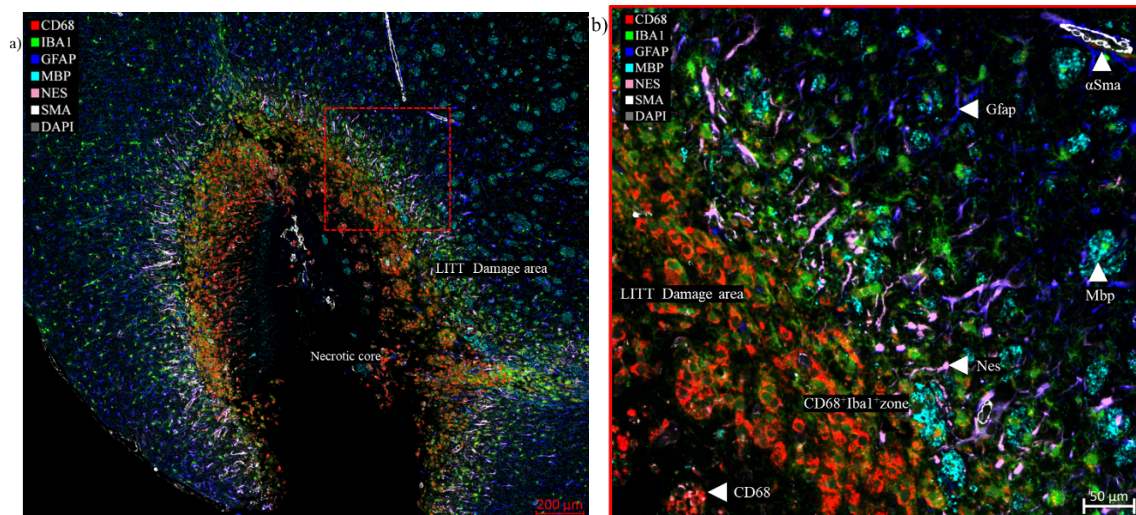


Figure 3.8 c) Representative image quantification of the number of CD11b⁺, Iba1⁺, and CD11b⁺Iba1⁺ cells in normal mouse brain at Day 6 post-LITT showing majority of Iba1⁺ microglia co-express with CD11b. Co-efficient of variation analysis was performed to calculate

variation between technical replicates (CV < 10% =Low variation relative to mean). The images shown are representative images of n=3.

The macrophage marker CD68 was also observed in LITT-damaged areas. A multiplex IF panel was formed to confirm the phagocytic phenotypes (**Figure 3.9a**). CD68⁺ phagocytic cells were co-localized with amoeboid-shaped Iba1⁺ activated microglia, whereas immunoreactive CD68 was absent in ramified or resting microglia. **Figure 3.9** displays the spatial gradient in the distribution of immunoreactive resident brain cells and macrophage markers at the LITT damage site. CD68⁺ macrophages (red) at the ablation core and co-expression with amoeboid-shaped CD68⁺, Iba1⁺ microglia cells confirmed a diverse phagocytic cell population at the innermost region of the LITT damaged site which was followed by astrocytes with a gradually decreasing activation state and intact myelin bundles in the periphery of the LITT damaged area (**Figure 3.9b**).



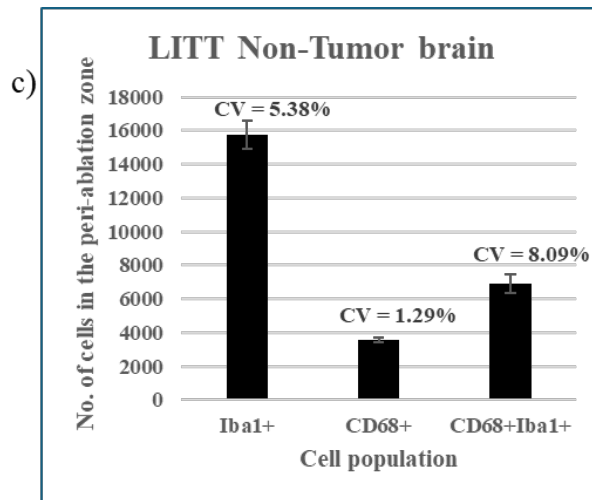


Figure 3.9 Macrophage and resident brain cells in LITT damaged area: **a)** Normal mouse brain treated with LITT was stained using a multiplex IF panel consisting of macrophage markers (CD68-red and Iba1-green), astrocytic markers (Gfap-blue, and Nes-pink), myelin bundles (Mbp-cyan), and blood vessels (α -Sma-white). The purpose of this mIF antibody panel was to study the spatial distribution of phagocytic macrophage phenotypes along the LITT damage region. **b)** Zoomed-in multiplex IF image **a)**. **c)** Representative image quantification of the number of CD68⁺, Iba1⁺, and CD68⁺, Iba1⁺ cells in the normal mouse brain at Day 6 post-LITT showing high microglial cell activation and dual CD68 and Iba1 co-expression in the peri-ablation zone. A laser ablation area with an ablation core in the center of 4.6 mm² was used for the quantification of phagocytic cells. Co-efficient of variation analysis was performed to calculate variation between technical replicates (CV < 10% =Low variation relative to mean). The images shown are representative images of n=3.

At the necrotic core, a CD68⁺ but Iba1-negative sub-population of phagocytic cells was detected, which was located adjacent to a tissue region with CD68⁺, Iba⁺ microglial cells and astrocytes with decreasing activation state farther from the core. To confirm the origin of the macrophages at the ablation core, monocyte-derived peripheral myeloid macrophage marker F4/80⁺ (Louwe et al., 2021; Sheng et al., 2017) was co-stained with Iba1 (**Figure 3.10a**). The

F4/80⁺ cell population co-localization with phagocytic Iba1⁺ microglia cells. In addition, and like CD68, an F4/80⁺, Iba1-negative phagocytic sub-population was identified in the ablation core. This F4/80⁺ cell population was also immuno-positive for CD68, suggesting the presence of myeloid-derived and possibly also monocytic macrophage sub-population in the LITT ablation core (**Figure 3.10b, 3.10d**). F4/80⁺ cells were also identified at the leptomeningeal layer of the mouse brain. The images shown were representatives of n=3.

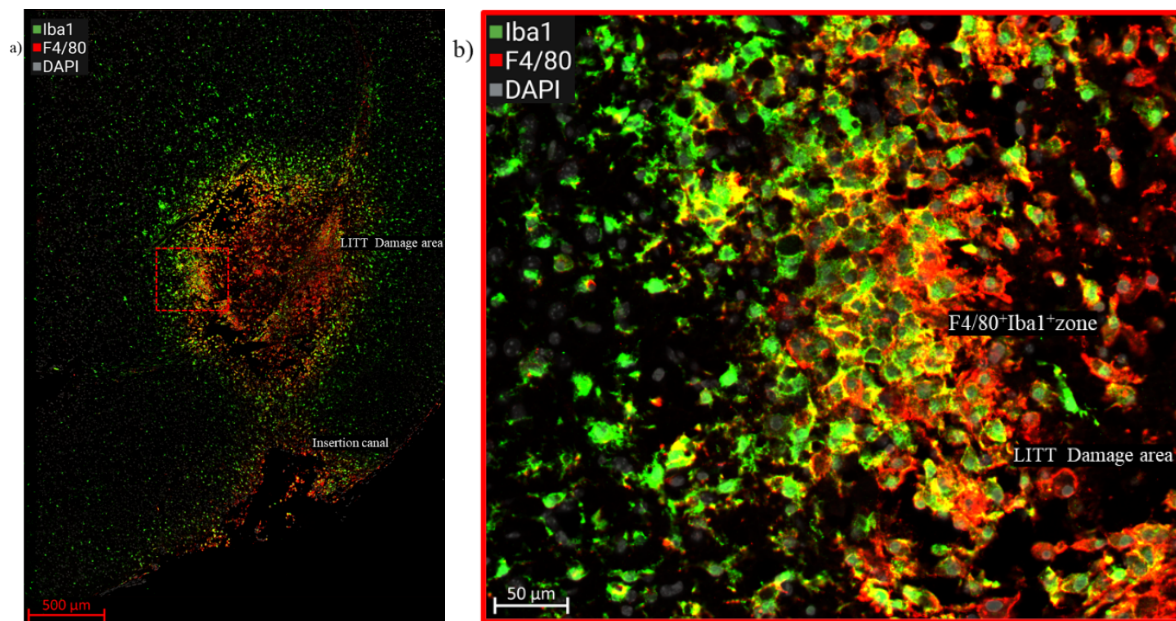
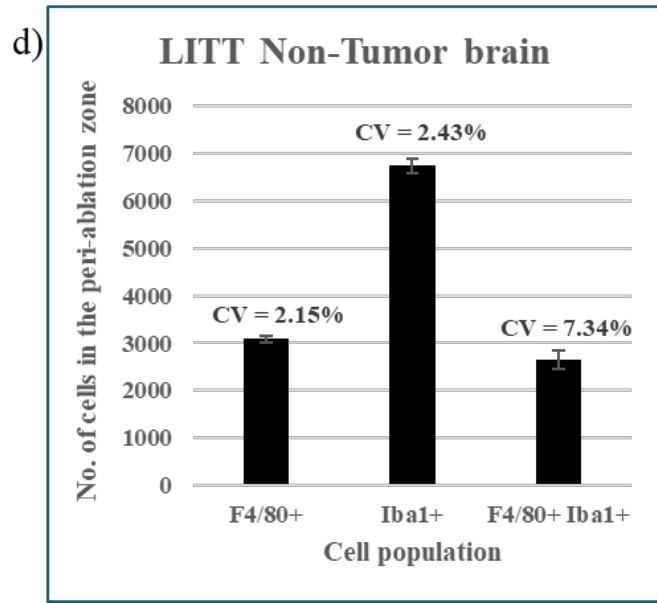
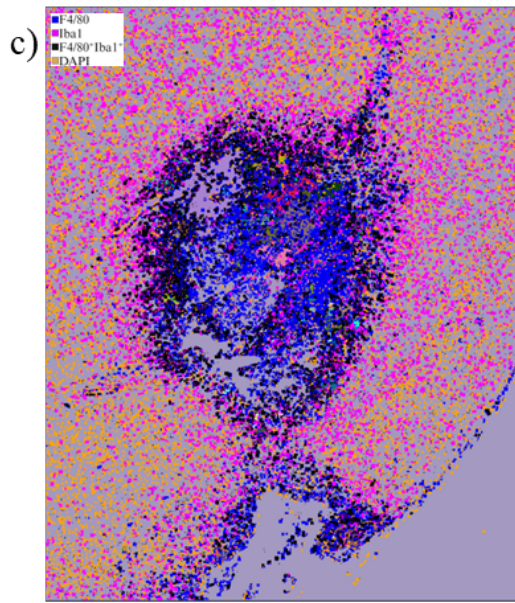
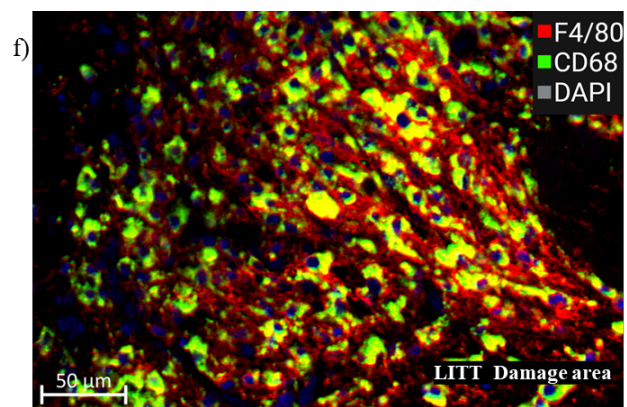
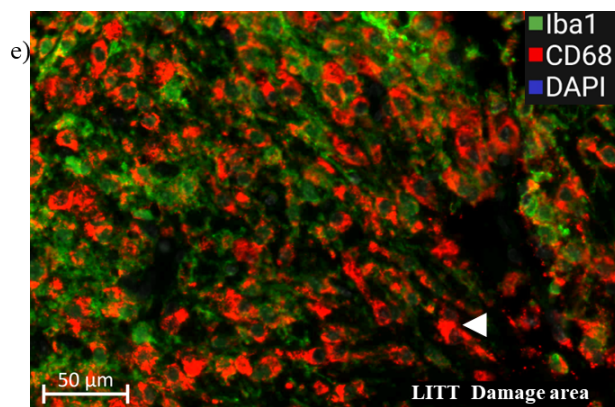


Figure 3.10 Identification of resident brain microglia and peripheral macrophages: a) Overview image of Iba1 and F4/80 co-IF in normal mouse brain at the LITT ablation site. F4/80⁺ macrophages were identified at the insertion canal and in the LITT damage area. Iba1⁺ and F4/80⁺ were identified in phagocytic microglia (amoeboid-shaped microglia) and exclusive F4/80 immuno-stained phagocytic cells devoid of Iba1 were identified in the ablation core. b) Zoomed-in image shows co-localization of Iba and F4/80 (yellow) and F4/80⁺, Iba1⁻ phagocytic cells in the LITT ablation core.



c) Segmentation Preview. Non-tumor brain segmentation preview shows the F4/80⁺ cells in blue, Iba1⁺ cells in pink, and the F4/80⁺Iba1⁺ co-localizing region in black. **d)** Representative image quantification of the number of F4/80⁺, Iba1⁺, and F4/80⁺Iba1⁺ cells in the normal mouse brain at Day 6 post-LITT showing high microglial/macrophage activity with intense F4/80⁺ monocyte-derived macrophage distribution in the core ablation region (blue). Co-efficient of variation analysis was performed to calculate variation between technical replicates (CV < 10% = Low variation relative to mean). The images shown are representative images of n=3.



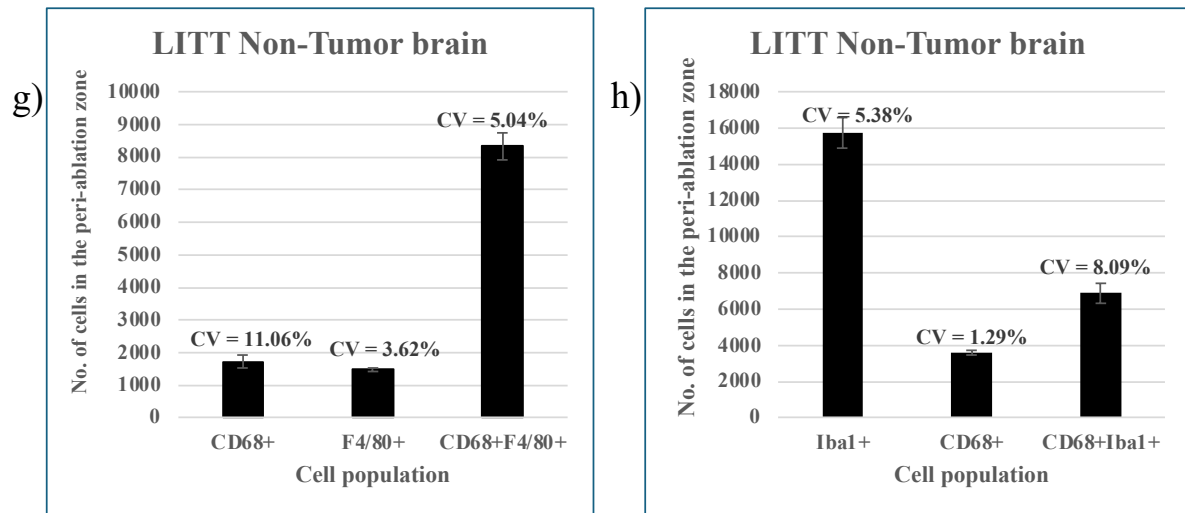


Figure 3.10: **e)** and **f)** show zoomed-in images from Iba1⁺ (green)/ CD68⁺ (red) and F4/80⁺ (red)/ CD68⁺ (green) IF using the same tissue section to identify the origin of the CD68⁺, Iba1⁻ cells. Weaker Iba1 positivity was observed closer to the core LITT damage region (pointed arrow region). **f)** The Iba1⁻ cells in the core LITT damage area were immune-positive for myeloid-derived macrophage marker F4/80 (yellow). **g)** and **h)** Representative image quantifications confirm the higher spatial distribution of CD68⁺F4/80⁺ macrophages in the core ablation region. A coefficient of variation analysis was performed to calculate variation between technical replicates (CV < 10% =Low variation relative to mean). The images shown are representative images of n=3.

3.5 NeuN, MBP, and Macrophages discriminate Different Zones of LITT-induced brain cell damage

The periphery of the necrotic core of the Day 6 post-LITT mouse brain was devoid of astrocytic activation but contained pycnotic neuronal nuclei and fragmented myelin bundles. To investigate the interaction between macrophages and damaged resident brain tissues/ cells, the neuronal marker NeuN and the myeloid marker Mbp were co-stained with macrophage markers CD68, CD11b, and F4/80 (**Figure 3.10**). At the LITT damage area, macrophages were

found in close proximity to Mbp⁺ fragmented myelin bundles and damaged neuronal nuclei with weak NeuN immunostaining and pycnotic neuronal nuclear morphology.

Different macrophage subpopulations (CD68, Iba1, CD11b, F4/80) were identified as seemingly engulfing heat-damaged fragmented myelin bundles (**Figures 3.11b, d-f**). Like with fragmented Mbp⁺ myelin, macrophages were identified as engulfing pycnotic neuronal nuclei (**Figures 3.11a, c**). CD68 exclusively stained all the phagocytic cells (cannot be used to identify the macrophage lineage). Mbp⁺ and NeuN⁺ structures co-stained with CD68 to particularly detect the macrophages engulfing the fragmented myelin bundles and damaged neurons. Different macrophage mIF panels like Mbp-CD68, NeuN-CD68, Mbp-Iba1-CD11b, and Mbp-Iba1-F4/80 were formed to understand the role of macrophage sub-populations in fragmented myelin bundle phagocytosis. Iba1⁺CD11b⁺ cells, i.e. The resident phagocytic microglia (amoeboid) have a higher phagocytic function than the F4/80⁺ peripheral macrophages (**Figures 3.11e-f**). As mentioned in section 3.4, F4/80⁺ peripheral macrophages are more infiltrative than the resident brain microglia. Thus, microglia were found in the zone of damaged resident brain cells (Mbp, and NeuN in the LITT damage zone) whereas highly phagocytic peripheral macrophages are found closer to the ablation core (the region with high ablation-induced cellular debris) (Grassivaro et al., 2020; Hsieh et al., 2013; O’Koren et al., 2016; Yamasaki et al., 2014). The images shown were representatives of n=3.

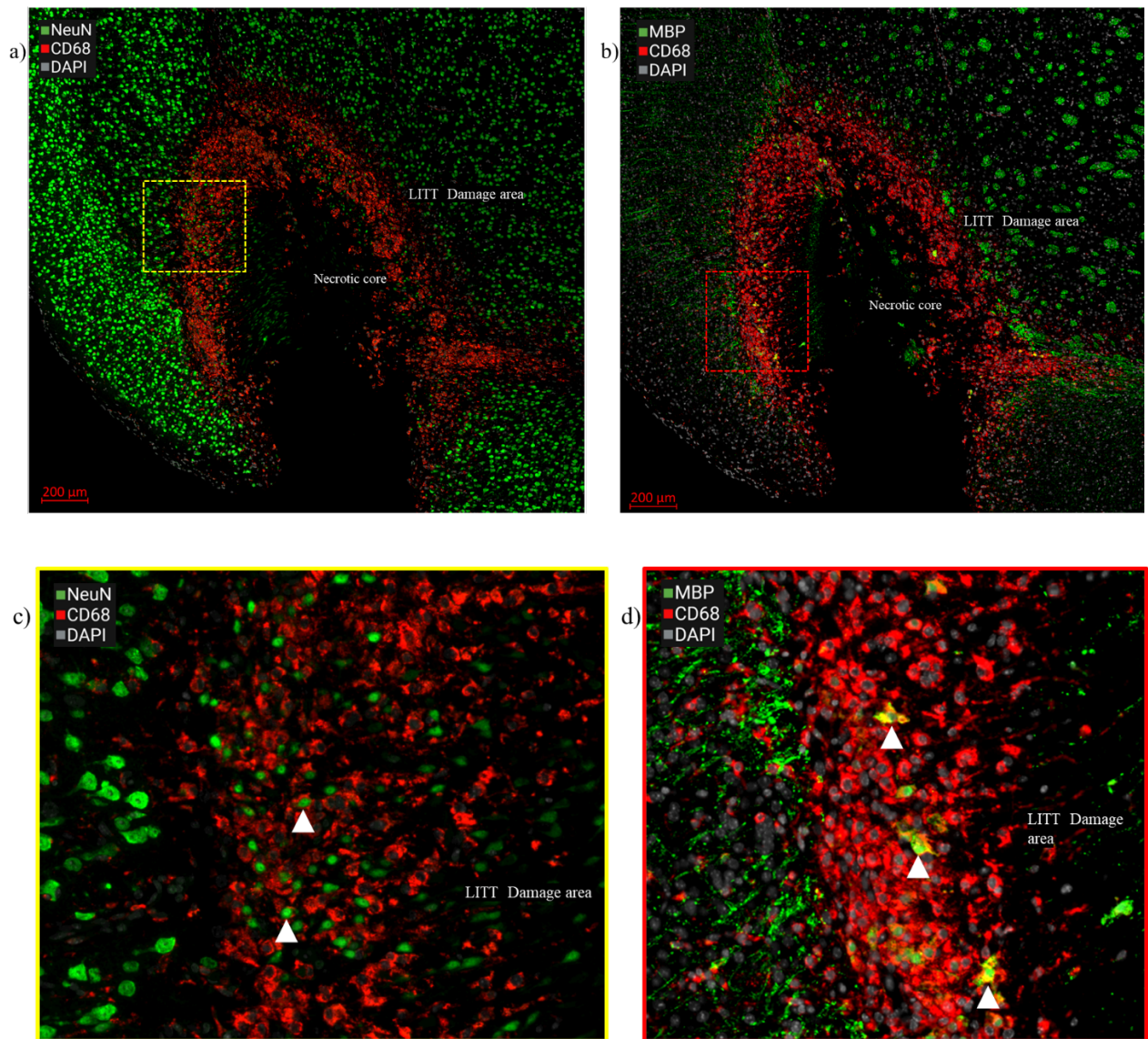


Figure 3.11 Role of macrophages in the LITT damage area: **a)** and **b)** were the overview images of NeuN (green)-CD68 (red) and MBP (green)-CD68 (red) co-stained LITT mouse brain. These images were formed by extracting the channels from a multiplex IF with all 4 markers (CD68, NeuN, Mbp, DAPI) used in the images **a)** and **b)**. Overview images showed the presence of CD68⁺ phagocytic cells in the LITT damaged area with pycnotic NeuN and fragmented myelin bundles. **c)** and **d)** are the zoomed-in images from **a)** and **b)** showing pycnotic NeuN⁺ neuronal nuclei engulfed by CD68⁺ phagocytic cells. CD68⁺ expression was identified in Mbp⁺ structures closer to the necrotic core.

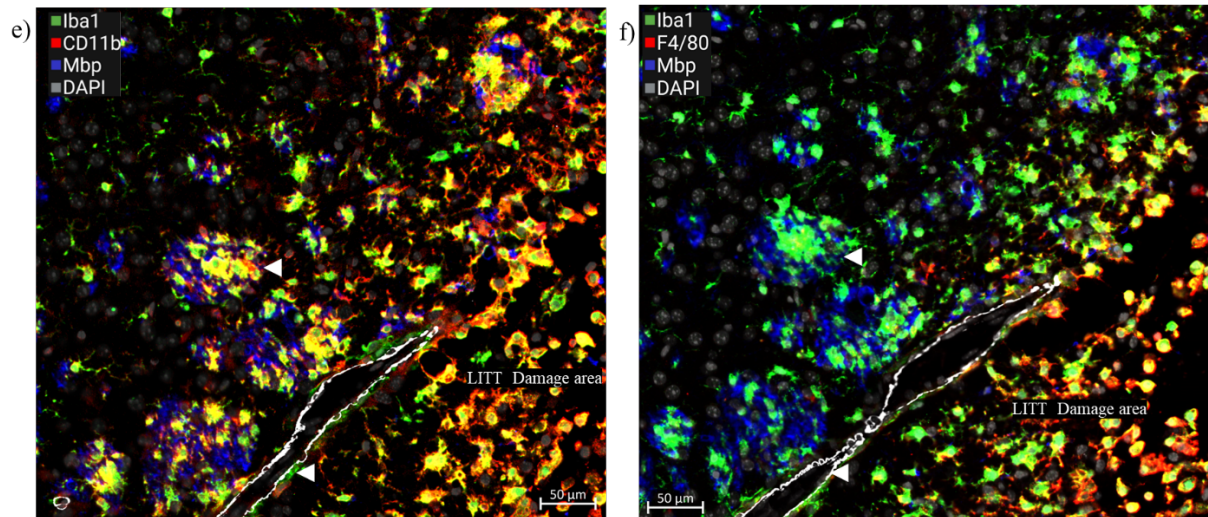


Figure 3.11: e) CD11b (red)-Iba1(green) and f) F4/80 (red)-Iba1 (green) co-staining with Mbp (blue) was used to study the distribution of phagocytic populations in the myelin bundles. Compared to F4/80⁺ macrophages, Iba1⁺/CD11b⁺ phagocytic cells had higher spatial distribution in the regions with fragmented myelin bundles.

3.6 Spatial Distribution of macrophage sub-populations (M1/M2)

There is no research on characterizing LITT-induced early changes in macrophage sub-populations. We identified innate immune cell sub-populations based on their ability to modulate inflammation, including M1-like pro-inflammatory and M2-like anti-inflammatory markers. High expression of pro-inflammatory markers was identified in Day 6 post-LITT models. Iba1, CD86, CD11b, and CD68 markers were used to detect an M1 pro-inflammatory macrophage phenotype. CD86 colocalization with CD68 and Iba1 was observed in the periphery of the LITT damage area (**Figure 3.12a**). Arg1 and CD206 are the two most common markers to identify M2-like macrophage sub-populations. We found that Arg1 antibody works in immunofluorescence than CD206. Hence, Arg1 was used to identify M2 sub-populations in the study. Arg1⁺ cells did not co-localize with Iba⁺ cells (**Figure 3.12b**). Comparing the spatial distribution of M1 and M2 macrophage subpopulations, a higher distribution of M1⁺ cells was found than macrophages positive for M2-like markers in the periphery of the LITT damage

area indicating the induction of pro-inflammation in the Day 6 post-LITT model. The combination of Iba1-CD86 and Iba1-Arg1 tissue regions showed the spatial distribution of macrophage sub-populations (M1/M2) across the ablated regions. The results shown in sections 3.4-3.6 established a strong relationship between LITT ablation and the macrophage sub-populations. The latter part of the section proved the higher distribution of pro-inflammatory environment in the 6 days post-LITT non-tumor mouse brains. The images shown were representatives of n=3.

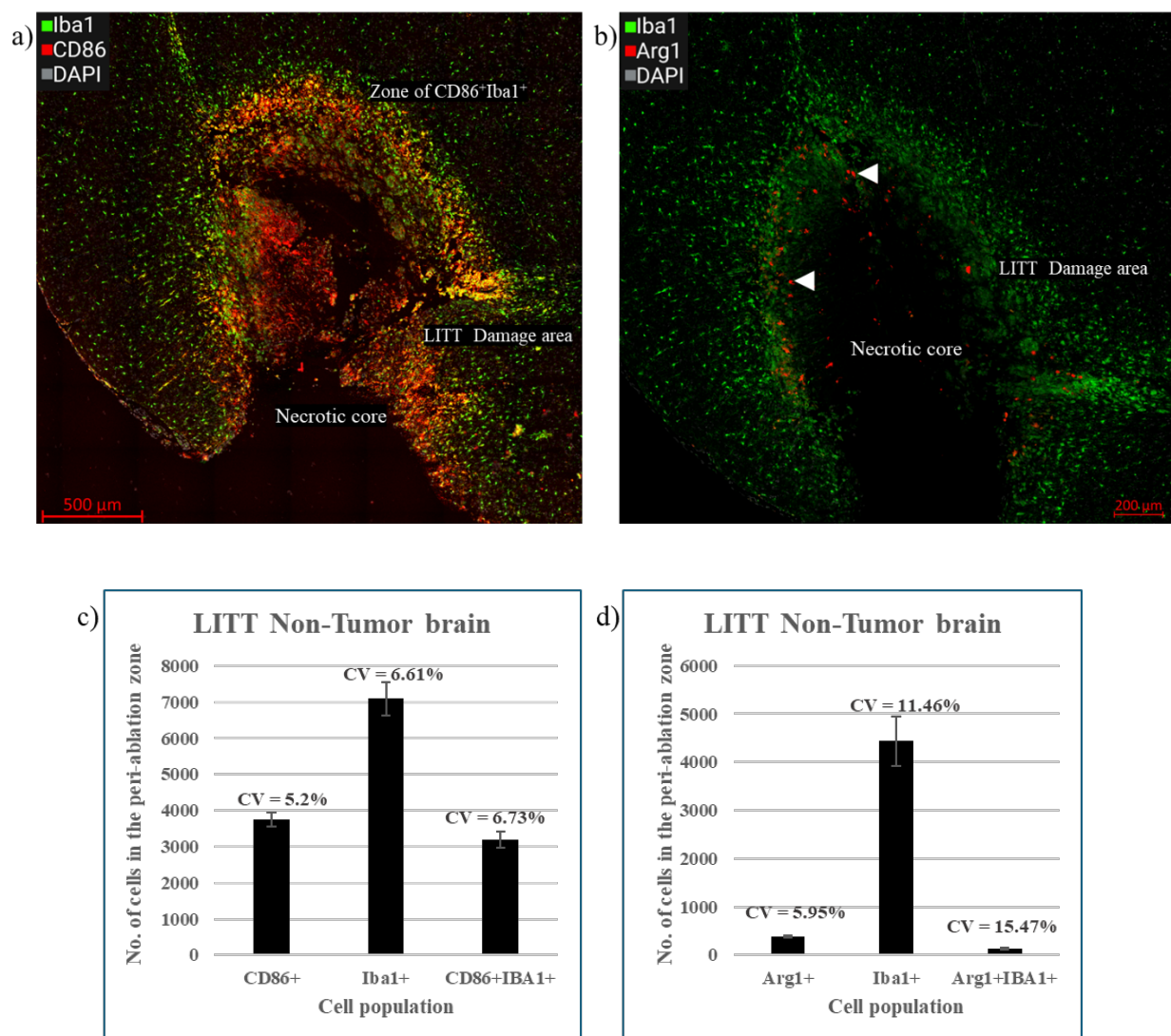


Figure 3.12 Spatial distribution of pro- and anti-inflammatory macrophages at the LITT site: Two mouse brains subjected to the same LITT parameters and collected at identical Day

6 post-LITT time points were used to study the spatial distribution of M1- and M2-like macrophage subpopulations. **a) M1 panel** with CD86 (red) and **b) M2 panel** with Arg1 (red) was co-stained with Iba1 (green) to distinguish the macrophages. Most CD86⁺ phagocytic cells were present in the LITT damaged area and colocalized (yellow) with Iba1⁺ cells, whereas significantly fewer Arg1⁺ positive phagocytic cells were identified in the LITT damaged area and these Arg1⁺ cells were distinct from Iba1⁺ microglia. **c) and d)** Representative image quantifications show higher co-expression of M1+ pro-inflammatory cells (CD86 and Iba1) than the M2-like anti-inflammatory macrophage (Arg1). Co-efficient of variation analysis was performed to calculate variation between technical replicates (CV < 10% =Low variation relative to mean). The images shown are representative images of n=3.

3.7 Pro-inflammatory Chromogranin A is Produced upon LITT

The characterization of macrophage sub-population upon LITT identified a pro-inflammatory microenvironment. We further investigated this pro-inflammatory microenvironment by employing the marker chromogranin A (CgA) for co-staining with macrophage markers. CgA is known to mediate the M1 and M2 macrophage subtypes and was detected at the necrotic core co-localized with markers for macrophages (Eissa et al., 2018; Stivanello et al., 2001). CgA co-stained with pro-inflammatory Iba1⁺ phagocytic cell populations (**Figure 3.13**). However, a few CgA⁺ and Arg1⁺ cells were also detected, suggesting that CgA may serve as a more general microglia/ macrophage marker spanning both M1- and M2-like phenotypes (**Figure 3.13c**).

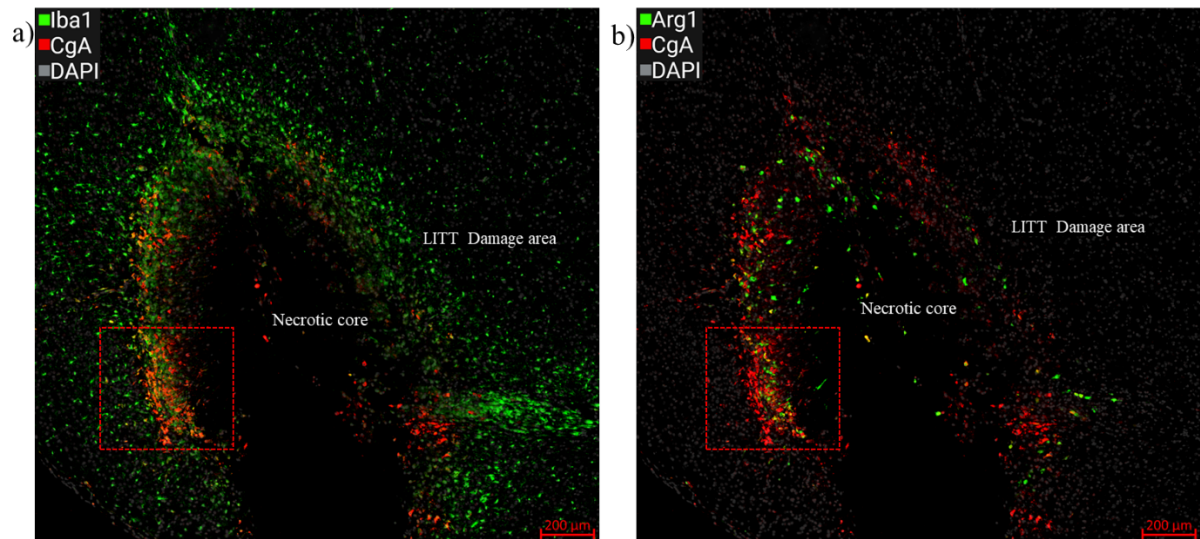
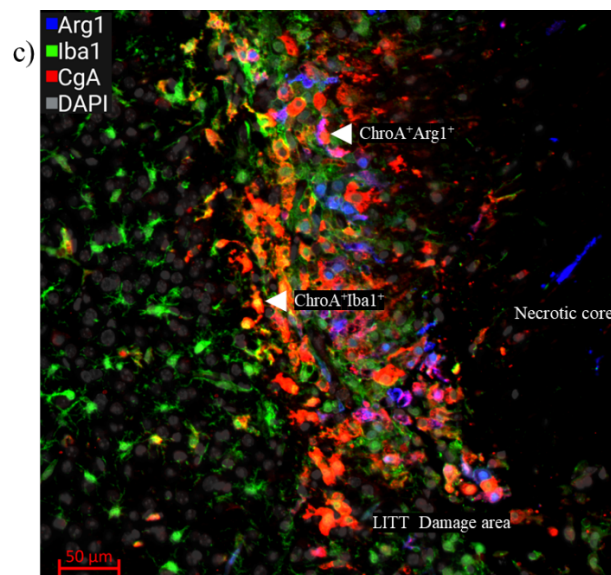


Figure 3.13: Chromogranin A co-stained with Iba1 and Arg1. Image a) shows the co-expression of Iba1 (green) with Chromogranin A (CgA; red) along the periphery of the necrotic core. b) Few CgA⁺ cells were co-localized with Arg1⁺ (green) anti-inflammation-promoting macrophages.



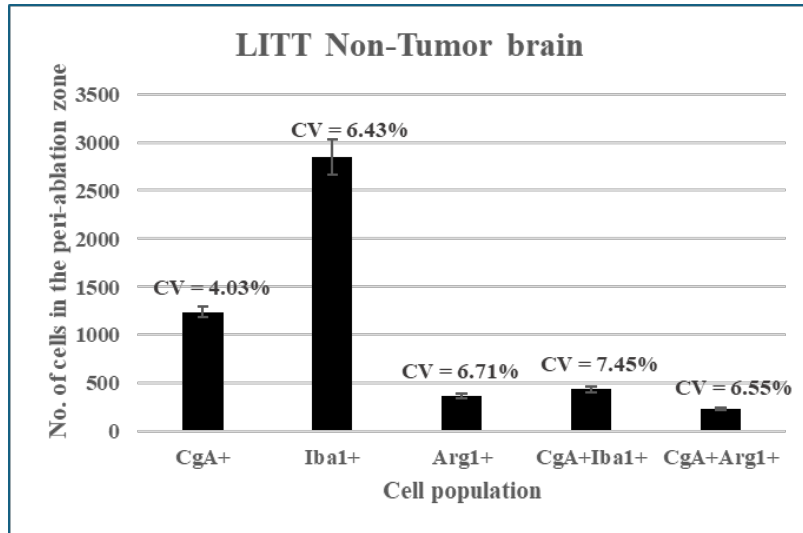


Figure 3.13: c) mIF image of the highlighted regions from **a)** and **b)** shows that Iba1⁺ (green) cells were co-localized (yellow) with CgA (red). A few Arg1⁺ (blue) cells were found to be colocalizing (pink) with CgA. Representative image quantifications show co-expression of CgA with both Iba1 (M1) and Arg1 (M2) macrophages. The role of CgA in neuroinflammation is yet to be studied. A coefficient of variation analysis was performed to calculate variation between technical replicates (CV < 10% =Low variation relative to mean). The images shown are representative images of n=3.

3.8 Inflammasome Marker NLRP3 and Pro-Inflammatory Signaling (pSTAT3) is

Induced Adjacent to LITT Damage

The presence of macrophage sub-populations with CD68⁺, Iba1⁺, CD86⁺, and potentially CgA⁺ M1-like phenotype confirmed the pro-inflammatory status at Day 6 LITT sites in mouse brain (Jensen et al., 2020; Jurga et al., 2020). We co-stained these LITT tissue sections with inflammasome marker NLRP3, (phospho-)STAT3 inflammation regulator, and resident brain macrophage markers to further characterize this pro-inflammatory brain microenvironment. NLRP3 overexpression co-localized with Vim⁺ intermediate filaments in

LITT-damaged tissue regions we had previously shown to contain plastic astrocytes (**Figure 3.14**). This physically linked VIM^+Gfap^+ astrocytes with inflammasome activation.

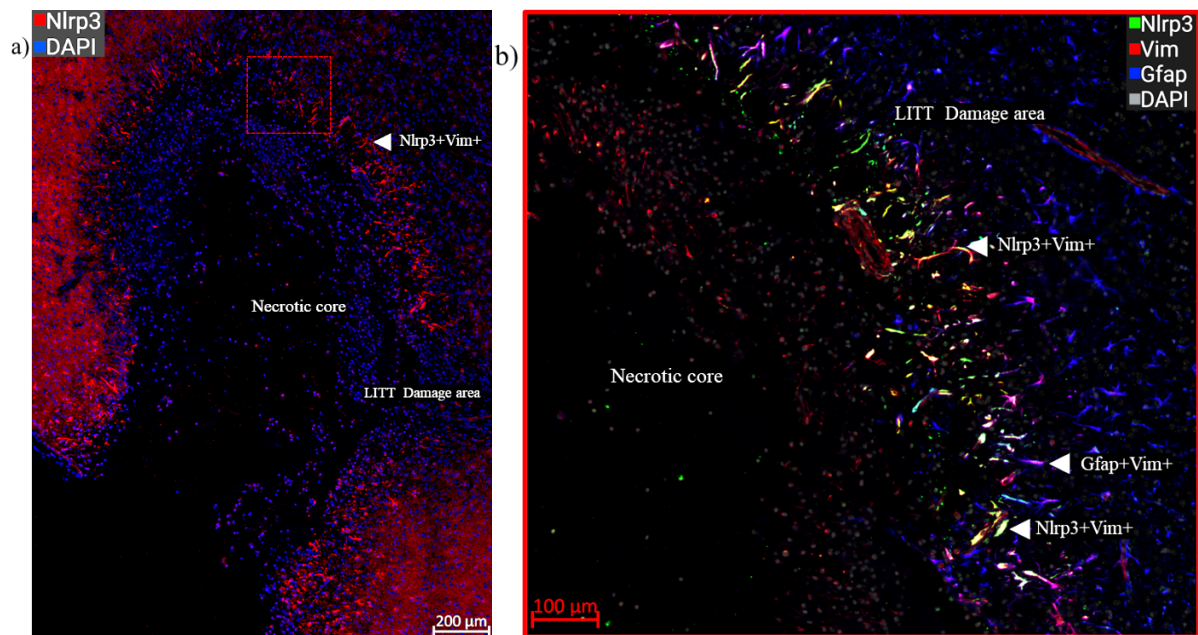


Figure 3.14 LITT induced Nlrp3 inflammasome response: a) Single IF image for Nlrp3 (red) and DAPI (blue) with $Nlrp3^+$ staining in the LITT damaged tissue area. b) mIF for Nlrp3 (green), Vim (red), Gfap (blue), and DAPI (gray). Nlrp3 and Vim co-expression (yellow) and $Gfap^+Vim^+$ (purple) were observed.

The optimization of the mIF protocol enabled the detection of phospho-proteins. This allowed us to visualize spatial cellular inflammatory responses to LITT that utilize the pro-inflammatory Stat3 signaling pathway by detecting total and phospho-Stat3 (pStat3) expressing cells at the LITT site. Stat3 and pStat3 were frequently co-localized in cells in the vicinity of LITT sites, with pStat3 immunostaining being more restricted to the periphery of the ablation core (**Figures 3.15a and b**). pStat3 partially co-stained with microglia marker Iba1 and F4/80, confirming the pro-inflammatory status of the microglia and monocyte-derived macrophages in the core LITT damage region (**Figure 3.15c and d**). pStat3 expression was also identified in the activated astrocytes in the LITT damage region. It has been identified that Stat3 plays a

critical role in modulating inflammation post-brain injury (**Figure 3.15e**)(Herrmann et al., 2008a).

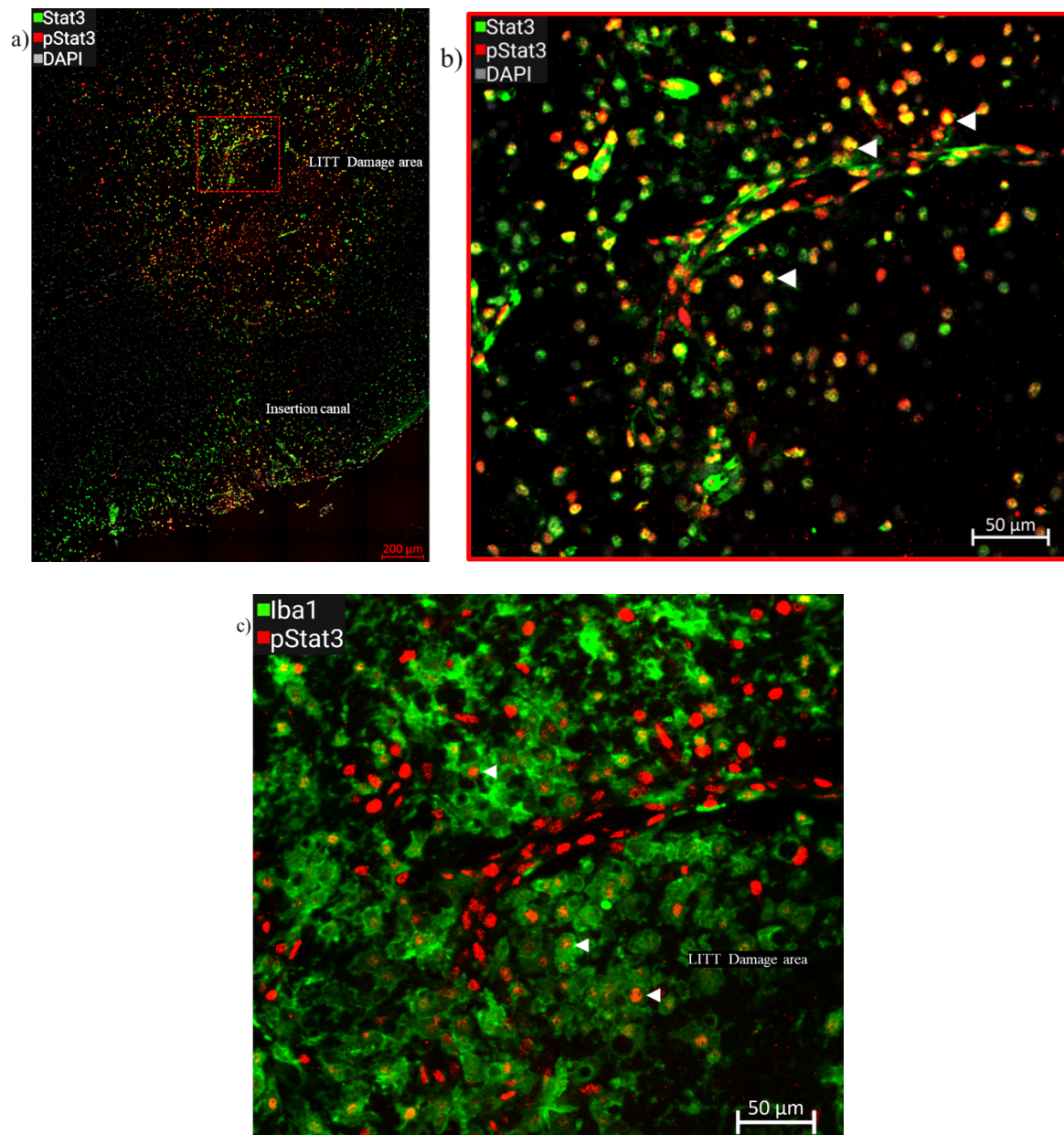


Figure 3.15 LITT induced phosphorylation of Stat3: a) Overview image - Total Stat3 (green) and phosphorylated Stat3 (red) co-presence (yellow) was detected in the LITT damaged area and closer to the insertion canal. The detection of pSTAT3 appeared to coincide with the cone-shaped laser heat emanating from the laser tip. b) Zoomed-in image of a) showing the Stat3-pStat3 co-presence. c) pStat3 (red) was identified to be upregulated in the Iba1⁺ (green)

microglial cells. DAPI stain intensity was adjusted in the histogram for better viewing of the nuclear Stat3 and pStat3 staining.

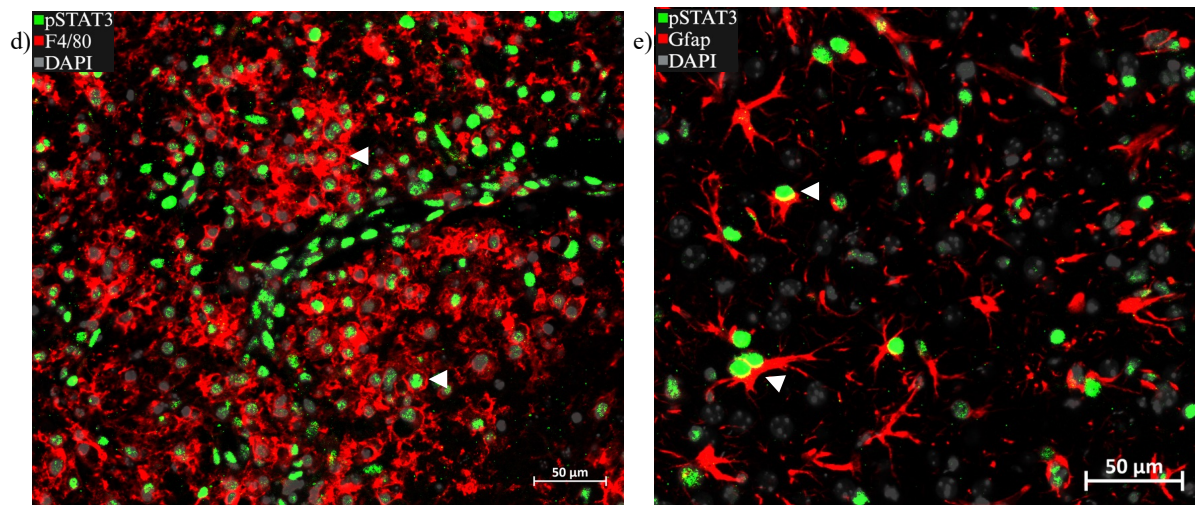


Figure 3.15: d) Similar to $Iba1^+pStat3^+$, pStat3 expression was also identified in F4/80 expressing monocyte-derived macrophages. Image e) shows the $pStat3^+$ astrocytes in the LITT damage region.

3.9 Endoplasmic Reticulum Stress is Induced Adjacent to LITT Damage

Endoplasmic Reticulum (ER) stress plays a significant role in the secondary injury cascade. Delayed cellular and molecular processes that exacerbate the initial mechanical damage, leading to further neuronal dysfunction and cell death is known as the secondary injury cascade. ER stress contributes to this cascade through its involvement in protein misfolding, inflammation, apoptosis, and other pathological mechanisms (Y. Yang et al., 2024). The heat damage induces significant stress in the surviving cells, and this can lead to improper protein folding. BiP (Binding immunoglobulin Protein), also known as GRP78 (Glucose-Regulated Protein 78) (Morris et al., 1997), and Pdi (Protein disulfide isomerase) (Parakh & Atkin, 2015; Perri et al., 2016; Xiao et al., 2018) are two important molecular chaperones involved in protein folding and act as an ER stress sensor. Antibodies to BiP and Pdi worked well in our

mIF tissue studies to study ER stress. To check the ER stress induced by the LITT, BiP, and Pdi were co-stained in Day 6 post-LITT mouse brains. High expression of BiP and PDI was identified in cells adjacent to the necrotic core (**Figure 3.16a**). Cells with high BiP expression had lower Pdi expression and vice-versa. A few Pdi⁺ and BiP⁺ co-expressing cells were also identified adjacent to the necrotic core. The results confirmed the upregulation of ER stress chaperones BiP and Pdi adjacent to the LITT ablation core.

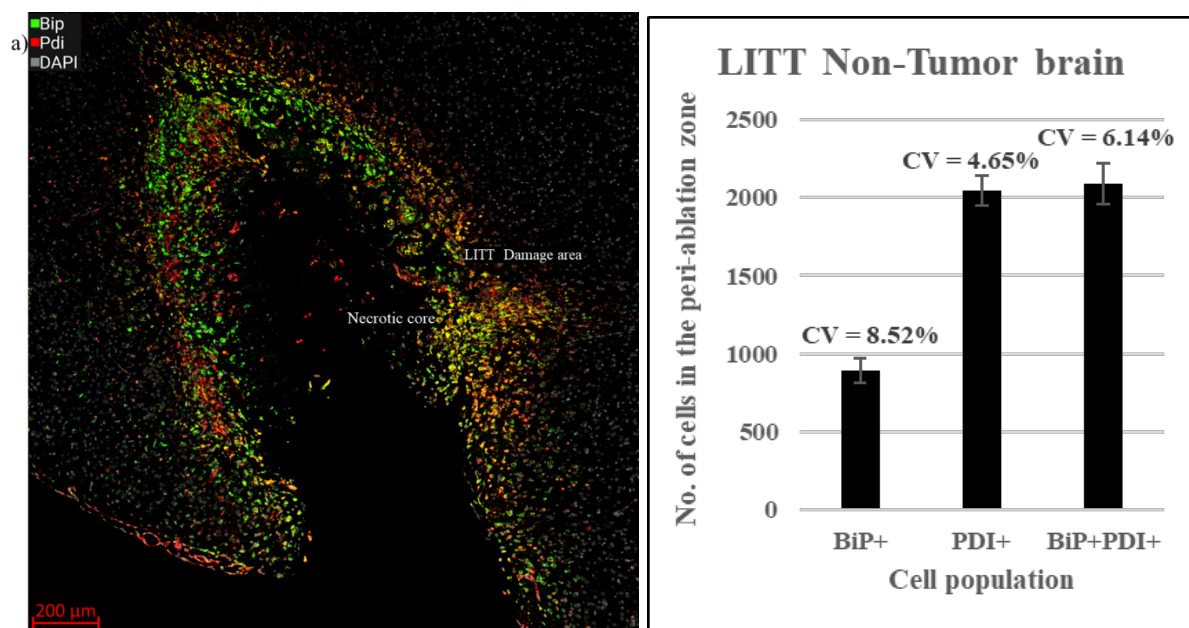


Figure 3.16a: Detection of Endoplasmic Reticulum Stress: ER stress activation markers BiP (green) and Pdi (red) co-expression (yellow) were detected in the LITT damage area and along the periphery of the necrotic core. Representative image quantifications show that the majority of the BiP⁺ cells co-express Pdi. Similar to BiP⁺Pdi⁺, high Pdi expression is due to their presence in the meningeal layer of the brain. Co-efficient of variation analysis was performed to calculate variation between technical replicates (CV < 10% =Low variation relative to mean). The images shown are representative images of n=3.

BiP and Pdi were not co-localized with resident brain cells and myelin structures, including NeuN⁺ neurons, Gfap⁺ astrocytes, or Mbp⁺ myelin bundles (data not shown).

However, BiP⁺ and PDI⁺ did co-express in cells expressing macrophage markers CD68 and F4/80 (**Figure 3.16b**). At LITT sites, these macrophage sub-populations also actively phagocytosed debris and promoted pro-inflammatory responses (**Figure 3.16c**). Hence, these macrophage populations likely experienced elevated ER stress at LITT sites as exemplified by upregulated ER stress markers BiP and Pdi.

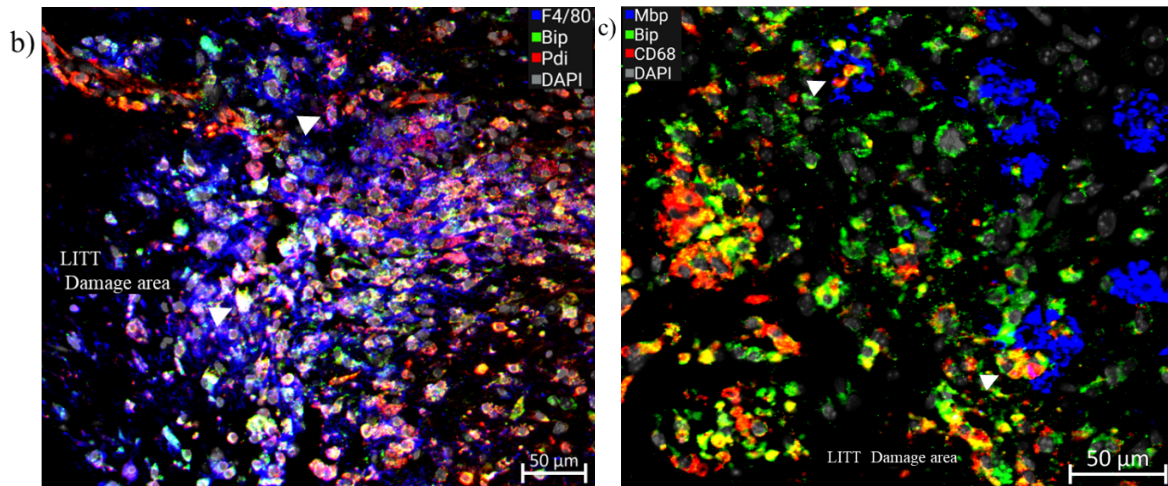


Figure 3.16b) BiP (green) and Pdi (red) were co-stained with a macrophage marker, F4/80 (blue) to identify the type of cells under stress. Co-expression of all three markers (white) confirmed the ER stress in F4/80⁺ macrophages. **c)** Co-expression of BiP (green) in CD68⁺ phagocytic cells and within fragmented myelin bundles (purple).

4. DISCUSSION

4. Discussion

Laser interstitial thermal therapy (LITT) has gained popularity as a promising neurosurgical procedure in patients (Holste & Orringer, 2020b). The applications of LITT are rapidly expanding due to LITT's minimally invasive nature, reduced surgical complication rate, and accelerated recovery time (Chiang et al., 2024). Over the past decade, numerous clinical studies have been conducted yielding successful outcomes (C. Chen, Guo, et al., 2021; Hect et al., 2024; Kamath et al., 2019; Menacho, 2024). LITT has emerged as a promising treatment option for conditions such as epilepsy, radiation necrosis, and certain types of brain tumors owing to its ability to minimize damage to the surrounding healthy brain tissue (Bastos et al., 2020; LaRiviere & Gross, 2016; Willie et al., 2014). The integration of real-time MRI guidance during surgery has further enhanced precision and significantly improved patient outcomes, fostering greater confidence and trust among patients and surgeons (De Groot et al., 2022). However, there remains a notable lack of information regarding the cellular modulations of LITT during the recovery phase. This knowledge gap is primarily attributed to the absence of an effective LITT animal model and a spatial immune-staining method. To address this limitation, we have developed a novel cost-efficient multiplex immunofluorescence protocol to investigate post-LITT cellular modulations within the mouse brain using our LITT mouse model (Spence et al., 2024).

Even though mouse models are the most commonly used research models, it is important to be cautious with the differences between a mouse brain and a human brain. The number of cell types among these species was found to be similar, but there is a significant difference in their organization and function. The human brains also have a much-advanced neocortex and high cognition related gene expressions. Yet, the mouse models remain a

valuable tool for neurological studies as the genetics can be modified to mimic human pathological conditions (Beauchamp et al., 2022; Hodge et al., 2019; Yao et al., 2023).

4.1 Successful combination of laser tissue ablation and multiplex immunofluorescence

We have established a comprehensive standard operating procedure for LITT which includes a post-LITT MRI to accurately verify the extent of LITT-induced tissue damage within brain tissue (Spence et al., 2024). Post-LITT MRI images provide crucial three-dimensional insights into the scope of thermal ablation after LITT treatment, thereby assisting in the optimization of LITT parameters. Additionally, *in-vivo* MRI imaging data help corroborate histopathology observed in sectional images collected from LITT mice brains. Hematoxylin and Eosin (H&E) stained LITT brain sections provide histological context. Hematoxylin stains the nuclei blue, whereas eosin colors the cytoplasm pink. LITT-induced heat damage gradually decreases away from the location of the laser probe, where the heat is most intense. The tissue damage pattern seen in H&E-stained and Gfap⁺ activated astrocyte IF images (**Figures 3.2 and 3.7**) showed that heat gradient is readily absorbed by the lipid-rich brain forming a trail of tissue damage (Lerner et al., 2022). The ablation core at the site of the laser fiber tip is marked by a zone of complete central tissue necrosis and eosinophilic protein coagulation with little hematoxylin blue stain composed of pyknotic and/ or nuclear fragmentation and layers of cells with varying degrees of damage adjacent to this ablation core (Lopez-Meraz et al., 2010; Petito et al., 1997; Sorian et al., 1993). Overly aggressive LITT treatment can lead to excessive brain tissue damage, irreparably laser probe damage, and may result in the loss of mice. Importantly, LITT treatment can cause fragility of brain tissue at the ablation site and the insertion canal, which can cause major challenges when collecting intact LITT mouse brains. Several pilot experiments were used to control the laser output and optimize a treatment parameter with minimal brain tissue charring. Minimal insertion canal damage and retention of the ablation

core region in FFPE sections are indicators of an optimized LITT treatment and brain tissue processing and sectioning protocol.

LITT damage gradually diminishes in the successive tissue sections, with each H&E-stained section, demonstrating slightly different laser-induced spatial tissue damage. To study the LITT-induced effects on the core ablation area (closer to the laser probe), we were able to obtain approximately 10 slides with 2 tissue sections per slide from each mouse brain. The development of a novel mIF protocol based on sequential staining enabled us to re-stain a single tissue section for several cycles and investigate the complex LITT-induced brain modulatory changes. An in-house developed cost-effective mIF protocol was employed to study the spatial interactions and co-expression patterns of multiple markers in LITT brains. Laser ablation induces coagulation of proteins which leads to false positive staining. The secondary antibodies interact with the coagulated regions leading to high background staining. Our mIF protocol uses individually IF-optimized antibodies and an autofluorescence quencher which produces reliable staining and reduced background staining. Upon LITT ablation, post-LITT imaging and brain collection, serial sectioning, and H&E staining of selected tissue sections have provided the foundation for the development of an optimized protocol for mIF. This approach significantly aided the spatial characterization of cellular and molecular changes resulting from LITT in normal and tumor mouse brain tissues.

4.2 LITT alters the composition and activation state of resident brain cells

Currently, no studies are looking at the early post-LITT resident brain cell modulations in human or animal models. Our study was the first to characterize the LITT effects on resident brain cell populations, including neurons, oligodendrocytes, myelination, astrocytes, and

microglia. In addition, we also were the first to capture phagocytic cell populations attracted to LITT sites to reveal an intriguing array of cellular responses in the mouse LITT brain.

The functionality of the brain neuronal network depends on resident oligodendrocytes forming myelin sheaths around neuronal axons and dendrites to enable expedient transmission of action potentials (J. F. Chen et al., 2022; Hughes & Appel, 2019; Stadelmann et al., 2019). The neuronal markers used in this study, nuclear NeuN and cytoplasmic neuronal marker Map2, highlighted neurons surrounded by glial cells in the mouse brain (DeGiosio et al., 2022; Matus, 1991; Mullen et al., 1992; Wolf et al., 1996). One of the most abundant proteins in the CNS is myelin basic protein (Mbp) which is a critical factor involved in myelin maintenance (Boggs, 2006). Serum levels of Mbp are clinically used as a biomarker in patients with brain injury (Wąsik et al., 2020) and a critical clinical parameter that reflects the extent of LITT-induced resident brain cell damage that translates into clinical recovery of patients. The combination of Mbp, NeuN, and Map2 provided a contextual view of the brain's structural framework. We noticed significant local damage to neurons and signs of demyelination at brain sites of laser probe insertion and the LITT core. The intense laser heat had destroyed neurons and myelin fiber tracts at the thermal ablation core, with morphologically altered myelin bundles displaying reduced immunoreactive Mbp. Similarly, a reduction in NeuN and Map2 immunostaining and pyknotic or fragmented neuronal nuclear morphology was typical at the LITT ablation core. Our optimized mIF technique was able to depict the close arrangement of Map2⁺ dendrites and Mbp⁺ myelin sheaths in healthy mouse brains (**Figure. 3.4a**). While immunostaining for Map2, NeuN, and Mbp revealed significant LITT-induced damage to those morphological structures along the trajectory of the LITT laser beam, progressive reduction in heat damage with increasing distance from the ablation core hinted at localized brain damage. We hypothesized that in regions farther from the destructive power of the laser beam, LITT can trigger a recovery process in astrocytes within the brain. Astrocytes are the most abundant glial

cells in the central nervous system and play a crucial role in maintaining blood-brain barrier integrity, providing nutrient support to neurons, and regulating extracellular ion homeostasis (Braun et al., 2017; Escartin et al., 2021; Nespoli et al., 2024; Shinozaki et al., 2017; S. Wang et al., 2020; Yuan & Wu, 2022). Alongside other resident brain cells and infiltrating immune cells, astrocytes contribute to inflammation, immune responses, and tissue repair (Lumpkins et al., 2008; Sofroniew, 2005). Pathological conditions such as glioma and traumatic brain injury induce astrocytes to undergo morphological changes and significantly upregulate Gfap expression, which is indicative of heightened astrocyte activation. Following LITT, Gfap⁺ astrocytes were abundantly observed at LITT sites, indicating astrocyte activation. Astrocytes express five intermediate filaments: Glial Fibrillary Acidic Protein (Gfap), Vimentin (Vim), Nestin (Nes), Synemin, and Lamin (Coelho-Rato et al., 2024; Potokar et al., 2020). These cytoskeletal proteins form filaments approximately 10 nm in diameter and are integral to providing structural and mechanical support, facilitating cell migration, regulating the cell cycle, and promoting cell proliferation. S100b, a calcium-binding protein, interacts with these intermediate filaments and plays a pivotal role in astrocytic cellular responses to injury and subsequent repair processes (Brozzi et al., 2009; Sorci et al.).

The neuroepithelial protein nestin is a type IV intermediate filament that is upregulated in neural progenitor cells and prominently detected during early developmental stages (Michalczyk & Ziman, 2005). Nestin plays a key role in mitotically active cells, and its re-expression is associated with enhanced plasticity and regeneration processes in the injured brain (Hendrickson et al., 2011; Kernie et al., 2001). Our mIF data revealed the co-expression of Gfap⁺ and Nes⁺ as well as Vim⁺ and S100b⁺, suggesting enhanced astrocyte plasticity and astrogliosis as part of the tissue repair process along the injection canal in non-tumor LITT brains. Vim⁺, and Nes⁺ cells, formed a first layer of cells with elevated plasticity at the necrotic core which was likely an initial recovery/ regeneration process in response to the LITT ablation

site. As heat damage progressively decreased with distance from the ablation core, the astrocyte activation marker Gfap became prominent. Co-expression of Gfap with Nes and/or Vim likely reflected a recovery process occurring in damaged mature astrocytes (Itoh et al., 2011; Sahin Kaya et al., 1999). Additionally, the co-expression of S100b with Gfap or Vim/Nes characterized the co-existence of mature and plastic astrocytic cell populations at the LITT site (Raponi et al., 2007b).

4.3 Post-LITT changes in brain phagocytic cell populations

Laser ablation generates cellular debris and damaged/ stressed cells, potentially exposing new antigenic sites that can immunomodulate resident brain cells and attract innate immune cells from outside the brain (Herzog, Garcia, et al., 2019). The latter include monocyte- and bone-marrow-derived phagocytic cells, and their function is to clear the debris field and possibly facilitate subsequent adaptive immune responses. The activation of resident brain cells as well as the infiltration of peripheral immune cells can contribute to an inflammatory brain microenvironment enriched in damage-associated molecular pattern (DAMP) signaling (Liesz et al., 2015). A primary function of microglia is the clearance of dead cells and debris following brain injury (Herzog, Pons Garcia, et al., 2019; Neumann et al., 2009). The presence of Iba1⁺ resident microglia is observed rapidly upon brain injury, and these cells may be considered the initiator of the innate immune cell response in the LITT-treated brain. CD11b and Iba1 co-staining identified the microglia population in the LITT-treated mouse brain (Michels et al., 2020). On Day 6 post-LITT, mIF images revealed highly activated Iba1⁺ microglia adjacent to the ablation core. Intense immunoreactive Iba1⁺ staining identified phagocytic microglia with a plump appearance, as opposed to microglia exhibiting

abundant and highly branched processes (Donat et al., 2017; Uff et al., 2022; G. Wang et al., 2013)

(Donat et al., 2017b) demonstrated that maximal cellular debris accumulates at the site of injury. The pan-macrophage marker CD68 is detectable on the surface of microglia as well as distinct phagocytic cell populations that have infiltrated the LITT brain. (Ngela et al., 1999). This suggests that CD68 may serve as a reliable marker of phagocytic macrophage subpopulations. We observed Iba1⁺ microglia within viable brain tissue surrounding the LITT core, whereas CD68⁺, Iba1⁻ phagocytic cells exhibited a broader distribution and were even detected within the debris field of the LITT core on Day 6 in post-LITT mouse brain. This finding established the periphery of the LITT necrotic core as the zone of active phagocytosis likely performed by several different phagocytic cell populations that included brain resident microglia and immigrated macrophages. To prove this point, mIF demonstrated a layer of Iba1⁺, CD68⁺ co-expressing cells alongside CD68⁺, Iba1⁻ cells. We consistently observed this pattern across all examined LITT mouse brains. Varying degrees of cellular mobility may influence the distribution of phagocytic cells at the LITT site. Cao et al., 2015 showed that the F4/80 marker expressing pro-inflammatory M1-like macrophages exhibit signs of enhanced phagocytic and tissue-infiltrative abilities when compared to other macrophage subtypes. Bordering the necrotic core, we identified a CD86⁺ and F4/80⁺ subpopulation of cells co-localized with fragmented myelin bundles. This evidence provides further support for CD68 as a sensitive and specific phagocytic macrophage marker.

Weakly stained regions in the H&E images (**Figure: 3.2**) containing pyknotic and fragmented cell nuclei from damaged resident brain cells, including neurons, were found to be engulfed by CD68⁺ phagocytic cells. Similar to Alam et al., 2020, we confirmed in Day 6 post-LITT brain tissue the presence of predominantly M1-like macrophages, with fewer M2-like macrophages occupying spatially distinct regions at the LITT site. Collectively, LITT-induced

brain injury initiates spatially complex repair and regenerative processes that are orchestrated by diverse resident and recruited cell types within the LITT brain. Studies at different time points would be essential to understand the changes in the post-LITT mouse M1/M2 populations.

4.4 LITT induces inflammation and ER stress

At Day 6 post-LITT, the non-tumor mouse brain had mounted a robust inflammatory response that included resident brain cells and had attracted macrophages from outside the mouse brain. A comprehensive review by Postolache et al., 2020 elucidated various brain damage-induced cellular modulations with a focus on the inflammatory response. Intriguingly, we identified co-expression of Nlrp3 and Vim within the zone of plastic astrocytes. Nlrp3 is a critical component of the innate immune system activated by DAMP signaling, involved in caspase-1 activation and pyroptosis initiation. (Dos Santos et al., 2015) Have demonstrated that Vim directly interacts with Nlrp3 and supports inflammasome assembly, corroborating our identification of Vim expression. From our results from astrocytic characterizations, we know that the zone of astrocytes is away from the periphery of the ablation core. Therefore, we tested several inflammatory markers to understand the LITT-induced inflammatory effects of cells in the periphery of the ablation core and confirm the pro-inflammatory status of the mouse brain 6 days post-LITT.

We examined the pro-inflammatory LITT environment using Stat3 and its phosphorylated form pStat3 (Zheng et al., 2022) to optimize our mIF protocol to immuno-stain the phosphorylated form of the transcription factor expressed in the cell nucleus. Stat3 plays an important role in regulating the response of astrocytes and macrophages to injury (Herrmann et al., 2008b; Lang, 2005; Oliva et al., 2012; Xia et al., 2023). Zheng et al., 2022 have shown

that Stat phosphorylation in microglia and astrocytes plays a significant role in promoting post-injury inflammation. Their upregulation is known to produce initial neuroprotective effects and neurodegenerative effects in prolonged-expression conditions (Reichenbach et al., 2019; Zhou et al., 2020). Hence, it is an important finding to understand the spatial distribution of Stat3. Stat3 was identified in the microglia/macrophages and activated astrocytes. The temporal expression pattern of the pStat3 can be studied using this mIF protocol optimized for phospho targets to investigate how Stat3 helps the LITT brain recover over time.

We are the first to identify the presence of chromogranin A (CgA) at LITT ablation sites. Previous studies by (Urushitani et al., 2006; Zhang et al., 2023) have described CgA as a crucial mediator of inflammation in neurological disorders. This is the first study to immunostain the co-expression of CgA and amoeboid-shaped Iba1⁺ cells in the brain. One of the early studies on CgA (Kingham et al., 1999; Taupenot et al., 1996) showed the role of CgA in the induction of the activated phenotype of microglia. Evidence also showed the potential contribution of CgA in Neuronal Death (Ciesielski-Treska et al., 2001) These studies tie the CgA to LITT-induced cellular modulations as one of the potential mediators of inflammation post-treatment. The preliminary evidence on the CgA expression at the periphery of the ablation opened a new perspective on understanding LITT-induced neuroinflammation.

Previous studies have shown that ER stress and inflammatory responses are interconnected (S. S. Cao et al., 2016). Interestingly, ER stress is induced by inflammatory factors and initiates Unfolded Protein Response (UPR) (Hasnain et al., 2012; Sukhorukov et al., 2020). We investigated the macrophages in the laser damage region to understand their endoplasmic reticulum (ER) stress levels. We used BiP/GRP78, ER chaperone protein, to study the activation of ER stress induction (Dorner et al., 1989; Kozutsumi et al., 1988; Lee, 1992). Morris et al. (1997) examined the role of BiP in maintaining ER stress in macrophages and proved that the overexpression of BiP alleviates the ER stress. Similarly, Xiao et al., 2018)

have explored the role of Protein disulfide isomerase (Pdi) in macrophages, identifying its role in preventing UPR and inducing pro-inflammatory effects. We identified BiP and Pdi co-expression in the macrophages around the ablation core. ER stress activation markers were not expressed in the surrounding healthy resident brain cells. This important finding revealed that macrophages experience greater ER stress than resident brain cells, likely due to their functional requirement for synthesizing more proteins. BiP and Pdi collectively support macrophage function related to protein folding and maintaining ER homeostasis.

4.5 Limitations

A significant limitation in classifying macrophage subpopulations was the fact that the majority of macrophage-targeting antibodies are derived from rabbit as the host species. Using our mIF protocol and not directly labeled antibodies, necessitates performing over 7 cycles of immunofluorescence to stain the same section with the majority of available macrophage markers. Given that most macrophage subtypes exhibited robust expression in the LITT-damaged area, concerns arise regarding potential false-positive signals due to incomplete antibody stripping or diminished antigen-antibody interactions. For future experiments, it would be ideal to identify potential macrophage markers from diverse host species to reduce the required number of staining cycles and characterize the activity of macrophage subpopulations within a single section. This approach may establish spatial tissue maps of different macrophage subpopulations at varying distances from the laser probe.

While we were interested in further studying inflammatory and ER stress signaling pathways, we faced limitations in the availability of immunostaining-compatible antibodies. Despite testing several members of these signaling pathways, we were unable to optimize the antibodies for immunofluorescence. Multiplex staining is currently one of the fastest-growing

assay techniques, owing to its wide range of applications. Leading antibody manufacturers, like Cell Signalling Technologies, Abcam, and Thermo Fisher, are actively developing immunostaining competent antibodies to stay competitive in the mIF field.

The biology of the LITT mouse brains equally contributes to the challenges we face in optimizing our mIF. The LITT damage zone, or the region followed by the ablation core, is the region with several cellular modulations taking place. It becomes impossible to study the LITT-induced resident brain cell damage, macrophage infiltrations, and their inflammatory responses at the same time using our mIF. We had to split them into different panels as they are expressed in the same brain regions (approx.). The intense macrophage population at the periphery of the ablation core forms clumps of cells. This makes it difficult to segment the cells using Zeiss Zen Intellesis as it segments based on the cell membrane. Our recent LITT protocol optimization has shown minimal laser probe insertion damage, which could significantly reduce the number of brain tissue damages and clumping of cell populations in the insertional canal border.

5. CONCLUSION & OUTLOOK

5. Conclusion and outlook

We have established an affordable in-house multiplex immunofluorescence protocol combined with our LITT animal model for the spatial visualization of early cellular and molecular changes in normal and tumor mouse brains exposed to LITT.

One of the key findings of our study is the alteration in the composition and activation patterns of the resident brain cells as a post-LITT effect. We studied the changes at the periphery of the necrotic core and across the LITT damage area. Our study on neurons, myelination, and astrocytes revealed that LITT damage is highly focused, with a gradual reduction in the heat damage away from the ablation core in all directions. The presence of Gfap, Vim, Nes, and S100b expressing astrocytes along the LITT damage gradient indicated the different states of cellular plasticity and recovery. These findings shed light on the brain's ability to initiate regenerative responses within 6 days of laser ablation.

Our study revealed the spatial distribution activation, expression pattern, and activation state of different phagocytic cell populations during inflammatory responses in the post-LITT brain. Multiplex immunofluorescence illustrated complex LITT-induced early repair and recovery mechanisms orchestrated by a diverse set of cells comprised of both resident brain cells and phagocytic cells recruited to the inflamed LITT brain environment. We have yet to fully elucidate the individual mechanisms of inflammatory and ER stress signaling pathways. This limitation stems from the scarcity of suitable antibodies for LITT brain immunostaining. Ongoing efforts focus on identifying appropriate antibodies targeting specific signaling pathway components.

5.1 Future Directions:

We optimized LITT conditions and generated preliminary results on Day 6 post-LITT non-tumor mouse brains. Future experiments on LITT glioma models will include tumor- and TME-specific immunomodulatory drug targeting and monitor LITT-induced spatiotemporal changes in glioma brains.

DAMPs activate Pattern Recognition Receptors (PRRs) on resident brain and immune cells to initiate pro-inflammatory cytokine signaling cascades (Klegeris, 2021). Our future direction includes comprehensive cytokine profiling in whole mouse brains at different time points post-LITT.

The sham control models were not characterized in this thesis work. Future experiments would consider including sham control models at different time points to identify the changes in the cell populations modulated by the LITT but not the laser probe insertion induced damage. The future experiments would be accompanied with significant advancements in the statistical analysis by including normalization algorithms to deduct the probe-induced cellular modulations and derive standard deviation comparing 3 or 4 mouse brains for each group.

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