4
Investigation of Cellular and Molecular Pathways of Innate Immunity in the Context of Glioma Pathophysiology

4.1 Abstract

Glioblastoma multiforme are considered the most severe gliomas are accounting for 50-60% of the primary brain tumors [Stupp *et al.*, 2005]. They are highly heterogeneous both at the cellular and genetic levels. The tumor microenvironment plays a pivotal role in the progression and heterogeneity of the gliomas. Glioma associated inflammation plays an integral part in the recruitment of infiltrating macrophages, angiogenesis, and metastasis. Microglia and tumor-associated macrophages are one of the major populations in the tumor cellular composition. These microglia and macrophages are the mediators of inflammation in and around the tumor [Perry *et al.*, 2010]. NLRs are the mediators of innate immunity and respond by recognizing several conserved pathogens, irritants, and damage-associated molecular patterns [Davis *et al.*, 2011]. NLRs activation has been seen in various cell types such as microglia and astrocytes during multiple sclerosis, Alzheimer's, and traumatic brain injury [Gharagozloo *et al.*, 2018]. Role of NLRs have also been defined in various cancers, yet their role in glioma remains largely unexplored [Davis *et al.*, 2011; Janowski *et al*, 2013]. Our main objective was to investigate the NLRs activated during glioma pathology. We confirmed the expression of NLRP3, NLRC4, NLRP12, AIM2, and ASC in grade IV glioblastoma tissue sections by immunofluorescence. An overall increase in the adaptor protein ASC and NLRP3 was also seen in the glioma tissues. A multiplex immunoassay was utilized to identify glioma-associated inflammatory, angiogenesis, and proliferative factors.

4.2 Introduction

World Health Organization (WHO) has classified glioma into four categories depending upon the level of malignancy (Grade I to Grade IV). Grade IV glioma or glioblastoma multiforme (GBM) are the most aggressive primary brain tumor with a median survival of 15 months from the time of diagnosis [Stupp *et al.*, 2005]. The standard treatment strategy mainly consists of surgical resection followed by radio- and chemotherapy. GBM is characterized by high infiltrative potential and hallmarked histologically by the presence of pseudopalisading necrosis, microvascular proliferation, perinuclear halos, and pleomorphic giant cells. The TCGA data is an assembly of genomic, epigenetic, and proteomic data for different cancers and their corresponding matched normal samples. These data are collected from different platforms and evaluated on a whole-tumor tissue level. Depending upon the current multimodal genetic analysis of TCGA glioma data, four subtypes of GBM has been identified, i.e., mesenchymal, classical, proneural and neural [Verhaak *et al.*, 2010](Figure 4.1). TCGA provides collective information regarding the variability of various transcription factors, metabolic and immune proteins. This information can be utilized to advance the understanding of glioma and its microenvironment

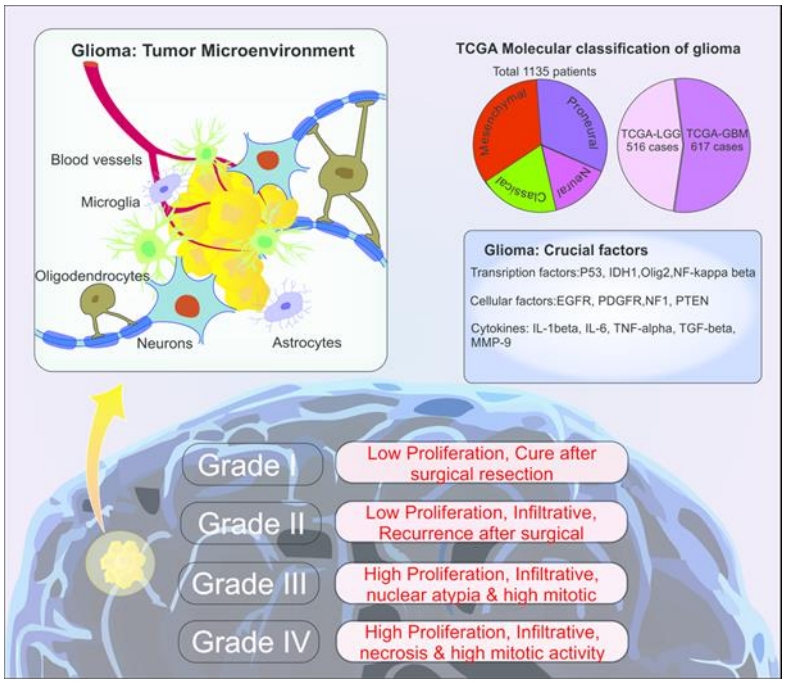


Figure4.1 Glioma classification and brain tumor microenvironment

The tumor microenvironment consists of the genetically altered cancer cells and contributing stromal cells, which are activated or recruited by cytokines and chemokines released by cancer cells. Glioblastoma associated microenvironment consists of glial cells, i.e., microglia, astrocytes, oligodendrocytes, immune cells along with endothelial cell (which form the blood vasculature). This tumor microenvironment not only participates in glioma growth but also leads to migration of tumor cells and invasion. Microglia are the resident immune cells of the brain, characterized by considerable plasticity and diversity [Hambardzumyan *et al.*, 2016]. Microglia are recruited in and

around the tumor by the factors secreted by the glioma cells such as monocyte chemoattractant protein (MCP-1). In the tumor vicinity, they get activated and acquire amoeboid shape.

Innate immunity in the glioblastoma microenvironment plays a central role in the disease pathogenesis. Inflammation intensely regulates the severity of glioma at different stages. Innate immunity relies on pattern recognition receptor (PRRs), which recognizes molecular patterns on the pathogens, foreign molecules, and irritants. NLRs are the cytoplasmic members of PRRs family have been shown to be involved in the various brain-related diseases. Activation of NLRC4 and AIM2 inflammasome has been identified in the different brain pathologies such as ischemic stroke, CNS associated bacterial infection and traumatic brain injury [Ge *et al.*, 2018; Hanamsagar *et al.*, 2014; Poh *et al.*, 2019]. Enhanced expression of NLRs and AIM2 was observed in the controlled cortical impact (CCI) mice model. The increase in NLRs and AIM2 expression contributed to apoptotic and pyroptotic cell death of brain microvascular endothelial cells (BMVECs) mediated by caspase-3 and caspase-1 respectively.

Until recently the role of NLRs in glioma pathogenesis remained largely unknown [Singh and Jha, 2018]. Recently, the elevated levels of *IL-33, ASC, NLRP1, AIM2, IL-18, CASP1,* and *NLRC4* transcripts were observed in human GBM samples as compared to the epilepsy control patients[Chen *et al.*, 2019]. They also confirmed that AIM2 immunofluorescence markedly increased in the core of the GBM tumor sections as compared to the corresponding unaffected peripheral tissue. In another study, Lim et al. stated the upregulation of *NLRC4* and *NLRP3* genes in glioma patients by TCGA data analysis[Lim *et al.*, 2019]. In an attempt to understand the role of NLRs in glioma pathology our lab has recently published an article in which we analyzed TCGA data and confirmed the differential expression and methylation of NLRs in glioma[Sharma *et al.*, 2019]. We also identified NLRP12 as a prognostic marker for glioma progression. We further extended the study by analyzing the expression of NLRs and AIM2 in the grade IV glioblastoma tissue sections via immunofluorescence. We also observed the expression of adaptor protein ASC and NLRP3 expression in human grade IV glioma tissue. Multiple cytokines, chemokines, growth factors, and angiogenesis factors were quantified via multiplex microarray to quantify their protein levels in glioma pathogenesis.

4.3 Materials and Methods

4.3.1 Human Glioma Tissue Samples

A total of 30 fresh human tumor samples and blood samples were obtained from patients who underwent surgery at Tata memorial hospital, Mumbai, India. Written informed consent was provided by the patients who underwent surgery. The study was conducted in accordance with recognized ethical guidelines and approved by the internal review board of Tata memorial cancer hospitals. Fresh tumor samples were frozen immediately in liquid nitrogen after removal and stored at −80°C. Representative tumor tissue specimens were evaluated by neuropathologists at the hospital according to the WHO grading guidelines.

4.3.2 Immunohistochemistry

Immunohistochemical staining was performed for the paraffin-embedded glioma and normal brain tissue sections. 5µm tissue sections were sectioned on Superfrost® Plus microscope slides (Fisher Scientific). Tissue sections were deparaffinized in xylene

and rehydrated by immersing them in graded alcohol 100%, 95%, 70% and 1X Phosphate buffer saline (PBS) respectively. Permeabilization of the tissue sections was performed for 20 minutes with 0.1% Triton-X PBS and blocked in 5 % fetal bovine serum (FBS) in 0.1%TritonX-100 in PBS for 1 hour at RT. To detect neurofilaments in the tissue sections were incubated with anti-neurofilament antibody (Sigma-Aldrich, N-4142, 1:300). To detect microglia/macrophages, the sections were incubated for 1 hour in Fluorescein labeled Ricinus Communis Agglutinin I (RCA I, Vector laboratories, FL-1081, 1:500). Thereafter the sections were incubated in primary antibodies of ASC (Cell-Signaling Technology,13833S,1:300), NLRP3 (Novus Biologicals,NBP-12446,1:300), AIM2 (Sigma-Aldrich, SAB-4503648,1:300), NLRP12 (GeneTex, GTX31418,1:300) , NLRC4 (Sigma-Aldrich,06-1125,1:300), Caspase-3 (Cell Signaling Technology,9664T,1:300) respectively Overnight at 4°C. After washing the sections with 1X PBS, the primary antibody was detected by incubation with goat anti-rabbit Alexa Fluor 594-conjugated antibody (A11012; Life Technologies, 1:1000) for 1 hour at RT. Finally, the sections were mounted with DAPI (Sigma, F6057). Fluorescence images were taken using a Leica Fluorescence Microscope (DM6000).

4.3.3 Histochemical staining (H&E staining)

The paraffin-embedded glioma and normal brain tissue sections were rehydrated by decreasing graded alcohol incubation. The sections were dipped in hematoxylin for 1 minute and washed with running water till the desired blue color appeared. This was followed by 1% eosin Y solution for 1 minute. Dehydration was performed by two changes of 95% alcohol and two changes of 100% alcohol for 30 second each. Finally, the alcohol was extracted with two changes of xylene. The sections were mounted with mounting medium and covered with a glass coverslip.

4.3.4 Bradford assay for protein estimation

For protein extraction from cultured cells, 0.25×10^6 cells were lysed in 0.25 ml RIPA buffer with freshly added protease inhibitors followed by centrifugation at 15000rpm for 20 minutes. The supernatants are used for further analyses. For protein extraction from frozen tissues, RIPA buffer was added (500µl/100mg of tissue) along with freshly added protease inhibitor and homogenizing it with handheld homogenizer. The supernatant was collected by centrifuging the tissue homogenate at 15000rpm for 20 minutes at 4°C. Protein concentrations are determined using a coomassie (Bradford) protein assay kit and Nanodrop Spectrophotometer by taking absorbance measured at 595nm.

4.3.5 Western Blotting

An equal amount of protein/lane was loaded and separated using 12% SDS-PAGE, then transferred to nitrocellulose membranes, and blocked 5% skimmed milk in TBST for 1 hour at room temperature. Subsequently, the membranes were incubated overnight at 4°C with primary antibodies of ASC (Cell-Signaling Technology,13833S,1:6000), NLRP3 (Novus Biologicals,NBP-12446,1:1500), AIM2 (Sigma-Aldrich, SAB-4503648,1:2500), NLRP12 (GeneTex, GTX31418,1:3000) and β-actin (Santa-Cruz Biotechnology, sc-47778,1:5000). β-actin protein was used as a loading control. Following primary incubation, the membranes were incubated with the anti-rabbit (Cell-Signaling Technology, 7074P2, 1:5,000) or anti-mouse (Cell-Signaling Technology, 7074P2, 1:10,000) IgG/horseradish peroxidase-linked secondary antibodies for 1 h at room temperature. To visualize protein bands, Luminata™ Crescendo western HRP substrate (Millipore, WBLUR0500) was used. The intensity of protein bands was analyzed using Alpha view SA software and normalized by β-actin.

4.3.6 Cytokine Microarray

The Bio-Plex pro-human cytokine 17-Plex immunoassay was utilized to detect the levels of IL-1β, IL-18, VEGF, PDGF-BB, IL-6, IL-2, IL-12, RANTES, IL-17, IL-1Ra, TNF-α, MCP-1, IFN-γ,IL-4, IL-8, GM-CSF, and G-CSF. Briefly, 50µl of the coupled beads were added in each well of the assay plate. The plate was washed two times with 100µl of Bio-Plex wash buffer per well. 50µl of the diluted standards, samples, and blank was added in duplicates and incubated at 850 rpm for 30 minutes at room temperature. The plate was washed three times, and 25µl of diluted 1X detection antibody was added. After 30 minutes of incubation, the plate was washed three times. 50µl of the streptavidin-PE was added and incubated for 10 minutes at room temperature. 125µl of the assay buffer was added in each well before reading. Biorad Bio-Plex 200 system was utilized for the immunoassay reading.

4.4 Results and discussion

4.4.1 Histopathological features of grade IV glioblastoma

The current standard evaluation and development of the glioblastoma are based on the histopathological analysis of glioma tissue specimens. Glioma tumors have been classified into grade I to grade IV according to the World Health Organization (WHO). Grade I glioma mainly occur in children and have a better prognosis as compared to corresponding higher grades[Gladson *et al.*, 2010; Wen and Kesari, 2008]. Grade II to grade III are considered low-grade glioma, and they are characterized by increased cellularity, nuclear atypia, and mitotic activity. The grade IV or glioblastoma being the most severe, have enhanced microvascular proliferation and necrosis. Glioblastoma tissue samples obtained from Tata Memorial Centre, Advanced center for treatment, research, and education in cancer (ACTREC) were classified based on the histopathological features. Cerebrum and corpus callosum sections of the normal brain obtained from All India Institute of medical science (AIIMS), Jodhpur were used as the controls (Figure 4.2). These histopathological classifications confirm that tissue sections obtained have grade IV glioblastoma features.

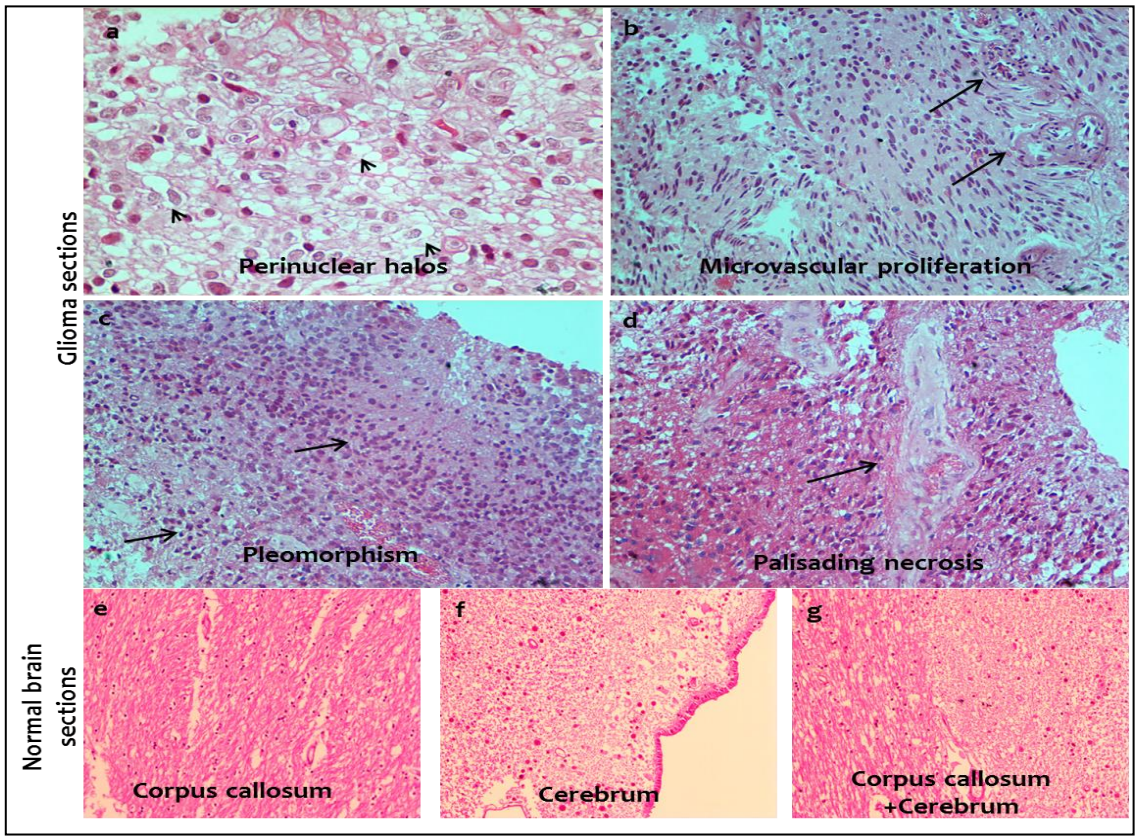
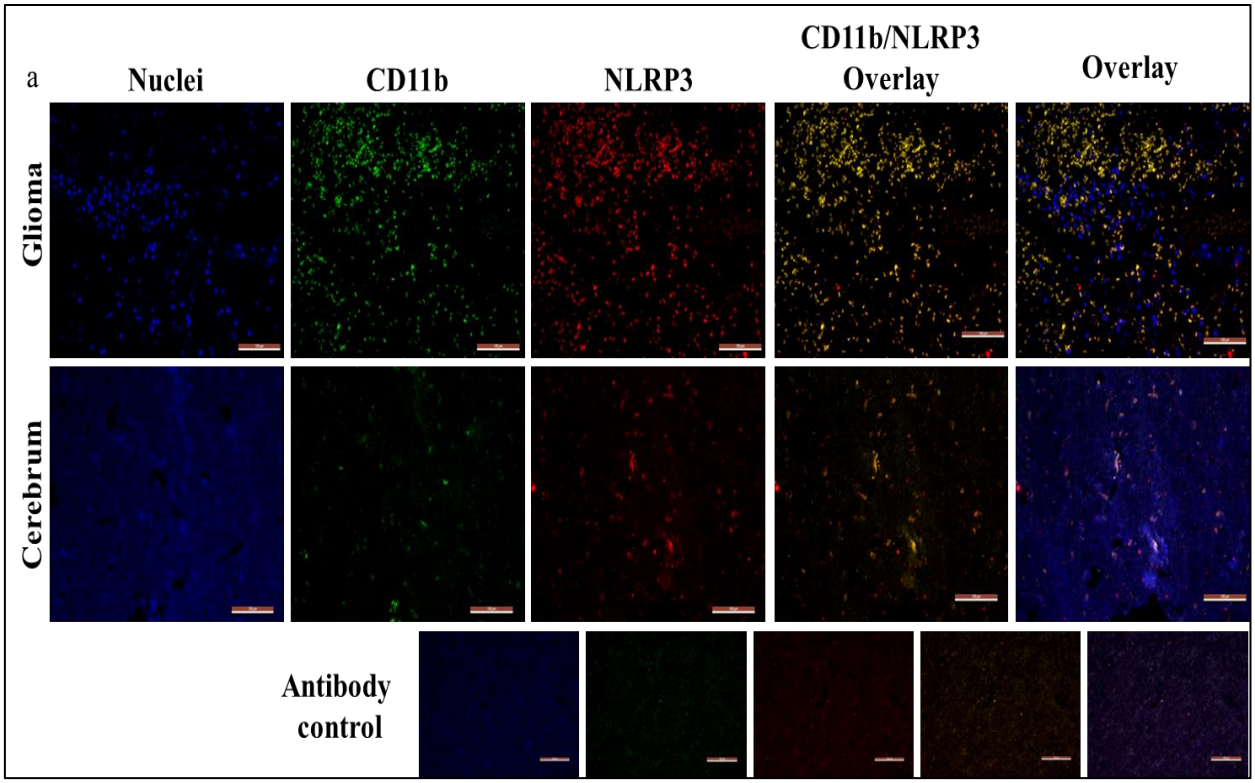


Figure 4.2: Histopathological hallmarks of grade IV glioblastoma tissue sections: Perinuclear halos, microvascular proliferation, pleomorphism, and palisading necrosis (panel a-d) are considered for the diagnosis and evaluation of glioblastoma. The normal brain was taken as the controls (panel e-g).

4.4.2 NLRP3 protein expression in normal brain and glioma tissue

The pattern recognition receptor NLRP3 is actively involved in the processing of IL-1β and IL-18 cytokines during various CNS inflammations such as multiple sclerosis and Alzheimer's diseases [Halle *et al.*, 2008; Jha *et al.*, 2010]. NLRP3 expression and function are well defined in the case of CNS associated inflammation, but its role in glioma pathogenesis is yet to be identified. NLRP3 inflammasome constitutive activation has been identified in primary glioblastoma cells as well as in U-87 glioblastoma cell line [Tarassishin *et al.*, 2014]. NLRP3 activation has also been shown to provide resistance to radiotherapy, and NLRP3 activation can act as a predictive biomarker for glioma progression [Li and Liu, 2014]. TCGA data analysis of low-grade glioma (grade II and grade III) and high-grade glioma (grade IV) have shown 5-10% altered *NLRP3* gene expression, respectively [Sharma *et al.*, 2019]. Along with concerted genomic and proteomics analysis of innate immune proteins via TCGA, we also analyzed the levels of NLRP3 at the cellular levels in the tumor microenvironment. NLRP3 protein levels were analyzed in astrocytes and microglial cells (Figure 4.3) in glioma tissue sections via immunofluorescence. Microglial cells and astrocytes both have an increased protein level as compared to the normal brain sections.



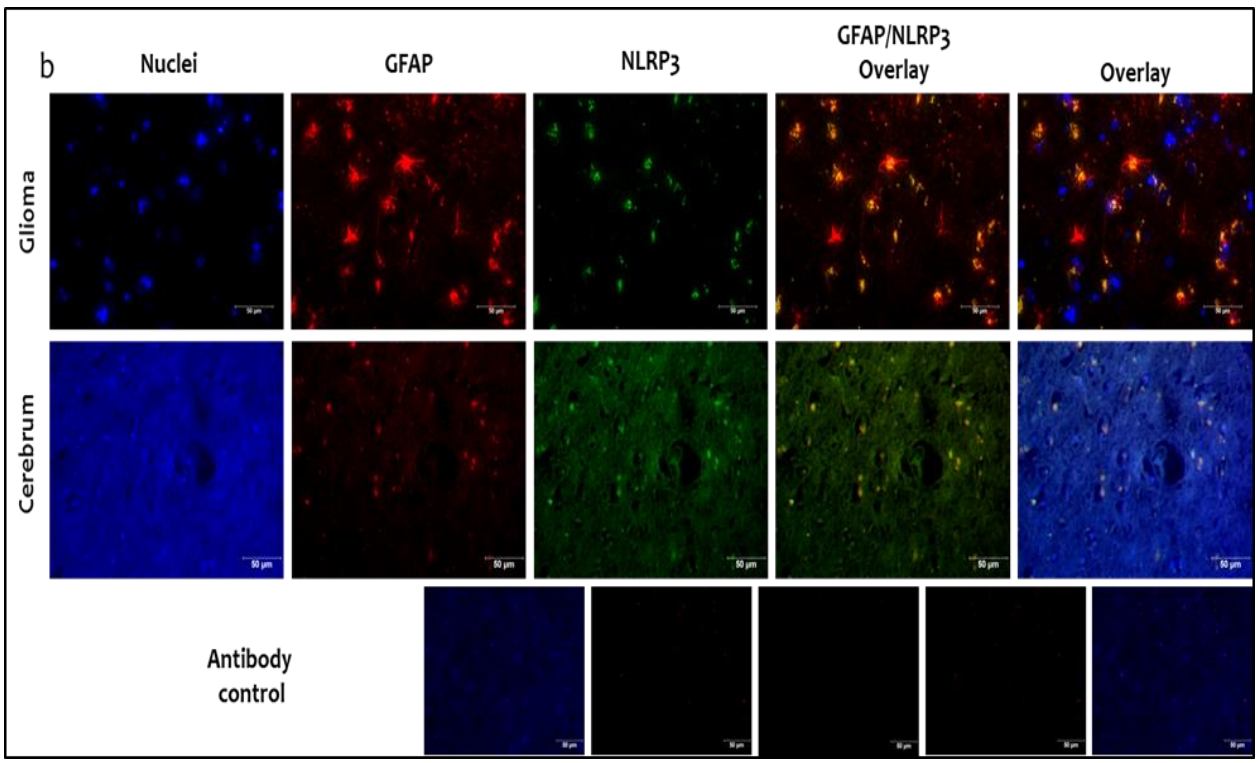


Figure 4.3: NLRP3 protein expression levels in different glial cell population: Panel a and b shows the NLRP3 protein expression in microglia (CD11b positive) and astrocytes (GFAP positive) in glioma and normal brain tissue sections.

4.4.3 ASC protein expression in normal brain and glioma tissue

ASC/ Target of Methylation-induced Silencing (TMS1) is an adaptor protein with bipartite domains, i.e., pyrin domain and caspase recruitment (CARD) domain. Functionally ASC has been involved in the regulation of apoptosis and NF-κβ pathway, cytokines release and as a tumor suppressor gene[Masumoto *et al.*, 1999; McConnell and Vertino, 2004; Srinivasula *et al.*, 2002]. Aberrant hypermethylation of CpG island at the promoter and first exon-level of *ASC* gene led to gene silencing in various cancers such as pancreatic cancer. TMS1 / ASC gene expression was also silenced by hyper-methylation in glioma patients, but it was not the only way by which the downregulation of ASC occurs. Downregulation of ASC may also depend upon the tumor microenvironment as well as the release of different cytokines such as IL-18[Stone *et al.*, 2004]. In this study, we observed increased expression of ASC protein in glioma and normal brain sections in astrocytes and microglia (Figure 4.4).

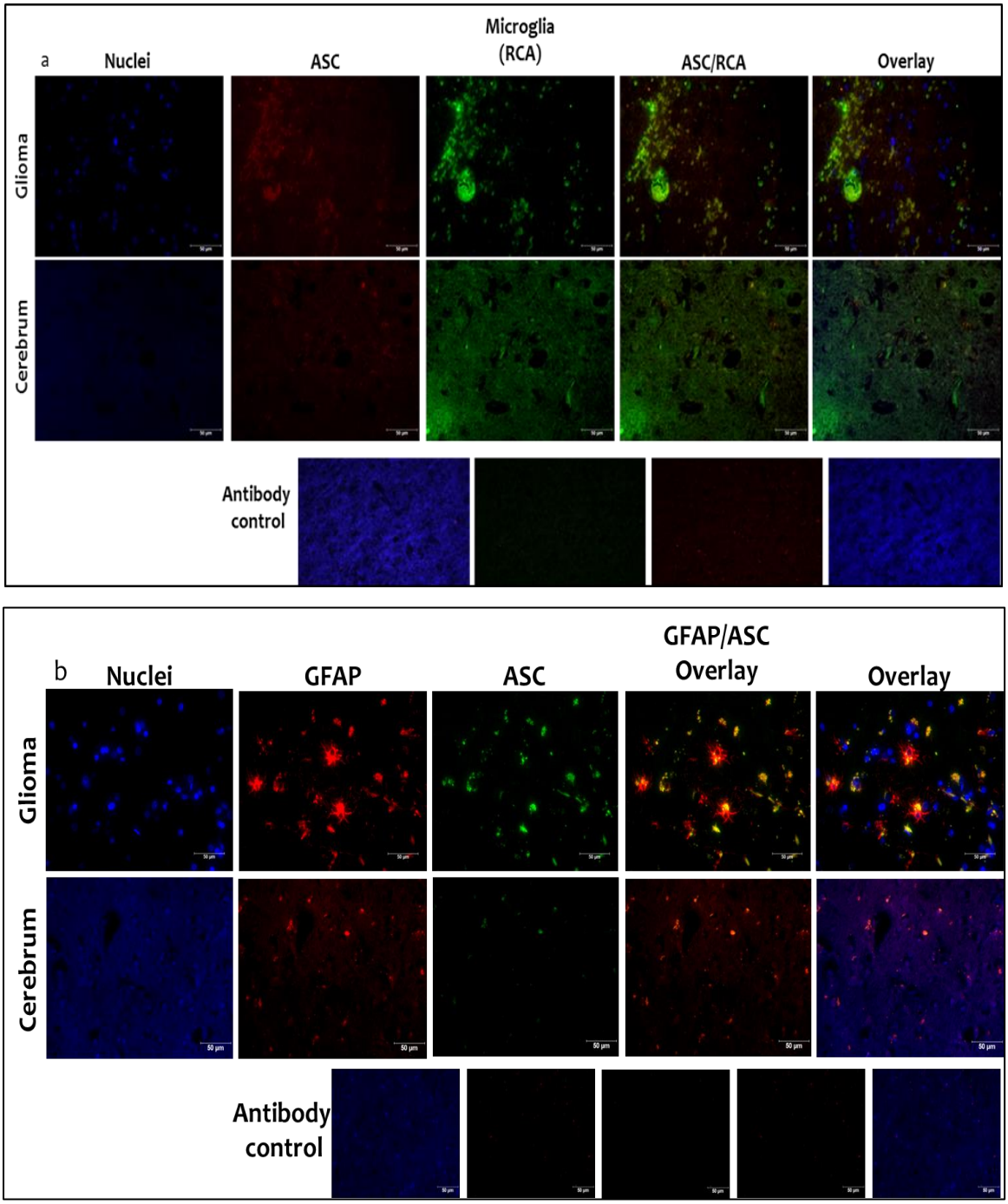


Figure 4.4: ASC protein expression levels in different glial cell population: Panel a and b shows the ASC protein expression in microglia (RCA positive) and astrocytes (GFAP positive) in glioma and normal brain tissue sections

4.4.4 AIM2 protein expression in normal brain and glioma tissue

Absent in melanoma 2 (AIM2) protein belongs to the family of IFI20X-IFI16 (PYHIN) protein family [Roberts *et al.*, 2009]. It consists of two domains HIN200 domain and a pyrin domain. HIN200 domain binds to the foreign DNA as well as endogenous self DNA [Bürckstümmer *et al.*, 2009]. AIM2 binds to ASC and procaspase-1 and forms a functional inflammasome and activates Il-1β cytokines [Fernandes-Alnemri *et al.*, 2009; Hornung *et al.*, 2009]. *AIM2* mRNA levels were high in the primary GBM cell lines isolated from GBM tumor samples [Liu *et al.*, 2004]. The role of AIM2 has been defined in various brain associated pathologies such as traumatic brain injury and Alzheimer's disease [Wu *et al.*, 2017]. In the case of 5XFAD mice, a model for Alzheimer's disease, the knockdown of *Aim2* gene led to the mitigation of Aβ deposition and microglial activation. The deletion of the *Aim2* gene also led to an increase in IL-1β, IL-18, and IL-6 cytokines. AIM2 expression also led to the processing of IL-1β in the cortical neurons following poly dA:dT stimulation[Adamczak *et al.*, 2014]. AIM2 expression in GBM cell line, U251 led to reduced cell proliferation and AIM2 knockdown cells were resistant to temozolomide mediated cell death [Chen *et al.*, 2019]. In the present study, we identified the presence of AIM2 in microglia and astrocytes in the tumor microenvironment. We also observed an increased expression of AIM2 in astrocytes as compared to microglia (Figure 4.5).

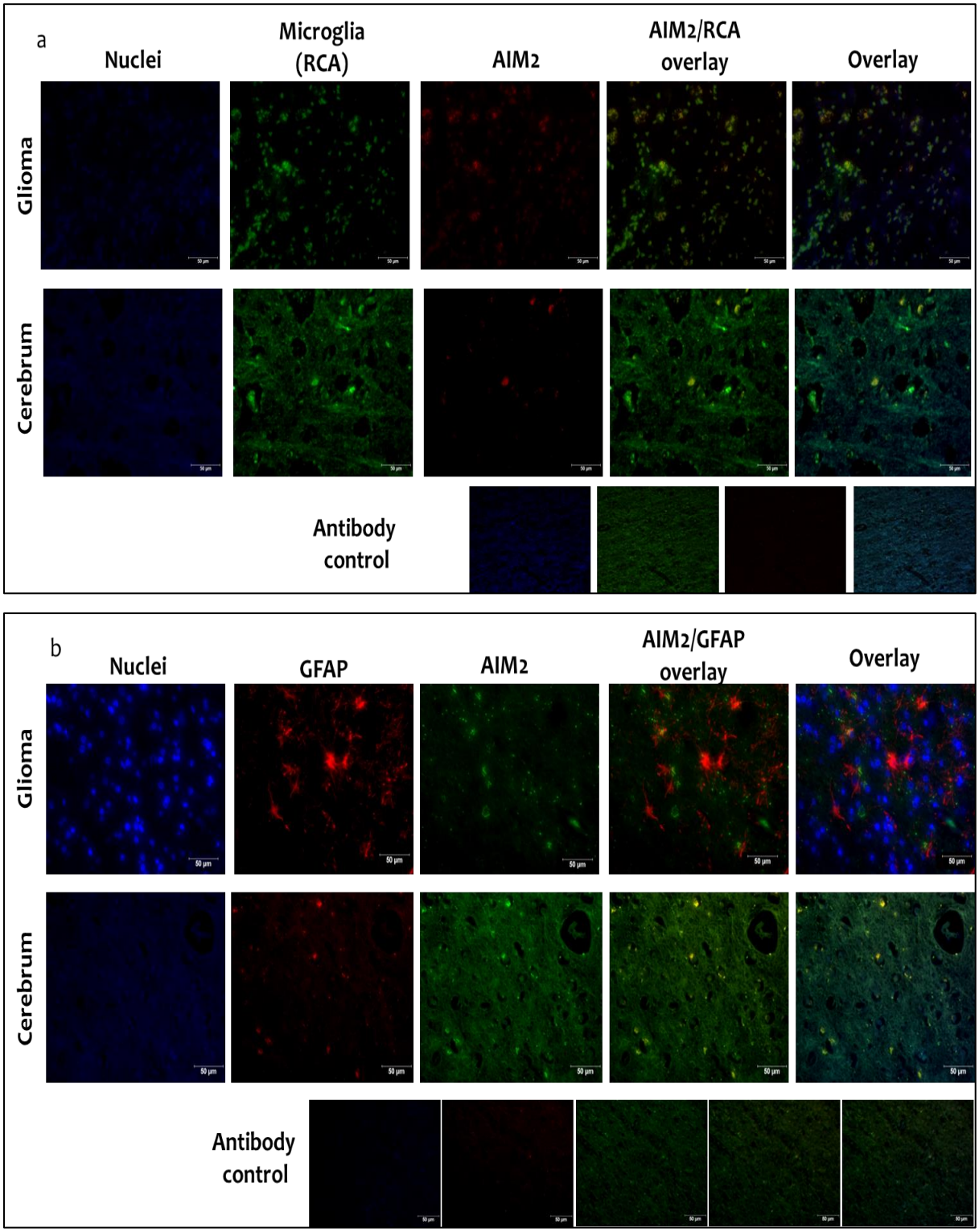
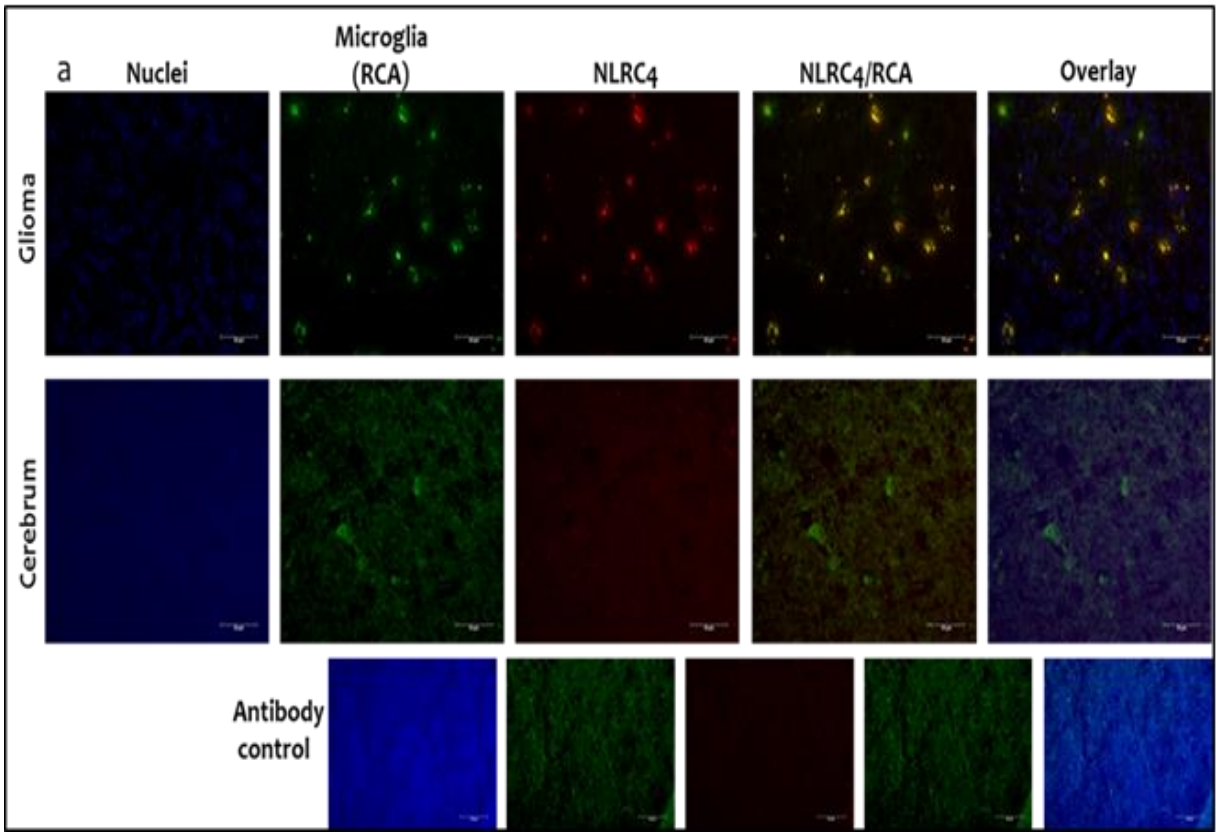


Figure 4.5: AIM2 protein expression levels in different glial cell population: Panel a and b shows the AIM2 protein(red) expression in microglia (RCA positive, green) and astrocytes (GFAP positive, green) in glioma and normal brain tissue sections.

4.4.5 NLRC4 protein expression in normal brain and glioma tissue

NLR family, Caspase Recruitment domain containing 4(NLRC4) also called as IPAF senses several intracellular bacteria such as *Salmonella typhimurium, Legionella pneumophila* via recognizing either flagellin or PrgJ component [Franchi *et al.*, 2006; Pereira *et al.*, 2011; Zhao *et al.*, 2011]. A heterozygous missense mutation in *NLRC4* gene has been identified to cause an autoinflammatory syndrome, i.e., Familial cold autoinflammatory syndrome (FCAS) without any mutation in *the NLRP3* gene [Kitamura *et al.*, 2014]. This study establishes the role of NLRC4 in the genesis of inflammatory diseases. The role of NLRC4 has also been recognized in the case of the mice model of ischemic brain injury[Denes *et al.*, 2015]. The absence of *Nlrc4* gene reduced the infarct volume and size in the mice ischemic brain injury. The study also establishes that NLRC4 can also recognize DAMPs in case of sterile inflammation. NLRC4 also recognizes lysophosphatidylcholine (LPC) as a DAMP [Freeman *et al.*, 2017]. LPC accumulates in case of multiple sclerosis, ischemia, and other brain disorders. LPC induces activation of NLRC4 and NLRP3 inflammasome activation through the canonical inflammasome activation pathway. NLRC4 activation also causes activation of both apoptosis and pyroptosis cell death of microglial cells in the ischemic brain injury mice model [Poh *et al.*, 2019]. Apoptosis was confirmed via activation of caspase-3 and pyroptosis was confirmed via activation of caspase-1 and -11 in mouse microglial cells. A recent study by Lim et al. confirms the expression and activation of NLRC4 inflammasome in case of glioma patients [Lim *et al.*, 2019]. This study supports the immunohistochemistry analysis performed by us in glioma patients (Figure 4.6).



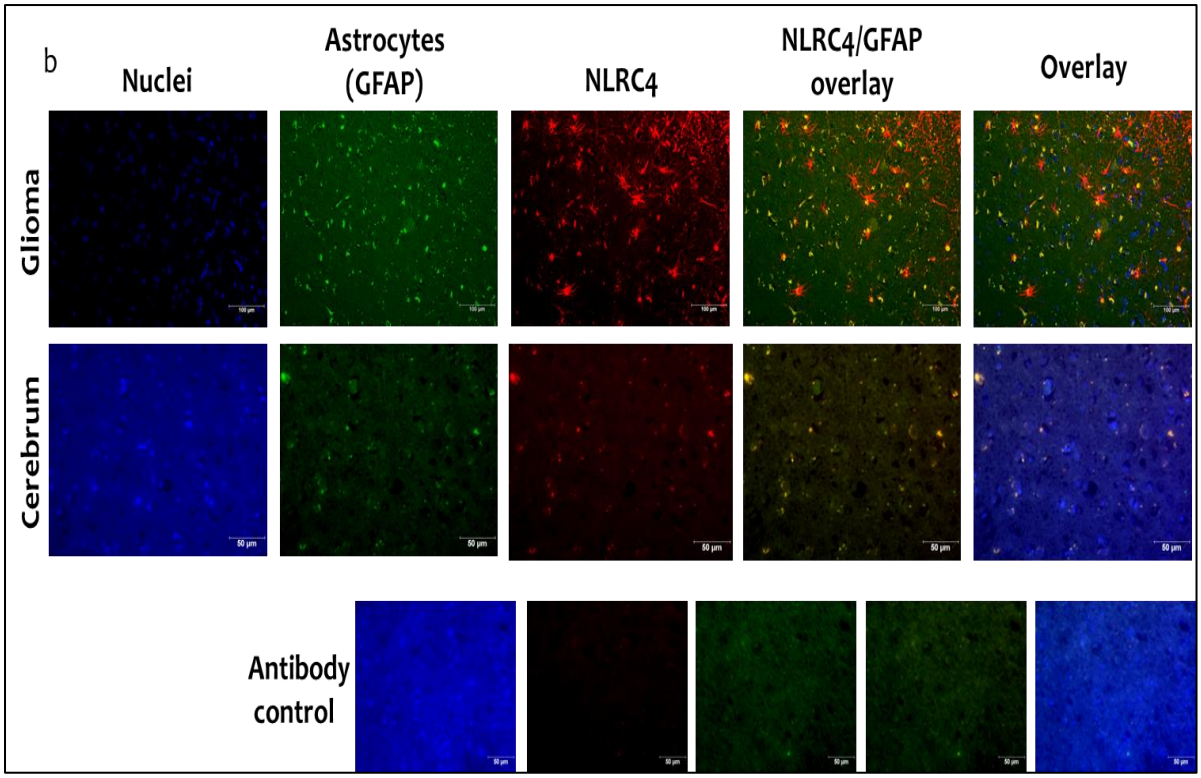


Figure 4.6: NLRC4 protein expression levels in different glial cell population: Panel a and b shows the AIM2 protein(red) expression in microglia (RCA positive, green) and astrocytes (GFAP positive, green) in glioma and normal brain tissue sections.

4.4.6 NLRP12 protein expression in normal brain and glioma tissue

NLRP12 also known as PYPAF-7, and Monarch-1 was discovered in 2002 as a member of the NLR gene family [Wang *et al.*, 2002; Williams *et al.*, 2003]. NLRP12 is mainly expressed in myeloid lineage cells as a 120kDa protein. NLRP12 attenuates inflammatory response by downregulating both canonical and non-canonical NF-κβ pathway [Lich *et al.*, 2007; Williams *et al.*, 2005]. It affects the canonical pathway by interfering with the TLR signaling molecules type I interleukin-1 receptor-associated protein kinase (IRAK1) and non-canonical pathway via association with NIK. Anti-inflammatory role of NLRP12 has been identified in the case of colon cancer and multiple sclerosis [Allen *et al.*, 2012; Gharagozloo *et al.*, 2015]. NLRP12 has also shown a protective impact on obesity via regulating gut microbiota [Truax *et al.*, 2018]. We recently identified the differential role of NLRP12 in glioma pathogenesis [Sharma *et al.*, 2019]. In the study, we characterized the expression of NLRP12 in glioma cell line LN-18 and microglial cell line BV-2. Inhibition of NLRP12 in BV-2 cells led to an increase in proliferation while the LN-18 cells had reduced proliferation. In figure 4.7, we show the co-localization of NLRP12 with microglial cells in glioma tissue (Figure4.7).

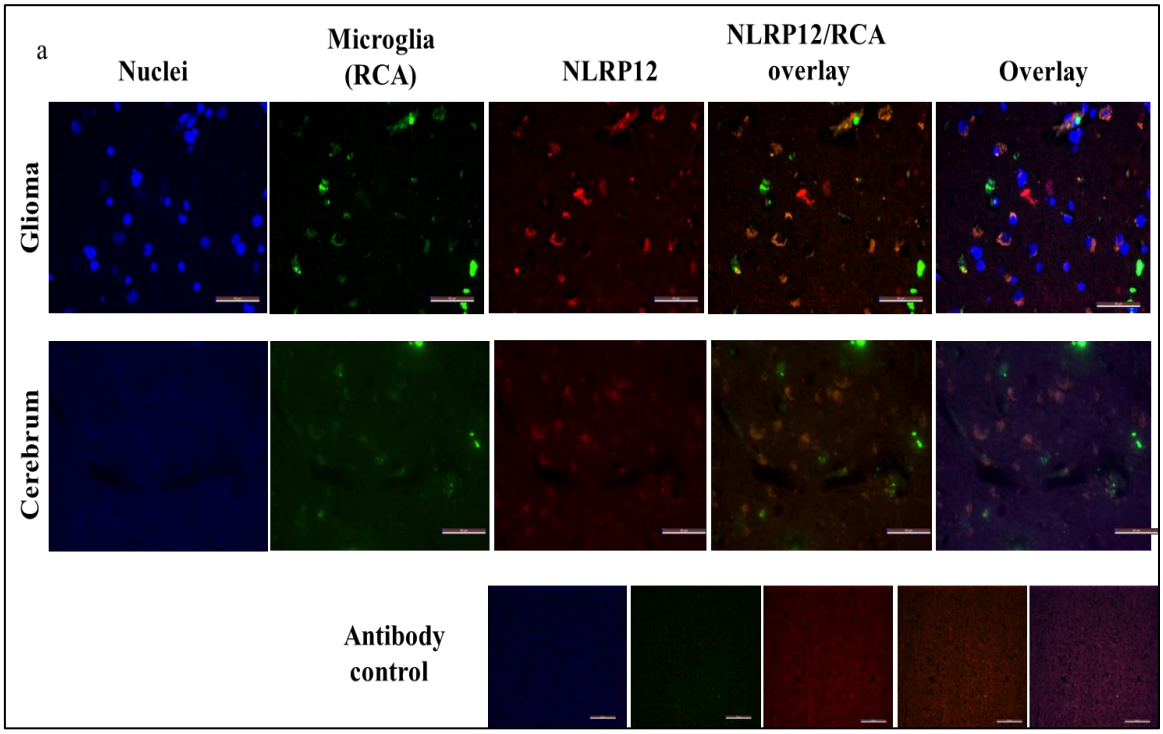


Figure 4.7: NLRP12 protein expression levels in different glial cell population: Panel a shows the NLRP12 protein (red) expression in microglia (RCA positive, green) in glioma and normal brain tissue sections.

4.4.7 Caspase-3 protein expression in normal brain and glioma tissue

Caspase -3 is widely considered as a marker for apoptosis. Caspase-3 is an executioner caspase and functions by degrading various structural and regulatory proteins within the cell and activates various DNases [Salvesen and Dixit, 1997; Slee *et al.*, 2001]. Caspase-3 inhibition via αB-crystallin, a heat shock protein, has been identified in normal and transformed glial cells [Stegh *et al.*, 2008]. αB-crystallin is expressed in >90% of the primary glioma specimens and acts pro-tumorigenic protein by neutralizing caspase-3 activation. Caspase-3 positive gliomas have a significantly higher survival rate [Kobayashi *et al.*, 2007]. Irradiated glioma cells having active caspase-3 has been shown to induce a pro-angiogenic effect on the nearby endothelial cells [Feng *et al.*, 2017]. Co-culture transwell set up of the microglia BV-2 cells and glioblastoma cell lines (U-87 and U-251MG cells) led to the reduced caspase-3 activity in microglial cells [Shen *et al.*, 2016]. Although we saw increased expression of caspase-3 in glioma tissue sections, we identified co-localization of caspase-3 with microglial cells but not in astrocytes (Figure 4.8).

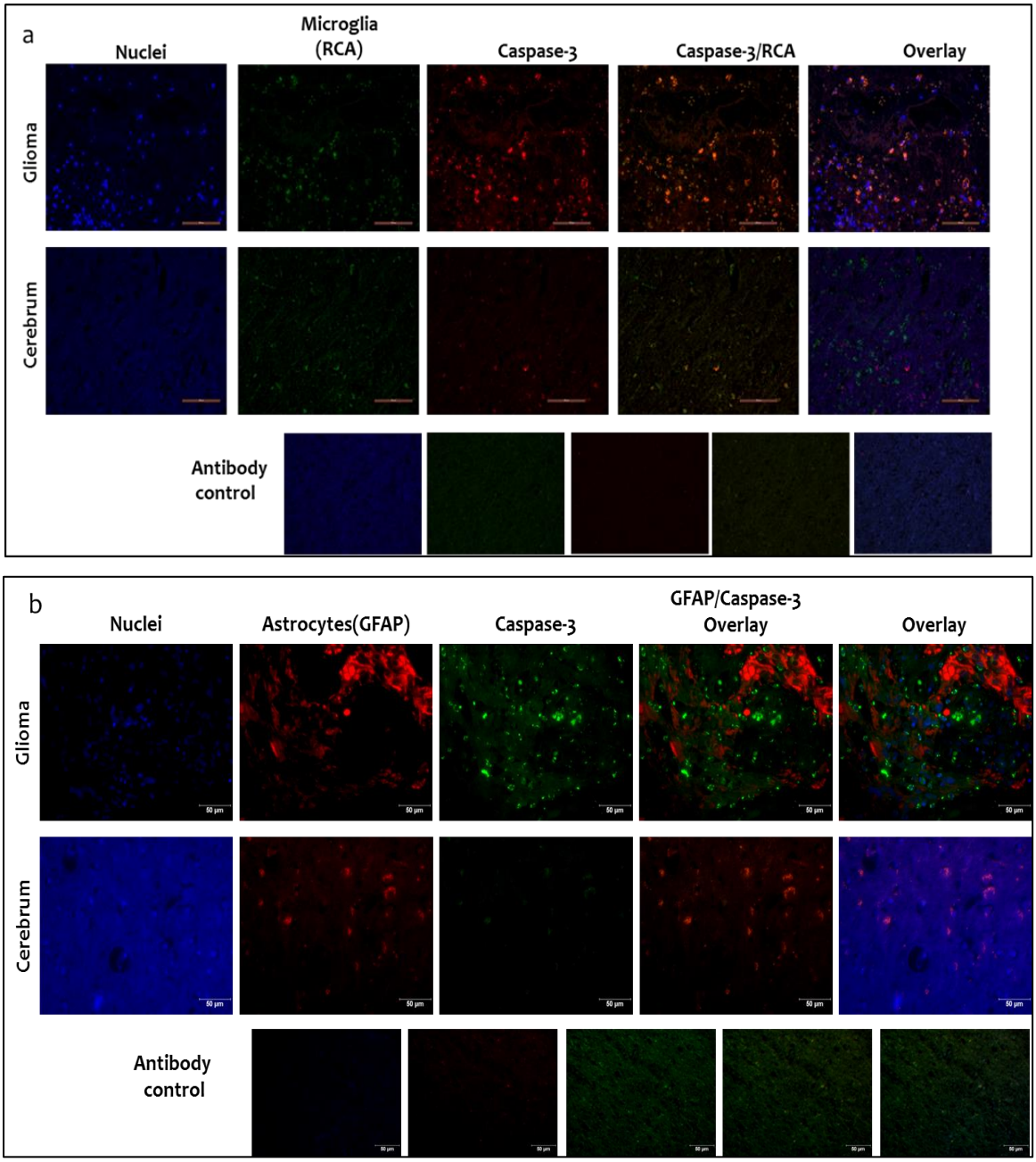


Figure 4.8: Caspase-3 protein expression levels in different glial cell population: Panel a and b shows the Caspase-3 protein(red) expression in microglia (RCA positive, green) and astrocytes (GFAP positive, red) in glioma and normal brain tissue sections

4.4.8 Increased expression of ASC and NLRP3 in low grade and high-grade glioma patients

Western blot analysis of inflammatory proteins showed an increased expression of NLRP3 and ASC in grade III and grade IV glioblastoma tissues as compared to normal brain tissue. We analyzed two normal brain, eight low-grade glioma tissue, and 13 high-grade glioblastoma tissues (Annexure 1). The high-grade glioblastoma tissues also showed increased ASC and NLRP3 expression as compared low-grade glioma tissue (Figure 4.9a and 4.9b).

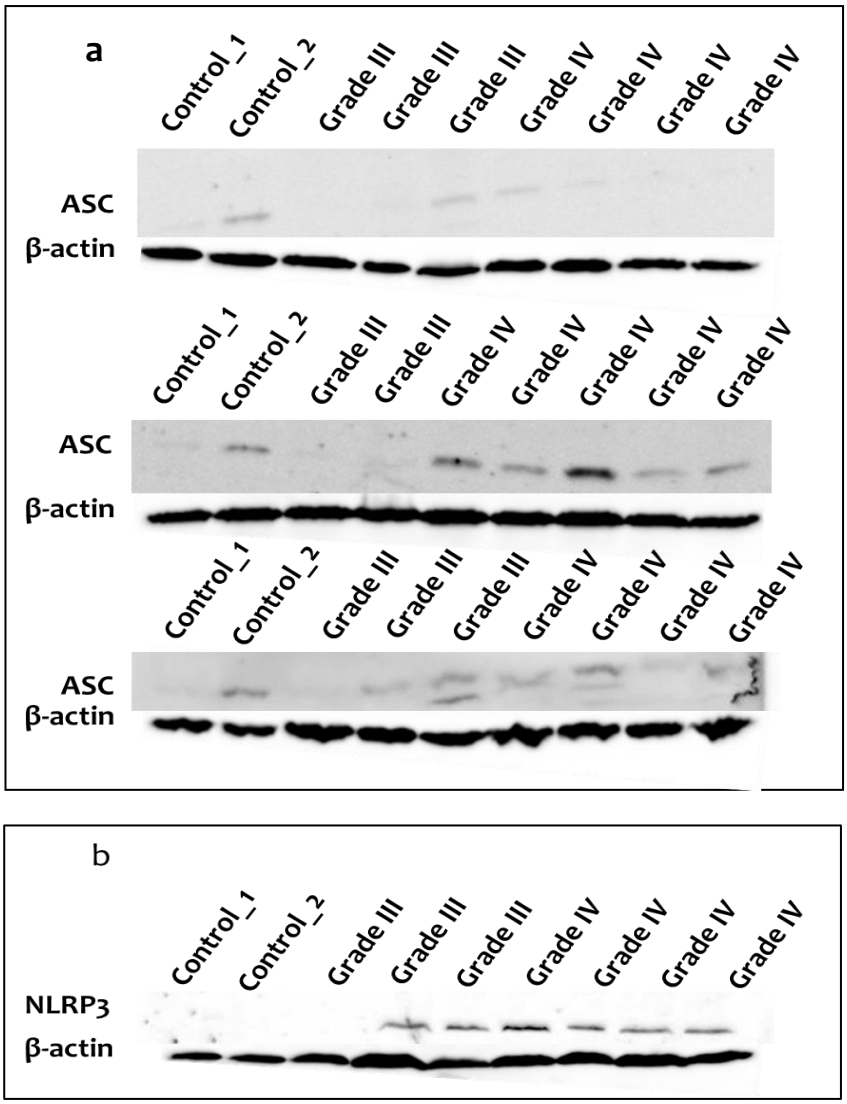


Figure 4.9: a) ASC protein expression grade III and grade IV glioma tissue, b) NLRP3 protein expression grade III and grade IV glioma tissue; β-actin as a loading control.

4.4.9 Differential expression of angiogenesis and proliferation promoting factors in glioma

GBM incorporates a number of different type non-neoplastic cells along with neoplastic tumor cells. The tumor microenvironment consists of microglia, reactive astrocytes, infiltrating macrophages, neural stem cells, and other immune cells. The brain tumor mass consists of approximately 30% of microglia and infiltrating macrophages. The tumor microenvironment supports glioma angiogenesis, proliferation, and invasion by secreting several chemokines and cytokines. Glioma tumor cells also release chemoattractant such as Monocyte chemoattractant Protein-1 (MCP-1) and Regulated on activation, normally T-expressed, and secreted (RANTES) which in turn activates surrounding glial and inflammatory cells. In Annexure 1, we have summarized the role of cytokines, growth factors, and chemokines measured in grade IV glioma tissue samples. Since malignant gliomas rarely metastasize outside the brain (only 0.5% cases), we have considered serum of glioma patients as the controls for cytokine assay [Sun *et al.*, 2017]. Our study includes 1 normal brain sample, 10 serum samples isolated from corresponding grade III and grade IV glioma patients, 10 grade III glioma samples, and 18 grade IV glioma samples.

Platelet-derived growth factor (PDGF) family has five members, depending on the dimer subunit combination. PDGF BB binds to PDGFRα and PDGFRβ [Fredriksson *et al.*, 2004]. During development, PDGF acts as a potent mitogen enabling the proliferation of undifferentiated mesenchyme [Hoch and Soriano, 2003]. PDGF-BB is involved in angiogenesis. PDGF-BB supports mesenchymal stem cell migration towards C6 glioma cells [Cheng *et al.*, 2009]. PDGF-BB regulated intercellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) expression for the migration of glioma cells such as C6 and U-87 cells [Hu *et al.*, 2013]. The concentration of PDGF BB in healthy serum comes in the range of 4758.2 ±1521.5 pg/ml [Kajizuka *et al.*, 2010]. Our results have an average concentration of 7235.03. ±1680.14 pg/ml of PDGF BB, which is a significant increase (p-value=0.001) considering patients undergo different modes of treatment before surgical removal of the tumor (Figure 4.10). The levels of PDGF BB in the brain for grade III glioma were measured to be 79.088±20.32 pg/ml, and in grade IV glioma samples, it was 138.94± 72.27pg/ml (Figure 4.10)

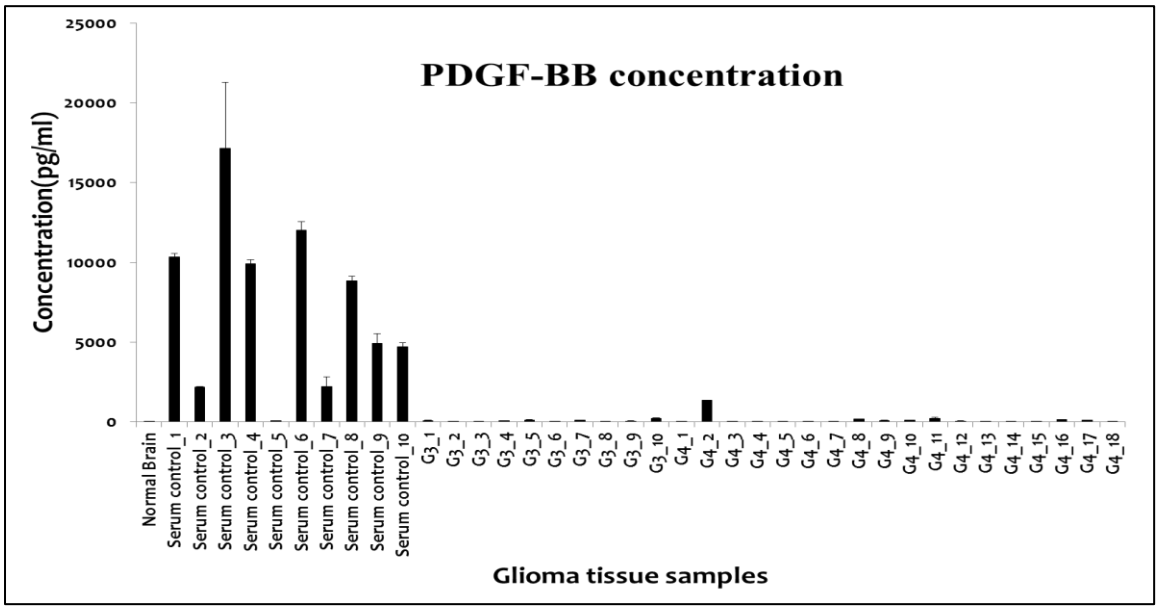


Figure 4.10: Levels of angiogenesis and proliferation factors in glioma: Cytokines levels of PDGF-BB in normal brain control, serum control, and glioma tissue protein samples.

Vascular endothelial growth factor (VEGF) is responsible for endothelial cell proliferation and permeability of blood vessels. PDGF and VEGF have a common 100 amino acid long homology domain. This domain has highly conserved residues domain, including the cysteine knot motif [Fredriksson *et al.*, 2004]. VEGF is considered to be a vital component for angiogenesis in various cancers. It is upregulated during hypoxia via HIF-1α expression [Breier *et al.*, 1992]. VEGF concentration was found to 200-300 times more in the cyst fluid of GBM patients [Takano *et al.*, 1996]. Region of pseudopalisading necrosis has been shown to have higher levels of VEGF, which in turn is responsible for microvascular proliferation [Brat and Van Meir, 2001; Kaur *et al.*, 2005]. Our results observed a significant increase in the levels of VEGF protein in grade IV glioblastoma tissue proteins as compared to the serum controls. The average concentration of VEGF in healthy human serum should be 222± 149 pg/ml, which was also confirmed in our results (279.51± 35.93 pg/ml)[Gagne *et al.*, 2003]. The average concentration of VEGF in grade IV glioma tissue increased up to 15 times i.e.1277.306±303.34pg/ml as compared to grade III 89.25±36.66pg/ml. Targeting VEGF by the use of various anti-angiogenic and cytotoxic therapies are under clinical trials and has achieved a considerable improvement in the survival of GBM patients[Reardon *et al.*, 2008) (Figure 4.11).

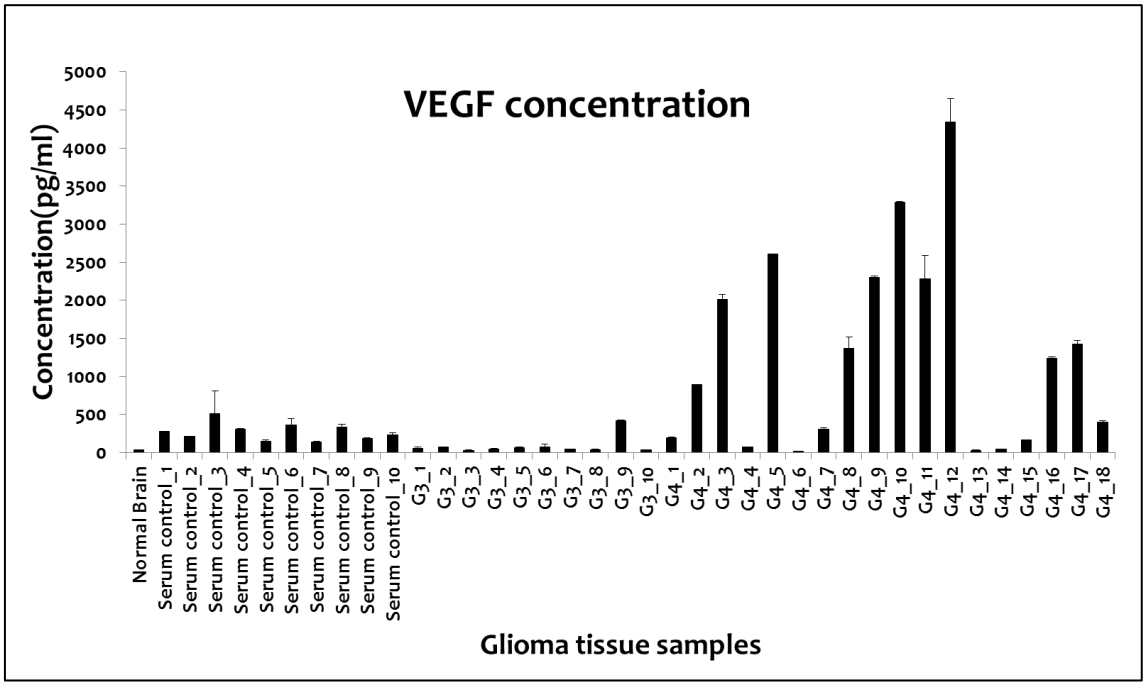


Figure 4.11: Levels of angiogenesis and proliferation factors in glioma: Cytokines levels of VEGF in normal brain control, serum control, and glioma tissue protein samples.

MCP-1 is a potent chemoattractant for microglial infiltration in glioma [Platten *et al.*, 2003]. MCP-1 was first isolated and characterized in glioma cell line U-125MG, which produced around 30- fold more MCP-1 as compared to other U373MG cell lines without any external stimuli [Kuratsu *et al.*, 1989]. Role MCP-1 in glioma has been identified in angiogenesis, chemotaxis, inflammation, and recruitment of microglia and macrophages [Vakilian *et al.*, 2017]. TCGA data analysis revealed that the median survival of glioma patients was more in low-MCP-1 expressing patients as compared to the high-MCP-1 expressing patients [Chang *et al.*, 2016]. Our study also confirms higher levels of MCP-1 in the grade IV glioma patients as compared to normal brain and serum controls. The serum controls have an average concentration of 66.577± 10.281 pg/ml while an average increased concentration of 929.72± 184.6 pg/ml in grade IV glioma tissue samples was observed. The highest concentration is 2424.21pg/ml. In grade III glioma tissue samples, we observed an average concentration of 929.72± 184.6 pg/ml. In a study conducted by Ueda et al., showed that the $(p65)_2$ and c-Rel /p65 NF-κβ dimer subunits bind to A1 and A2 sites of *MCP-1*gene and leads to its transcription in LPS mediated activation of THP-1 monocytes cells[Ueda *et al.*, 1997]. NF-κβ also leads to the constitutive MCP-1 production in glioma cell lines U-105MG, and U-373MG, and its inhibition led to decreased production of MCP-1[Yoshimura, 2018] (Figure 4.12)

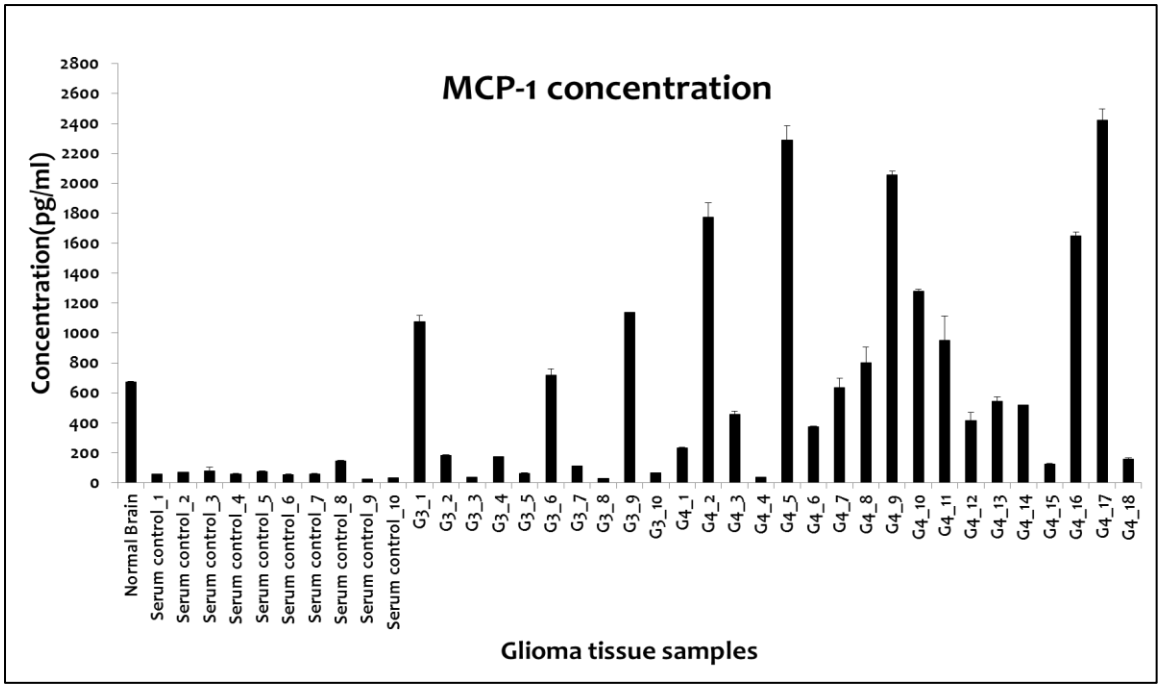


Figure 4.12: Levels of angiogenesis and proliferation factors in glioma: Cytokines levels of MCP-1 in normal brain control, serum control, and glioma tissue protein samples.

RANTES also called CCL5 is a chemokine that binds to CCR5 and CCR1 receptor [Pham *et al.*, 2012]. CCL5 stimulates glioma cell proliferation and invasion (U87 and U251 cells) in a dose-dependent manner [Zhao *et al.*, 2015]. Mesenchymal glioma tissue expressed the highest levels of *CCL5* mRNA out of the four molecular subtypes. Pro-neural subtype had the least expression of RANTES. shRNA mediated knockdown of *Ccl5* gene in mouse mesenchymal glioblastoma cells, 1861, led to a reduction in cell number and cell growth via an increase in apoptosis[Pan *et al.*, 2017]. CCL5-CCR5 interaction activates AKT/PI3k pathway. In the study, we observed increased levels of RANTES in glioma patients as compared to normal brain control. The level of RANTES in the serum of healthy subjects ranged from 5147-6089 pg/ml, while we observed 9744.14±280.25 pg/ml. The level of RANTES in case of traumatic brain injury is reported to be in range 8.47-895.25pg/ml in the contused brain tissue and 5.43-226.80 pg/ml in the cerebrospinal fluid (CSF) [Albert *et al.*, 2017]. Our results show elevated levels of RANTES in both grade III (5479.28± 1771.43 pg/ml) and grade IV glioma (5686.78± 1146.95pg/ml) as compared to the normal brain which has only 239.74pg/ml (Figure 4.13).

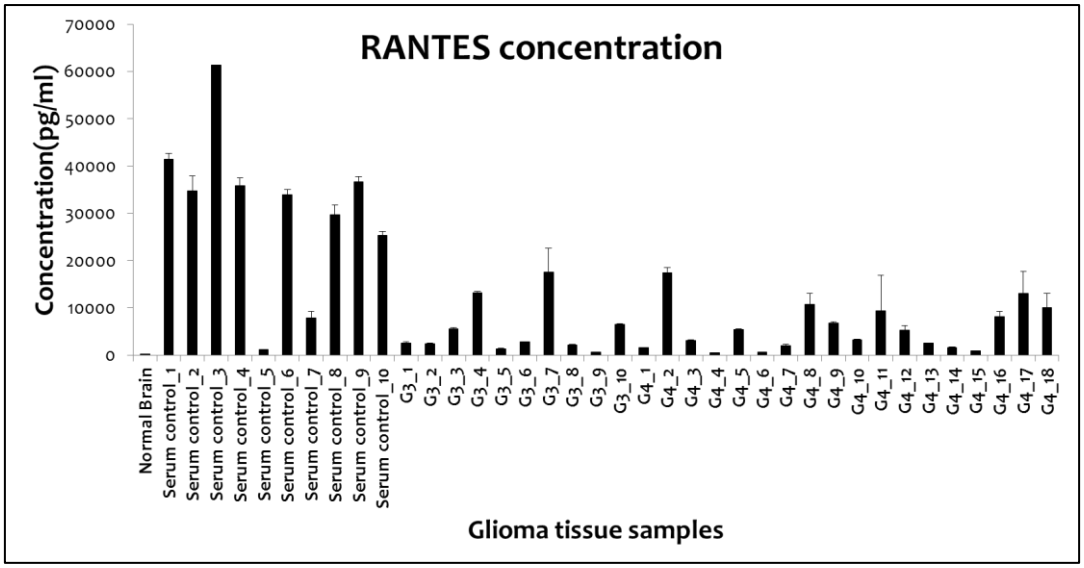


Figure 4.13: Levels of angiogenesis and proliferation factors in glioma: Cytokines levels of RANTES in normal brain control, serum control, and glioma tissue protein samples.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) phosphorylate JAK kinases and recruits STAT5 for the subsequent maturation of granulocytes and macrophages [Aliper *et al.*, 2014]. GM-CSF and G-CSF have immunosuppressive effects by recruiting antigen-presenting cells and producing cytotoxic T-cell response [Butowski, 2010]. Low levels of GM-CSF in glioma cell lines A-172 and U87-MG led to decreased survival of eosinophils during a conditioned media experimental setup [Curran *et al.*, 2011]. Levels of G-CSF in plasma derived from glioma patients were elevated, and it supported the myeloid-derived suppressor cells accumulation [Aliper *et al.*, 2014; Raychaudhuri *et al.*, 2011]. Levels of GM-CSF and G-CSF were not affected in our cytokine assay. The average serum concentration of G-CSF and GM-CSF ranges from 34-53.6 pg/ml and 26.3-63.8pg/ml respectively [Kleiner *et al.*, 2013]. In our study also the serum concentration of G-CSF was in the normal range, i.e., 47.3± 7.14 pg/ml, while a lower concentration of GM-CSF was observed i.e.4.7±1.14pg/ml (Figure 4.14a and 4.14b).

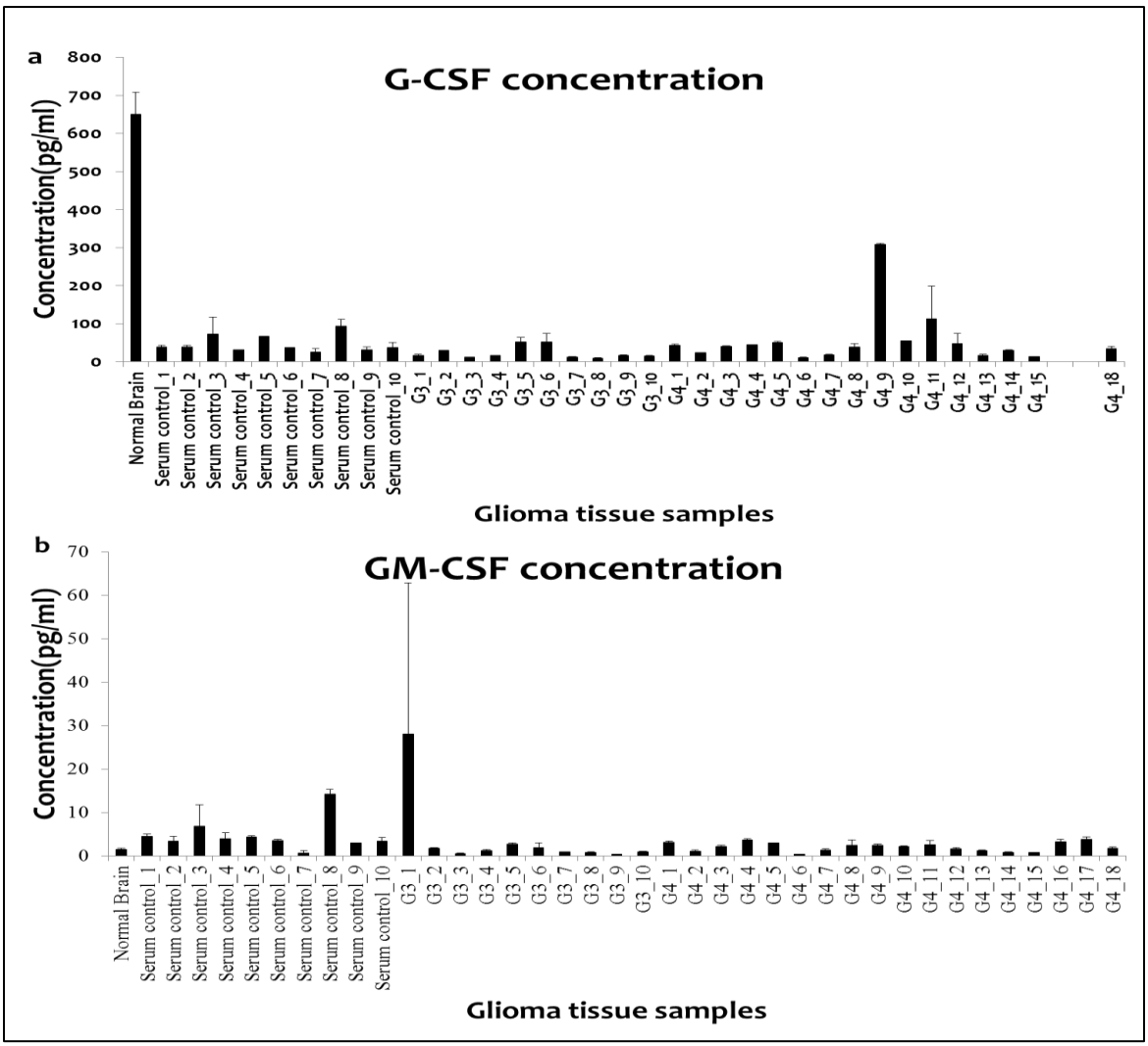


Figure 4.14: Levels of angiogenesis and proliferation factors in glioma: Panel 4.14a-b Cytokines levels of G-CSF and GM-CSF in normal brain control, serum control, and glioma tissue protein samples.

4.4.10 Differential expression of inflammatory cytokines in glioma

We previously observed the increase in the expression of several NLRs and associated proteins via immunofluorescence and western blotting at both cells specific and tissue levels. We further utilized Bio-plex human cytokine assay to determine the concentrations of inflammatory cytokines in grade III and grade IV glioma tissues. IL-1β and IL-18 elevated levels have found in case various neurodegenerative diseases such as Multiple sclerosis, Alzheimer's disease, and focal ischemia [Alboni *et al.*, 2010; Freeman and Ting, 2016]. IL-1β level in the body is very tightly regulated, and it is not constitutively active in healthy humans [Garlanda *et al.*, 2013]. The average level of IL-1β was 2.9±0.6 pg/ml in serum controls, while 12 out of 18 patients showed above 5pg/ml with an average of 7.14±1.47pg/ml. IL-18 is also involved in the interferon-gamma (IFN-γ) activation. In regards to glioma pathogenesis, the aberrant and constitutive activation of IL-1β has been observed in both patient-derived glioma cell line and U-87 glioma cell line [Tarassishin *et al.*, 2014]. The study also confirmed that knockdown of *NLRP3* by siRNA led to the alleviated release of IL-1β in U-87 glioma cells. Figure 4.15a and 4.15b show an increased IL-1β and IL-18 cytokines level in grade IV glioma tissue, which in turn confirms the role of inflammation in glioma tumor and its surrounding microenvironment. IL-18 levels in serum range from 0-492 pg/ml,which is comparable to the serum concentration of glioma patients in our study, i.e., 246.909±42.98 pg/ml [Colafrancesco *et al.*, 2012]. However, the levels of IL-18 in grade

III glioma were 29.85±7.18 pg/ml and 56.5. ± 31.9 in grade IV glioblastoma (Figure 4.15a and 4.15b).

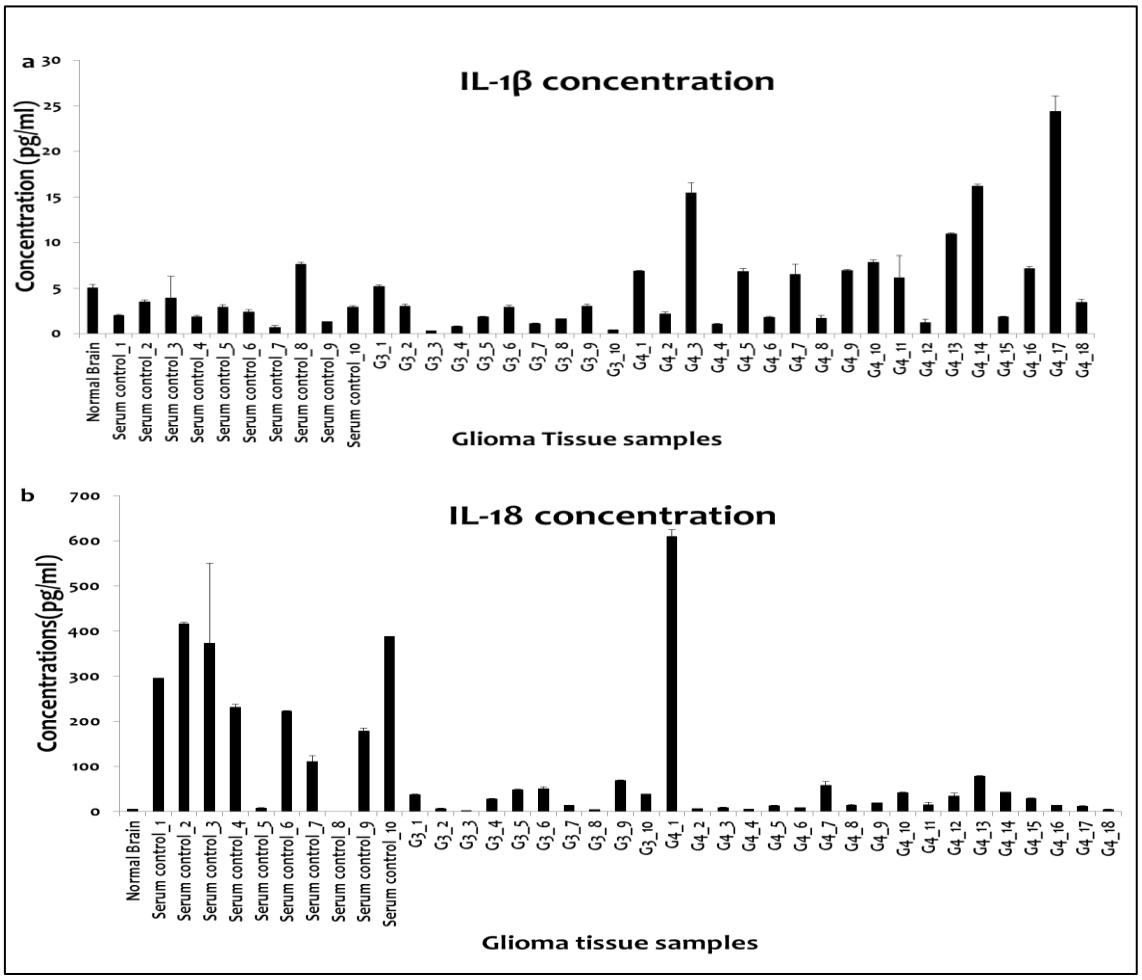


Figure 4.15: Differential expression of inflammatory cytokines in glioma: Levels Panel 4.15 a-b: Cytokines levels of IL-1β and IL-18 in normal brain control, serum control, and glioma tissue protein samples

Interferon-gamma (IFN-γ) is suggested to have anti-tumorigenic effects by the activation of apoptosis and inhibiting dividing cells [Horiuchi *et al.*, 2006]. Reduced levels of IFN-γ were found in peripheral lymphocytes and primary glioma cells [Kane and Yang, 2010; Zisakis *et al.*, 2007]. IFN-γ induces production of interferon-6 (IL-6) in glioma cell lines [Hotfilder *et al.*, 2000]. In our study, we observed that IFN-γ production in grade III glioma tissues was 13.23±2.44pg/ml, and in grade IV, it comparably increased, i.e., 33.14±4.81pg/ml. The corresponding serum controls have an average of 102.10± 7.78 pg/ml, which is within the normal range, i.e., 100-200 pg/ml (Figure 4.16a). Higher levels of IL-6 were measured in serum and cerebrospinal fluid of glioma patients as compared to the healthy controls. Induction of IL-6 cytokine in U-87 glioma cell line markedly increased their invasion properties [Shan *et al.*, 2015]. IL-6 from tumor-associated endothelial cells lead to alternate macrophage polarization and tumor cell survival [Wang *et al.*, 2018]. IL-6 levels in the multiplex immunoassay performed showed significant increase in grade IV glioma tissue (583.2± 296.90 pg/ml) as compared to normal brain (8.16pg/ml) and serum samples (6.49± 0.87pg/ml) (Figure 4.16b). The levels of serum controls were also in the normal range 5-15 pg/ml.

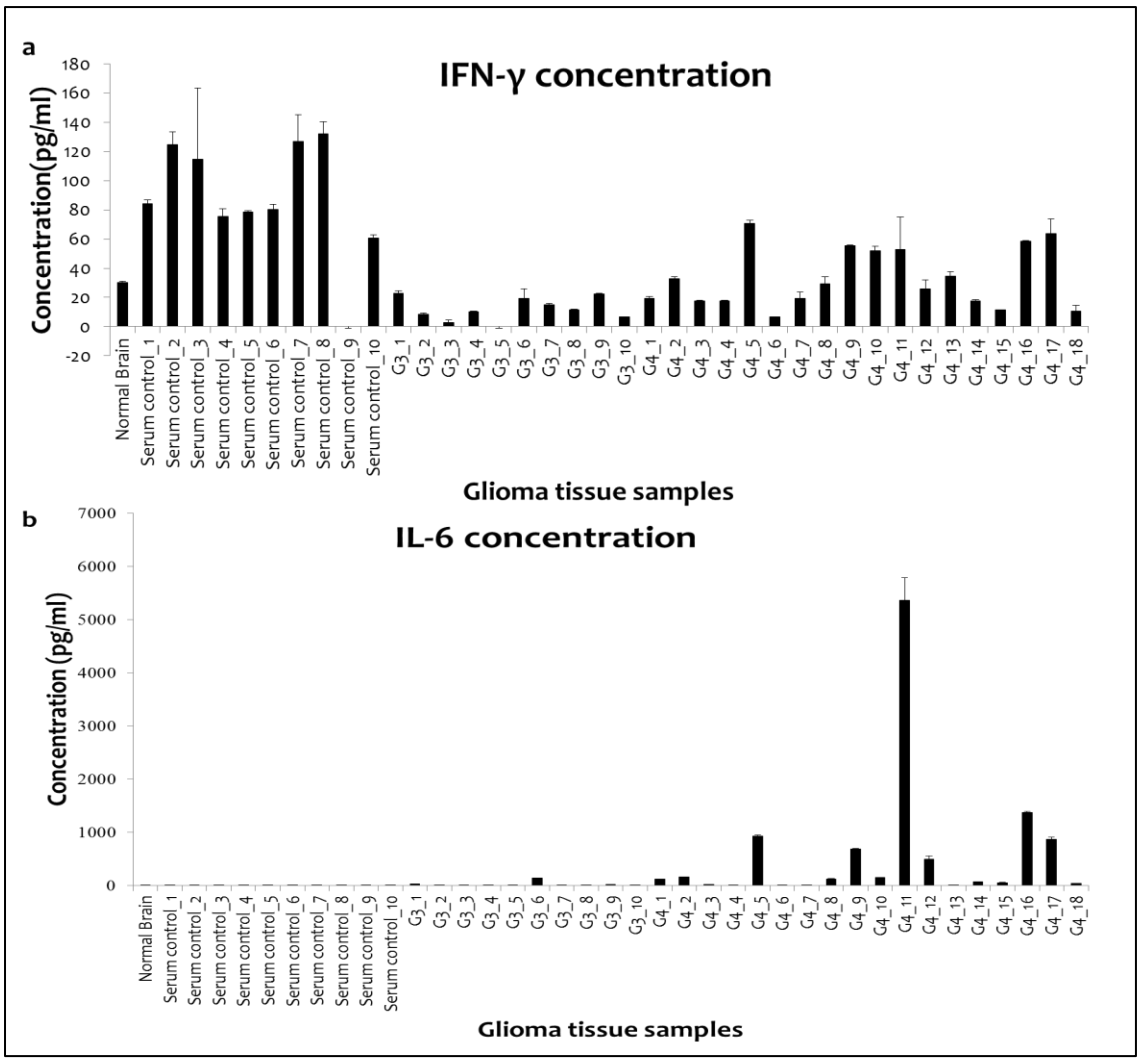


Figure 4.16: Differential expression of inflammatory cytokines in glioma: Levels Panel 4.16a-b: Cytokines levels of IFN-γ and IL-6 in normal brain control, serum control, and glioma tissue protein samples

Interleukin 1 receptor antagonist (IL-1Ra) acts as an anti-inflammatory cytokine by competitive inhibition of IL-1β. It alleviates inflammation in case of various brain disorders such as stroke [Pradillo *et al.*, 2012]. It also reduces angiogenesis and metastasis of tumor in case of fibrosarcoma mice model, which constitutively secretes IL-1β [Bar *et al.*, 2004]. In our study, we observed increased levels of IL-1Ra levels in the corresponding serum controls (4137± 608.71 pg/ml) as compared to the normal range (101-172 pg/ml). The IL-Ra levels in grade III glioma samples were 316± 4.44 pg/ml, and it 634± 118.533 pg/ml in grade IV glioma tissue samples (Figure 4.17a). IL-17 is secreted by inflammatory T cells, Th17. IL-6, IL-1β, and IL-23 cytokines are critical in recruiting Th17 cells and hence producing IL-17 in case cancer such as ovarian cancer[Aggarwal *et al.*, 2003; Miyahara *et al.*, 2008]. IL-17 cytokines further induce the production of other inflammatory cytokines such as IL-6, G-CSF, MCP-1. Although in the context of glioma, IL-17 acquires an anti-cytotoxic role and supports tumor growth [Paladugu *et al.*, 2013; Parajuli and Mittal, 2013]. Levels of IL-17 were 18.389±3.79 pg/ml in the serum of the patients while in grade III, and grade IV glioma tissues were 3.2±0.18 pg/ml and 4.69±0.33 pg/ml, respectively(Figure 4.17b).

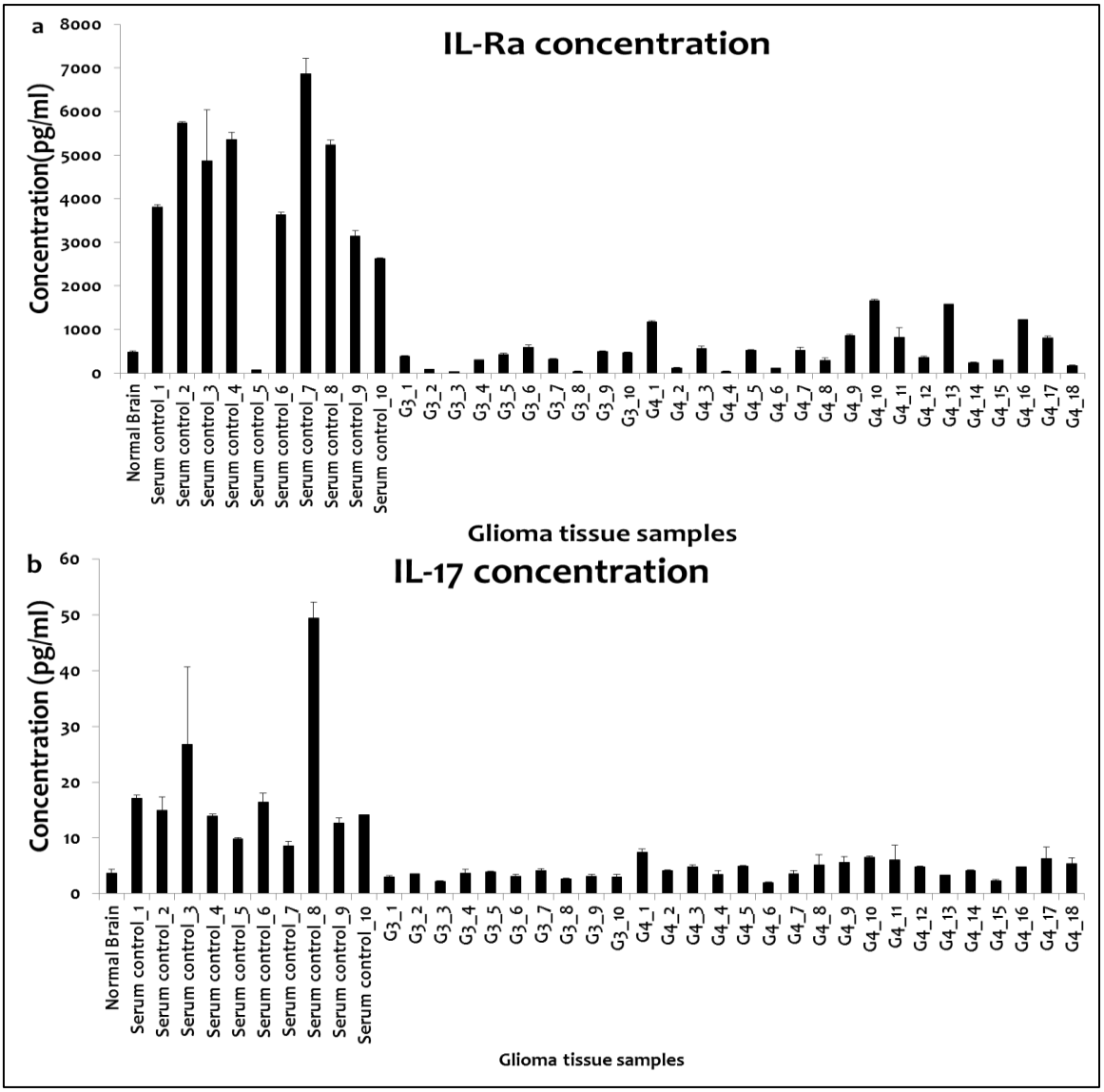


Figure 4.17: Differential expression of inflammatory cytokines in glioma: Levels Panel 4.17a-b: Cytokines levels IL-Ra and IL-17 in normal brain control, serum control, and glioma tissue protein samples

Tumor necrosis factor-alpha (TNF-α) is a potent inducer of IL-6 and IL-8 cytokine in case of astrocytoma and glioblastoma cell lines [Van Meir *et al.*, 1990]. TNF-α also strongly induces the activation of NF-κβ pathway in glioma cells [Otsuka *et al.*, 1999](Figure 4.18a). TNF-α level in grade III glioma samples were 20.89±6.55pg/ml while in grade IV glioma it was 18.45± 2.98 pg/ml. IL-8 is induced by various factors such as IL-6, IFN-γ, IL-1, and LPS. Many GBM cell lines constitutively express IL-8 has a pro-angiogenic and growth-promoting effect on glioma cells [Yamanaka *et al.*, 1995]. The average serum levels of IL-8 is 29.3 pg/ml; we observed an average of 55.63±17.38 pg/ml of IL-8. We also observed an increase in IL-8 levels in 7 out of the18 patients with an average of 264.89±116.17pg/ml (Figure 4.18b).

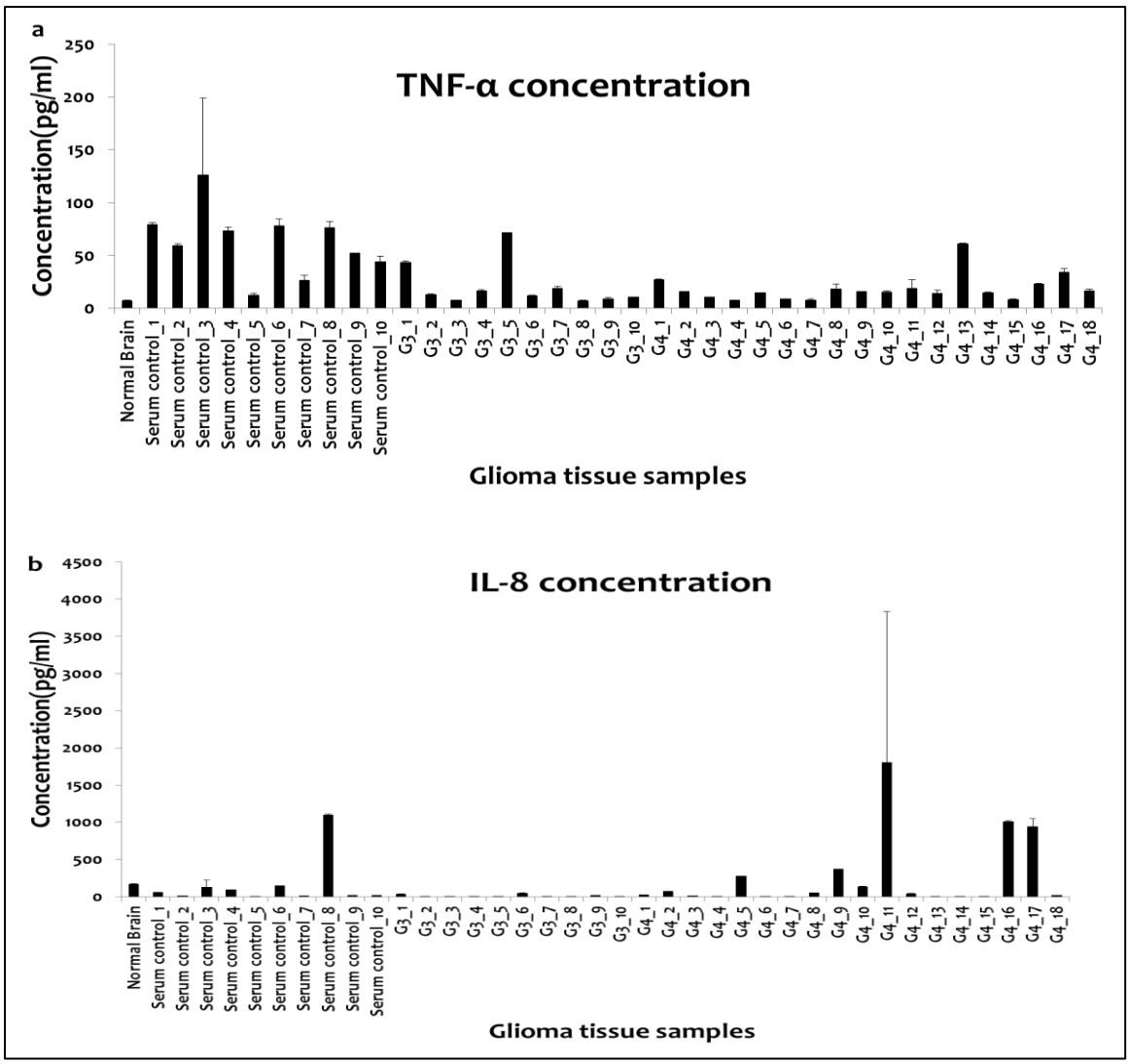


Figure 4.18: Differential expression of inflammatory cytokines in glioma: Levels Panel 4.18 a-b: Cytokines levels of TNF-α and IL-8 in normal brain control, serum control, and glioma tissue protein samples

4.5 Concluding Remarks

Glioblastomas are the most aggressive and infiltrative primary brain tumors consisting of 54% of gliomas [Ostrom *et al.*, 2013]. The current therapy consists of temozolomide and radiotherapy, followed by temozolomide for glioblastoma patients. Due to its extensive invasiveness and radical tumor resection, the median survival is less than 15months in a newly diagnosed glioblastoma [Stupp *et al.*, 2005]. Glioblastoma consists of tumorigenic cells and brain resident cellular population, all of which maintains a tumor microenvironment. Understanding the functions and properties of the tumor microenvironment can assist us in developing a better treatment modality. Inflammation and angiogenesis particularly play an important part in glioma pathogenesis [Aldape *et al.*, 2019].

Role of NLRs as a sensor for PAMPs, DAMPs, and irritants has been defined in the case of various cancers, infections, and brain disorders [Davis *et al.*, 2011]. The role of NLRs in glioma pathogenesis remains unresolved. We have already identified the differential gene expression and methylation levels of various NLRs during glioma. This study further confirms cell-specific increased expression of NLRs and AIM2 in glioma. We comprehensively analyzed the co-localization of NLRP3, NLRC4, NLRP12, and AIM2 in both microglia and astrocytes by immunofluorescence. We also observed an overall increase of NLRP3 and ASC in glioma tissue samples by western blotting.

Angiogenesis is an integral part of tumor growth and proliferation. Gliomas are highly vascularized tumors which require angiogenesis beyond 1-2mm in size [Kim and Lee, 2009]. We have quantified the protein levels of VEGF, MCP-1, RANTES, and various other pro-angiogenic factors required for tumor growth and invasion. We observed an overall increase in the levels of VEGF, RANTES, and MCP-1 in the glioma tissue samples. We also quantified various pro-inflammatory cytokines such as IL-6, IL-4, IL-1β, IFN-γ, and IL-18. The study confirms increased levels of pro-inflammatory cytokines in the glioma tumor microenvironment.