Isabele Fattori Moretti

**Toll like receptor 4 among the plasmatic membrane Toll like receptors plays a
role in astrocytoma malignancy**

**Receptor do tipo Toll 4 dentre os TLRs de membrana plasmática possui um
papel na malignidade de astrocitomas**

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Medicina da Universidade de São Paulo
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Programa de Neurologia

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“I remembered that the real world was wide, and that a varied field of hopes and fears, of sensations and excitements, awaited those who had the courage to go forth into it’s expanse, to seek real knowledge of life amidst it’s perils.”

Charlotte Brontë

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Abbreviation list

µg	Micrograma
µM	Micromolar
AGII	astrocytoma grade II
AGIII	astrocytoma grade III
AGIV	astrocytoma grade IV
AIF1	allograft Inflammatory factor 1
AKT	serine/threonine kinase 1
AKT1	AKT serine/threonine kinase 1
ARHGAP6	rho GTPase activating protein 6
ATCC	American Type Culture Collection
BCL2L1	apoptosis regulator Bcl-X
CASP	Caspase
CCND1	cyclin D1
CD	cluster of differentiation
CD133	prominin-1
cDNA	complementary DNA
CHUK	inhibitor of nuclear factor kappa-B kinase subunit alpha
CNS	central nervous system
CS	Classical
Ct	cycle threshold
C-terminal	carboxi-terminal
DAB	3'-diaminobenzidine
DAMP	danger associated molecular pathern
DAPI	4',6-diamidino-2-phenylindole
DMEN	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence
ECM	extra-cellular matrix
EDTA	ethylenediamine tetraacetic acid
ER	endoplasmic reticulum
EZR	ezrin
FADD	fas associated via death domain
FITC	Fluorescein
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GBM	Glioblastoma
GFAP	glial fibrillary acidic protein
GUSB	glucuronidase Beta
H3	histone 3
HCL	hydrochloric acid
HE	hematoxylin and eosin

HEPES	hydroxyethyl piperazineethanesulfonic acid
HPRT	hypoxanthine phosphoribosyltransferase
HRP	horseradish peroxidase
hrs	Hours
HSP	heat shock proteins
IBA1	ionized calcium-binding adapter molecule 1
ID	Identification
IgG	immunoglobulin G
IKBKB	inhibitor of nuclear factor kappa-B kinase subunit beta
IL	Interleukin
ILS	immunolabelling score
IRAK	interleukin 1 receptor associated kinase
IRF	interferon regulatory factor
JUN	proto-Oncogene C-Jun
LPS	Lipopolysaccharide
LY96	lymphocyte Antigen 96
MES	Mesenchymal
ml	Mililiter
MLKL	mixed lineage kinase Domain Like pseudokinase
mM	Milimolar
Mops	(3-(N-morpholino)propanesulfonic acid
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
MYC	avian myelocytomatosis viral oncogene homolog
MYD88	myeloid differentiation primary response 88
NaCl	sodium chloride
NF1	neurofibromin 1
NF-κB	nuclear factor kappa B
ng	Nanogram
NLRP	NLR family pyrin domain containing
NN	non-neoplastic
N-terminal	amino-terminal
<i>p</i>	Significance
PAMP	pathogens associated molecular patherns
pb	base pair
PCR	polymerase chain reaction
pH	power of hydrogen
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
PN	Proneural
PTEN	phosphatase and tensin homolog
PTK2	protein tyrosine kinase 2
PYCARD	PYD And CARD domain containing
qRT-PCR	quantitative reverse transcription PCR
<i>r</i>	Coeficient
RB1	retinoblastoma-associated protein

REL	V-Rel reticuloendotheliosis Viral Oncogene Homolog
RIPA	radioimmunoprecipitation assay buffer
RIPK	receptor Interacting serine/threonine kinase
RNA	ribonucleic acid
RPKM	repeats per million
rpm	rotations per minute
SCR	Scramble
SDS	sodium dodecyl sulfate
shRNA	short hairpin RNA
SNC	sistema nervoso central
SRF	serum response factor
STR	short tandem repeats
TBP	TATA-Box Binding Protein
TCGA	The Cancer Genome Atlas
TE	Tris-EDTA
TICAM2	toll like receptor adaptor molecule 2
TIR	toll IL-1 receptor
TIRAP	TIR domain containing adaptor protein
TLR	toll-like receptor
TMA	tissue microarray
TRADD	TNFRSF1A associated via death domain
TRAF	TNF receptor-associated factor
TRAM	translocation associated membrane protein
TRIF	TIR-domain-containing adapter-inducing interferon- β
WHO	World Health Organization

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Abstract

Moretti I.F. *Toll like receptor 4 among the plasmatic membrane TLRs plays a role in Astrocytoma malignancy* [dissertation]. São Paulo. “Faculdade de Medicina, Universidade de São Paulo”; 2018.

Toll-like receptors (TLRs) are the first to identify disturbances in the immune system, recognizing pathogens such as bacteria, fungi, and viruses. Since the inflammation process plays an important role in several diseases, TLRs have been considered potential therapeutic targets, including treatment for cancer. However, TLRs' role in cancer remains ambiguous. This study aims to analyze the expression levels of plasmatic cell membrane TLRs (TLR1, TLR2, TLR4, TLR5, and TLR6) in different grades (II-IV) of human astrocytoma, the most prevalent tumor of CNS. We demonstrated that TLR expressions were higher in astrocytoma samples compared to non-neoplastic brain tissue, by qRT-PCR. The genes and proteins expressions were observed in U87MG and A172 GBM cell lines, proving their presence in the tumor cells. Associated expressions between the known heterodimers TLR1-TLR2 were found in diffusely infiltrative astrocytoma. In GBM, the mesenchymal subtype showed higher levels of TLR expressions in relation to classical and proneural subtypes. Aiming to identify the role of TLRs in tumor cells, we chose the highest TLR expressed in GBM cells, the TLR4, and performed functional assays stimulating U87MG-GBM cell line with LPS, a natural agonist for TLR4. The proliferation rate was similar in treated and non-treated cell with LPS. However, NF- κ B activation was detected after 12hrs of LPS stimulation. When TLR4 signaling pathway was inhibited by a chemical compound (VGX-1027) a decrease in the proliferation rate was observed. Additionally, *in silico* analysis revealed a strong association of TLRs upregulation with increased expression level of genes related to cell cycle, inflammasome and ripoptosome pathways, further highlighting TLRs as interesting targets for cancer complementary treatment.

Descriptors: astrocytoma; glioblastoma; toll like receptors; cell proliferation; inflammation; nuclear factor – kappa B; lipopolysaccharides.

Resumo

Moretti I.F. *Receptor do tipo Toll 4 dentre os TLRs de membrana plasmática possui um papel na malignância de astrocitomas* [dissertação]. São Paulo. “Faculdade de Medicina, Universidade de São Paulo”; 2018.

Os receptores do tipo Toll (TLRs) são as primeiras proteínas do sistema imune a identificarem distúrbios, reconhecem patógenos como bactérias, fungos e vírus. Como o processo inflamatório possui um importante papel em diversas doenças, os TLRs foram considerados potenciais alvos em estratégias terapêuticas, incluindo o tratamento de câncer. No entanto, o papel dos TLRs permanece ambíguo. Esse estudo teve como objetivos analisar os níveis de expressão dos TLRs presentes em membrana plasmática, *TLRs* (*TLR1*, *TLR2*, *TLR4*, *TLR5*, *TLR6*) em astrocitomas de diferentes graus de malignidade (grau II-IV), tumor mais prevalente do Sistema Nervoso Central (SNC). Nós demonstramos que a expressão dos TLRs foi mais alta em amostras de astrocitomas comparadas com tecido cerebral não-neoplásico, por qRT-PCR. A expressão gênica e proteica foi observada em células de linhagem de glioblastoma (GBM) U87MG e A172, mostrando sua presença em células tumorais. Foi observada expressão associada entre os heterodímeros *TLR1-TLR2*. Em GBMs, o subtipo mesenquimal mostrou maior nível de expressão dos TLRs comparados aos subtipos clássico e proneural. Com o objetivo de identificar o papel dos TLRs nas células tumorais, foi selecionado dentre os TLRs o que apresentou maior nível de expressão, o *TLR4*, e realizamos ensaios funcionais estimulando a U87MG com LPS, um agonista natural para *TLR4*. A taxa de proliferação da célula tratada com LPS foi similar a não tratada. No entanto, foi observado a ativação do NF- κ B após 12hrs do estímulo com LPS. Quando a sinalização do receptor foi inibida por um composto químico (VGX-1027), o nível de proliferação da U87MG decaiu. Adicionalmente, análise *in silico* revelou uma forte associação dos TLRs hiperexpressos com aumento da expressão de genes relacionados à sinalização do ciclo celular, inflamassoma e ripoptossoma. O que sugere serem os TLRs alvos para complementação do tratamento do câncer.

Descritores: astrocitoma; glioblastoma; receptores Toll-like; proliferação celular; inflamação; fator nuclear – kappa B; lipopolissacarídeos.

1. Introduction

For the past decade, the inflammatory response in the tumor microenvironment has been in the spotlight due to the controversy regarding its ultimate effect. Despite the expectation that inflammation would wield a positive effect against tumorigenesis, relevant studies about the tumor-promoting effects of immune cells have been published. The resulting bioactive molecules from inflammation can provide the tumor microenvironment with growth factors that sustain proliferative signaling, with survival factors that impair apoptosis, along with proangiogenic and extra-cellular matrix-remodeling factors that facilitate invasion, metastasis, and the formation of new blood vessels (1).

Microglia are specialized cells that guarantee tissue homeostasis, in Central Nervous System. The microglia main functions are the phagocytic ability to remove dead or dying cells, preventing the tissue of an out of proportion inflammatory response (2, 3). Microglia has a role in regulation of neurogenesis (4-8), apoptosis (9-11), vessels formation (12-14), protection against neuronal damage (15-17), secretion of neurotrophic factors (18, 19), neuronal networks maturation (20), and synaptic pruning (21-25).

The homeostatic microglia have a very small soma and long branches to sense the environment, presenting a unique gene expression profile to this function (26, 27). When activated for an immunoinflammatory role, microglia present an unramified and ameboid form, and are proliferative and phagocytic (22, 27, 28). The homeostatic and active states define the microglia as plastic cells. Microglia present diverse response according to tissue environment (29). Microglial proliferative response in early phase of neurodegeneration and immune response in late-phase is one example of the plasticity of these cells (30, 31). Also, aging microglia present decreased gene expression for axonal guidance, cell adhesion and decreased sensory phenotype which are shaped by aging brain environment (32, 33).

Brain tumor is known to be heterogeneous presenting non-tumor cells like neuron, astrocytes, microglia and infiltrated macrophages in addition to

tumor cells. Interestingly, activation of microglia and macrophages may support tumor growth and my impact in the tumor biology (34). Microglia, in glioblastoma (GBM), present a pro-tumorigenic and pro-survival role due to the production of cytokines, growth factors and chemokines to attract monocytes (35-37). In the beginning of the neoplastic transformation, microglia induce tumor cells apoptosis. Some tumor cells are capable to evade the immune system and alter microglia functions to support tumor growth (38). Walentynowicz *et al* have observed microglia morphological transformation to phagocytic and tumor supporting profile after treatment with GBM conditioned medium (39).

Toll-like receptors (TLRs) act as the first line of defense, recognizing non self-molecules and activating inflammatory processes and their presence are classically described in immune cells (40). Therefore, TLRs have been considered as potential targets for tumor therapeutics (41, 42).

There are two subgroups of TLRs, based on the sub cellular location of these proteins and their respective pathogen-associated molecular pattern (PAMP) ligands. In humans, cell surface TLR1, TLR2, TLR4, TLR5, and TLR6 comprise one group, recognizing mainly microbial membrane components such as lipids, lipoproteins, and proteins. The other group consists of TLR3, TLR7, TLR8, and TLR9, which are expressed exclusively in intracellular vesicles, including endoplasmic reticulum (ER), endosomes, lysosomes, and endolysosomes, and recognizing microbial nucleic acids. TLR2 and TLR4 also identify endogenous molecules rising upon tissue injury, the damage-associated molecular patterns (DAMPs), such as heat-shock proteins, including HSP70, HSP60 Gp96, HSP22, and HSP72, and high-mobility group box-1, protein, as well as extra-cellular matrix (ECM) molecules such as biglycan, tenascin-C, versican, and fragments of ECM molecules (oligosaccharides of hyaluronic acid and heparan sulfate) (43).

Structurally, TLRs are integral membrane receptors, presenting a N-terminal ligand recognition domain, with a leucine-rich repeat motif, a single transmembrane helix, and a C-terminal cytoplasm signaling domain—known as Toll IL-1 receptor (TIR) domain due to the homology of signaling domains of IL-1R family members (44).

TLR1, TLR2, TLR4, TLR5, and TLR6 are involved in a diversity of cellular responses, ranging from cell proliferation to cell death, and as such they are the targets of the current study in astrocytomas. Cell surface TLRs may present distinct signaling pathways. Below is a brief summary of their signaling pathways and a schematic figure (Figure 1):

1) *Canonical signaling pathway* of plasmatic membrane TLRs involves myeloid differentiation primary response 88 (MYD88), which activates nuclear factor-kappa B (NF- κ B) by a protein complex with kinases that recruit tumor necrosis factor receptor associated factor 6 (TRAF6) and leads to pro-inflammatory cytokines production, such as interleukin-6 (IL-6), interleukin-1 β (IL-1 β) and α , tumor necrosis factor (TNF), interleukin-8 (IL-8) and interleukin-18 (IL-18). The final result of this pathway is a cell survival profile (45). NF- κ B canonical activation after TLR signaling is constituted by the heterodimerization of p65 and p50 subunits. After activation of the I κ B kinase, I κ B is phosphorylated, ubiquitinated, and degraded by the proteasome that enables NF- κ B translocation to nucleus, triggering transcriptional activity (46, 47). Several of such activated transcripts lead to the up regulation of positive regulators for cell cycle as cyclin D1, with a fundamental role in cellular division and DNA synthesis (48), c-Myc, responsible for enhancing expression of genes related to proliferation (49, 50), c-Jun (51), and serum response factor (SRF) (52, 53).

2) *Pathway through TLR2 dimerization with TLR1 or TLR6*. TLR2 can form a heterodimer complex with both TLR1 and TLR6 when activated. The heterodimerization of TLR2 with TLR1 or TLR6 expands the ligand spectrum, enabling the innate immune system to recognize different structures of pathogens associated molecular patterns. The downstream signal pathways remain the same for both heterodimers (54).

3) *Pathway through TLR4 endocytosis*, independent of MyD88, activating TIR-domain-containing adapter-inducing interferon- β (TRIF), with interferon type 1 response (55).

4) *Pathway through TLR4 activating ripoptosome complex formation*, composed of receptor-interacting serine/threonine kinase 1 and 3 (RIPK1/3) and mixed lineage domain kinase like pseudokinase (MLKL), tumor necrosis

factor receptor type 1-associated death domain protein (TRADD), and fas-associated protein with death domain (FADD). Ripoptosome activation can lead to apoptosis when caspase 8 (CASP8) is present, or to necroptosis when it is absent. TLR5 and TLR2 are indirectly involved in ripoptosome activation by TNF secretion (56-58).

5) *Pathway through TLR4, TLR2, and TLR5 forming inflammasome complex*, with the production of Caspase-1 and the pro-inflammatory cytokines IL-1 β and IL-18. The inflammasome complex is constituted by NLR family pyrin domain containing 3 (NLRP3), PYD and CARD domain containing (PYCARD), and procaspase 1 (pro-CASP1) (59, 60).

Figure 1. Schematic plasma membrane TLR signaling pathways. 1) *TLR canonical signaling pathway* involves MYD88 that activates, through a signaling cascade, NF- κ B and leads to pro-inflammatory cytokines production, such as IL-6, TNF, IL-8, IL-1 β and IL-1 α and IL-18 resulting in cell proliferation and cell survival profile. 2) *TLR4 endocytosis by endosome complex* signals through TIR-domain-containing adapter-inducing Interferon- β (TRIF), culminating in interferon type I secretion. 3) *TLR4 activates the ripoptosome complex formation*, which is composed of receptor RIPK1/3 and MLKL, TRADD and FADD. Ripoptosome leads to apoptosis when CASP8 is present or to necroptosis when it is absent. 4) The signal that activates TLR leading to its interaction with the ripoptosome can form an *inflammasome complex*, with the

production of IL-1 β , and IL-18. The latter two are pro-inflammatory cytokines. The inflammasome complex is composed of NLRP3, PYCARD, and CASP1.

The present study was performed in astrocytoma, a brain tumor with an astrocytic phenotype. The 2007 World Health Organization (WHO) Classification of Tumors of the Central Nervous System (CNS) divided astrocytomas in four malignant grades, I to IV. The astrocytoma grade I also known as pilocytic astrocytoma, present no infiltrative characteristics, the tumor cells present low proliferative profile and a good outcome after the cirurgical resection. Astrocytomas grade II to grade IV are known as diffusely infiltrative tumors: astrocytoma grade II is a diffuse astrocytoma, although presenting a low proliferative level with cytological atypia, it tends to progress to higher tumor malignancy. The presence of anaplasia and mitotic activity characterize the grade III astrocytoma, known as anaplastic astrocytoma. Presence of neovascularization and/or necrosis are observed in astrocytoma grade IV, glioblastoma, which is the most frequent malignant CNS tumor, with a median overall survival of 15 months under current standard of care treatment of total macroscopic surgical resection combined to radiotherapy and chemotherapy with the alkylating agent, temozolomide (61, 62).

With the onset of next generation sequencing, it was possible to assess the molecular landscape characterizing GBMs. In an attempt to better elucidate the involved molecular tumorigenic processes, and to better guide the adjuvant therapeutic strategies, three main molecular patterns, associated to somewhat distinct clinical outcomes, were identified: 1) the proneural subtype with molecular markers related to progenitor neuronal cells; 2) the classical subtype with markers of proliferative cells; and 3) the mesenchymal subtype with markers of epithelial-mesenchymal transition. Patients exhibiting molecular markers for the mesenchymal subtype presented poor prognosis and the worst response to standard of care, and proneural markers showed better prognosis (63-66). The result of this initiative prompted a new WHO classification, in 2016, which partially includes molecular aspects of GBMs as segment of its official characterization (67)

This study aims to analyze the presence of TLR (1, 2, 4, 5, and 6) expressions in astrocytoma tumor cells and to explore *in silico* the signaling

pathways under their up regulation. In particular, the phenotype alteration of U87MG-GBM cells by TLR4 activation and the involved signaling pathway was analyzed. The differential expression of TLR4 in the tumor cell and microglia compartments was explored aiming to better understand the microenvironment impact in tumor aggressiveness.

.

2. Aims

General aims:

- 1) to analyze plasmatic membrane TLRs expression levels in diffusely infiltrative astrocytomas WHO grades II to IV and their presence in GBM cell lines.
- 2) to analyze the signaling pathways involved in TLR4 activation in U87MG-GBM cell line

Specific aims:

- 1) to analyze the gene and protein expressions of TLR1, TLR2, TLR4, TLR5, TLR6 in astrocytomas, in GBM cases, according to molecular stratification and GBM cell lines, and to validate the findings in The Cancer Genome Atlas (TCGA) dataset.
- 2) to analyze the putative signaling pathways related to the TLRs in the TCGA-RNASeq dataset.
- 3) To analyze the TLR4 protein expression in tumor cell and microglia compartments and their modulation according to the tumor microenvironment.
- 4) analyze U87MG phenotype alteration after TLR4 activation by LPS.
- 5) to analyze TLR4 signaling pathway related to TLR4 activation by LPS stimulation through *TLR4* chemical inhibition.
- 6) to explore the impact of the microglia compartment in the TLR4 expression resulted of the astrocytoma samples.

3. Materials and Methods

3.1. Tumor samples and ethical statement

The studied cases were composed of 140 astrocytoma grade II-IV samples, collected during therapeutic surgical intervention in the Neurology Department of the Hospital das Clinicas of University of Sao Paulo School of Medicine, by the institutional neurosurgery group. The cases were stratified according to the WHO classification as: 22 non-neoplastic (NN) cases from epilepsy surgery, 26 astrocytoma grade II (AGII) cases, 18 astrocytoma grade III (AGIII) cases, and 96 astrocytoma grade IV (GBM) cases, this cohort has been previously published (68, 69). The procedures were performed with informed and approved consent according to the Institutional Ethical Committee guidelines at the Hospital das Clinicas of University of Sao Paulo School of Medicine (691/05). The present study was approved by the same institution (059/15) to use the biorepository.

3.2. Sample preparation and RNA extraction

The samples were macrodissected and frozen in liquid nitrogen right after the surgical removal, then cryosectioned for RNA extraction. For the tumor tissue analysis, a 6 µm thick section and hematoxylin-eosin staining was made to guarantee the absence of necrotic, gliosis, non-neoplastic areas, and more than 80% of tumor cells in all tumor tissue specimens (70, 71).

The RNA extraction was accomplished by the RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer instructions. The RNA concentration and purity were evaluated by NanoDrop, and 1.8-2.0 values for 260 nm and 280 nm absorbance ratios were considered satisfactory. RNA quality was checked by electrophoresis in agarose gel. Reverse transcription was performed with 1 µg of RNA treated with DNase I (FPLC-puro, GE Healthcare, Uppsala, Sweden), and amplified with random primers and oligodT

oligonucleotides, RNase inhibitor, and the SuperScript III reverse transcriptase (Thermo Fisher Scientific, Carlsbad, CA), following the manufacturer instructions. Finally, the cDNA was treated with RNase H (GE Healthcare, Uppsala, Sweden), diluted in TE (Tris/EDTA) buffer and stored at -20°C for posterior use by quantitative real-time PCR (qRT-PCR) analysis.

3.3. Quantitative Real-Time PCR (qRT-PCR)

TLR1, *TLR2*, *TLR4*, *TLR5*, and *TLR6* mRNA levels were evaluated by qRT-PCR, using Power SYBR Green. The results were normalized with the geometrical mean of three reference genes for each sample, as previously described (72): hypoxanthine phosphoribosyltransferase (*HPRT*), glucuronidase beta (*GUSB*), and TATA box-binding protein (*TBP*). The primers were designed to amplify 80-120pb, with melting temperature around 60°C and synthesized by Exxtend (Campinas, Brazil) and IDT (Coralville, IA). The primers were designed as described in Table 1.

Table 1. Used primer sequences

Gene	forward primer (5'-3')	reverse primer (5'-3')
<i>TLR1</i>	GGCACCCCTACAAAAGGAATC	GATAATGGCAAAATGGAAGATGCT
<i>TLR2</i>	TGTGGGTTGAAGCACTGGAC	TTGCGGTCACAAGACAGAGAAG
<i>TLR4</i>	TTTATCCAGGTGTGAAATCCAGAC	TCCAGAAAAGGCTCCCAGG
<i>TLR5</i>	CATACTCCTGATGCTACTGACAACG	GCAGATGAGAGTAGGGAAGTCCA
<i>TLR6</i>	AAACGGCACATTCTTCCACAA	TTTGTCGTTGTTGTTACTGTGGTTG
Reference gene		
<i>HPRT</i>	TGAGGATTTGGAAAGGGTGT	AGCACACAGAGGGCTACAA
<i>GUSB</i>	GAAAATACGTGGTTGGAGAGCTCATT	CGAGTGAAGATCCCCCTTTTA
<i>TBP</i>	AGGATAAGAGAGCCACGAACCA	CTTGCTGCCAGTCTGGACTGT

Primers were optimized to the minimum concentration for the minor cycle threshold (Ct), the maximum amplification efficiency, and minimum nonspecific amplifications. The mix was composed by cDNA (3 µl), Power SYBR Green PCR Master Mix (Thermo Fisher Scientific, Carlsbad, CA) (6 µl), and the reverse and forward primers (3 µl of each). The qRT-PCR was done in duplicate using the ABI Prism 7500 (Thermo Fisher Scientific, Carlsbad, CA) following the protocol: 2 minutes at 50°C, 10 minutes in 95°C, and 40 cycles of 15 seconds at

95°C, and 1 minute at 60°C. The expression values were assessed by the formula $2^{-\Delta C_t}$ as ΔC_t is: the C_t of analyzed gene – geometric mean C_t of the reference genes.

3.4. Immunofluorescence

The presence of TLR proteins in tumor cells was analyzed by immunofluorescence. A172 and U87MG human GBM cell lineages were acquired from ATCC and authenticated by short tandem repeats (STR) analysis using GenePrint 10 System (Promega, Madison, WI). Cells were cultured in monolayer with DMEM medium (Dulbecco's Modified Eagle's Medium, (Thermo Fisher Scientific, Carlsbad, CA), 10% fetal bovine serum and 100 µg/ml streptomycin and 100 IU/ml penicillin.

Cells were fixed with methanol and acetone (1:1), the membrane was permeabilized with Triton-X-100 (0.1%), and, to avoid unspecific reactions, the cells were treated with 2% bovine serum albumin. The primary antibodies anti-TLR1 (ab180798, rabbit polyclonal, 1:400 diluted), anti-TLR2 FITC-conjugated (AB_945852, ab59711, mouse monoclonal, 1:50 diluted), anti-TLR4 (AB_446735, ab22048, mouse monoclonal 1:200 diluted), anti-TLR5 (AB_793183, sc-57461, mouse monoclonal, 1:50 diluted, Santa Cruz, CA), and anti-TLR6 (AB_2205406, ab37072, rabbit polyclonal, 1:50 diluted) (Abcam, Cambridge, UK) were incubated overnight at 4°C. The secondary antibodies (Thermo Fisher Scientific, Carlsbad, CA) goat anti-Rabbit IgG H&L (Alexa Fluor 568) and goat anti-Mouse IgG H&L (Alexa Fluor 568 and 488) were incubated for one hour, and nuclei were stained with DAPI (Thermo Fisher Scientific, Carlsbad, CA). The preparations were analyzed in confocal microscopic Zeiss 510 LSM META and Zeiss 780-NLO (Thornwood, NY). The retrieved images were analyzed by Image J/Fiji (73).

3.5. Immunohistochemistry

Paraffin embedded tissue of five representatives cases of GBM, and five non-neoplastic brain tissue from surgical epilepsy cases were stained for the

five TLRs, IBA1, CD68 and GFAP by immunohistochemistry using the Novolink kit (Novolink; Novocastra, Newcastle-upon-Tyne, UK), following the manufacture guide. The sections were processed to antigen retrieval, by citrate buffer (10mM, pH6.0) for 3 minutes at 122°C, using an electric cooker (BioCare Medical, Walnut Creek, USA). After protein blocking, the tissue was incubated with antibodies: anti-TLR1(ab180798, rabbit polyclonal, 1:800 diluted), anti-TLR2 (AB_307008, ab9100, mouse monoclonal, 1:50 diluted), anti-TLR4 (AB_446735, ab22048, mouse monoclonal 1:800 diluted), anti-TLR5 (AB_793183, sc-57461, mouse monoclonal, 1:500 diluted, Santa Cruz, CA), anti-TLR6 (AB_2205406, ab37072, rabbit polyclonal, 1:200 diluted) (Abcam, Cambridge, UK), anti-CD68 (AB_2687454, M0718, mouse monoclonal, 1:1600 diluted, DAKO, CA), anti-IBA1 (AB_2224403, ab15690, mouse monoclonal, 1:700 diluted, Abcam, Cambridge, UK), anti-GFAP (AB_10013382, Z0334, rabbit polyclonal, 1:600, DAKO, CA) at 20° for 16 hours. The reaction performed by the kit uses diaminobenzidine (DAB), and for nuclear staining Harris hematoxylin was used. To obtain optimal dilution tonsil sections was used. The immunoreactions for the five TLRs were analyzed according to a semi-quantitative score system considering the intensity of staining (0: negative, 1: weak, 2: moderate and 3: strong), and percentage of immune-positive cells (0: no cells stained, 0.5: 1-10%, 1: 10-25%, 2: 26-50% 3: 51-75% and 4: 76-100%). An immunolabeling score (ILS) was obtained by the product of the intensity of staining and the percentage of stained cells, by two independent investigators (IFM, SKNM), and simultaneous revision were performed to obtain the final score in case the concordance was not achieved. Digital photomicrographs of representative fields were captured and processed using Adobe Illustrator CS6 (Adobe System, San Jose, CA).

3.5.1. Immunohistochemistry in tissue microarray (TMA) preparations

Three representative areas of the core of each tumor tissue were extracted with diameter of 0.6mm using an array machine (MTA-1, Beecher Instruments Inc., Sun Prairie,WI, USA). A TMA block was produced by the

insertion of the cores in predetermined areas and produced 3µm thickness sections that was paraffin coated and store in -20°C until use (74). The tissue cohort is composed of 142 GBM cases.

For TMA sections, the immunohistochemistry was performed as described above and prepared with anti-TLR4 (AB_446735, ab22048, mouse monoclonal 1:800 diluted) and anti-IBA1 (1:1000 diluted, 019-19741 Wako Chemicals USA, Inc.).

3.6. TLR4 activation in U87MG cell

U87MG cells were stimulated with lipopolysaccharide (LPS) from *Escherichia coli* O127:B3 (Sigma-Aldrich, St. Louis, MO, USA) a known agonist for TLR4 (75). For the proliferation assays the concentration used was 1µg/ml, 10µg/ml and 100µg/ml and the stimulation was done for up to 96 hours. An optimal concentration of 10µg/ml was chosen for the following assays: TLR4 inhibition by chemical compound and NF-kB activation assay.

3.7. Cellular proliferation assay

U87MG cells were plated in 96 wells (2×10^3 cell/well), stimulated in three different concentrations of LPS 1µg/ml, 10µg/ml and 100µg/ml and compared to non-stimulated group. All groups were evaluated in sextuplicate. The plates were observed in four time periods of 24hrs, 48hrs, 72hrs e 96hrs. The cell proliferation was analyzed by the Presto Blue reagent (Thermo Fisher Scientific, Waltham, MA, US), following the manufacture instructions. An additional spot with only the Presto Blue reagent was evaluated and used to normalize the fluorescence results. The fluorescence of each well was measured by the Glomax (Promega, Madison, WI, US). Presto Blue reagent is a resazurin based assay, it indicates the cell viability by the ability of living cell to reduce the non-fluorescent blue dye to resorufin, a red highly fluorescent component.

3.8. NF-kB translocation to nucleus

To analyze NF- κ B activation by TLR4 stimulation, the translocation of the subunit p65 to nucleus was evaluated by Western Blot. Cytoplasm and nuclear protein extractions were performed of U87MG cells after LPS treatment. The cells were lysed with a buffer solution containing light detergents to separate the cytoplasm proteins: Igepal-CA-630 (0.5%) (Sigma Aldrich, St. Louis, EUA), Triton X-100 (0.25%) (Sigma Aldrich, St. Louis, US), Glycerol (10%) (*Thermo Fisher Scientific*, Waltham, MA, US), 1mM EDTA, 50mM Hepes, 140mM de NaCl. After centrifugation, nuclei were lysed with a buffer containing strong detergent and sonicated with 50mM Tris-HCl, 10mM EDTA e SDS (1%). For the Western Blotting, 40 μ g of protein was loaded in each lane of 4-12% polyacrylamide gel (Invitrogen, Carsbald, CA, US), in a buffer solution NuPAGE MOPS SDS (Invitrogen, Carsbald, CA, US). The proteins were transferred electrophoretically to a polyvinylidene fluoride membrane through the Trans-Blot® SD semi-dry system (*Trans-Blot® Transfer Cell*, Biorad, Hercules, CA, US). For the protein identification, p65 (Abcam, ab32536, Cambridge, UK, 1:500 dilution) and Histone H3 total (ab12079, Abcam, Cambridge, UK, 1:20000) antibodies were used. The secondary antibodies were anti-rabbit and anti-goat (Sigma-Aldrich, St. Louis, MO, EUA). The bands were revealed by the chemiluminescent reagent ECL (*Western Lightning Chemiluminescence Reagent Plus*, Perkin Elmer, Waltham, MA, US) in the ImageQuant LAS4000 (GE Healthcare, Little Chalfont, UK).

3. 9. TLR4 chemical inhibition

TLR4 was also blocked by a commercial immune modulator VGX-1027 (Git-27) (Abcam, Cambridge, RU) currently used for the treatment of immune-mediated inflammatory diseases (76). 1 μ M of this compound from a previous 1mM dilution in ethanol was used for the inhibition assays.

3.10. Statistical analysis

The statistical analysis was performed in the program SPSS version 23.0 (IBM, Armonk, NY). The data distribution was evaluated by Kolmogorov-

Smirnov test. The non-parametric results were evaluated by Kruskal-Wallis test, followed by post hoc Dunn's test. The correlations between each two receptors gene expression levels were assessed by Spearman-rho. The associations are considered strong when $r \geq 0.7$, moderate when $0.4 < r < 0.7$, and low when $r \leq 0.4$. Differences were considered significant for $p < 0.05$.

The GBM RNAseq dataset from *The Cancer Genome Atlas* (TCGA - <http://cancergenome.nih.gov/>) was downloaded from *.rsem.genes.normalized_ results address. The data was analyzed by DE-seq and the RPKM values were normalized by the z-score.

For the proliferation assay was performed the test Two-way Anova followed by the post hoc test Bonferroni. Differences were considered significant for $p < 0.05$.

4. Results

4.1. *TLR1*, *TLR2*, *TLR4*, *TLR5*, and *TLR6* mRNAs were up-regulated in diffuse astrocytomas

Genes coding the five receptors was analyze by qRT-PCR, *TLR1*, 2, 4, 5, and 6, and presented higher expression levels in astrocytoma samples compared to non-neoplastic brain tissues, with statistical significance ($p < 0.05$) (Figure 2A). Only *TLR2* expression in AGII did not present any statistical difference compared to NN tissue, in contrast to the statistical difference observed in AGIII and GBM ($p < 0.05$). *TLR1*, *TLR2*, and *TLR6* mRNA expression median values showed an increase in parallel to the increase of malignancy. On the other hand, the median *TLR4* expression in GBM cases was lower than in the other astrocytoma grades (AGII-AGIII).

4.2. Associated *TLRs* expressions were observed in GBM

The correlation between each gene was analyzed. All *TLRs* expressions were correlated between themselves in GBM, with statistical significance of $p < 0.05$ (Figure 2B). Expression levels of *TLR2* and *TLR1* presented the strongest correlation ($r = 0.8448$, $p < 0.0001$ by Spearman-rho test). The correlation indicates the activation and heterodimerization for *TLR1* and *TLR2* in the tumor tissue.

Figure 2. *TLR1*, *TLR2*, *TLR4*, *TLR5*, and *TLR6* expression levels in astrocytomas of different malignant grades. (A) The analyzed samples consisted of 22 non-neoplastic (NN) cases, 26 astrocytoma grade II (AGII) cases, 18 astrocytoma grade III (AGIII) cases, and 96 glioblastoma (GBM) cases. Data are represented by box and whisker plots, with the median represented by the line in the middle of the boxes, and top and bottom boxes represent the first and third quartiles. qRT-PCR values are normalized by three housekeeping genes (*HPRT*, *GUSB*, *TBP*). For statistical analysis, Kruskal-Wallis and Dunn's tests were applied, wherein (*) $p < 0.05$ when compared to NN cases and (†) $p < 0.05$ when compared to AGII (Dunn test), all the genes present $p < 0.01$ (Kruskal-Wallis). (B) Correlation between *TLR2*-*TLR1*, *TLR2*-*TLR6*, *TLR2*-*TLR4*, *TLR2*-*TLR5*, *TLR4*-*TLR1*, *TLR4*-*TLR5*, and *TLR4*-*TLR6* are demonstrated in GBM cases. Statistical analysis was made by the Spearman-rho correlation, and $p < 0.05$ were considered significant.

4.3. Higher *TLR4* and *TLR6* median expression levels were observed in mesenchymal GBM compared to other molecular subtypes

The analysis of TLRs expression levels for GBM molecular subtypes showed that mesenchymal GBM subtype presented higher *TLR4* and *TLR6* mRNA median levels than other molecular subtypes in our cohort (Figure 3) (77). However, given the reduced sample number, particularly for the proneural and mesenchymal GBM cases in this cohort, statistical significance was not achieved. Therefore, we performed a similar analysis in molecular GBM subtypes of TCGA public dataset. In this larger cohort, all five TLRs presented higher expression in mesenchymal subtype with statistical significance compared to proneural and classical subtypes (Figure 4).

Figure 3. *TLR1*, *TLR2*, *TLR4*, *TLR5*, and *TLR6* expression levels in the GBM subtypes of our cohort composed of 14 proneural (PN), 38 classical (CS) and 16 mesenchymal (MES) subtype cases. Horizontal bars indicate the mean value of each group.

Figure 4. *TLR1*, *TLR2*, *TLR4*, *TLR5*, and *TLR6* expression levels in GBM molecular subtypes from TCGA dataset. Data are represented by box and whisker plots, with the median represented by the line in the middle of the boxes, and top and bottom boxes represent the first and third quartiles. The dataset was divided into 37 proneural (PN) cases, 40 classical (CS) subtype cases, and 55 mesenchymal (MES) subtype cases, in which (*) and (†) are $p < 0.05$ when mesenchymal group was compared to proneural cases and to classical cases, respectively (Dunn's test) and $p < 0.01$ (Kruskal-Wallis).

4.4. *TLR1*, *TLR2*, *TLR4*, *TLR5*, and *TLR6* were present in tumor cell lines

Aiming to identify the distribution of the TLR proteins in specific cell compartment we performed an immunofluorescence in pure tumor cells of two GBM cell lineages, U87MG and A172. The five TLR proteins were identified in the cells, both GBM cell lineages with mutational status consistent for the mesenchymal subtype (U87MG and A172): RB transcriptional co repressor (*RB1*) missense mutation in A172, and neurofibromin (*NF1*) missense and deletion mutation in U87MG (78). For the immunofluorescence observation (Figure 5), TLR5 presented lower protein expression in U87MG compared to A172. The evaluation of these *TLR* mRNA levels by qRT-PCR corroborated

their presence in both cell lineages; *TLR5* mRNA expression level was not detected in U87MG cells by qRT-PCR (Figure 6).

Figure 5. Immunofluorescence of TLR1, TLR2, TLR4, TLR5, and TLR6 in GBM cell lines. A172 (A) and U87MG (B). TLR1, TLR4, and TLR6 are stained in red, TLR2 and TLR5 in green, and nuclei in blue by DAPI. The presence of all five TLRs was detected in both cell lines. Expression of TLR5 was more intense

in A172 compared to U87MG. TLR4 and TLR5 positivity were detected in both tumor lineage cells nuclei. Magnification of 400x.

Figure 6. *TLR1*, *TLR2*, *TLR4*, *TLR5*, and *TLR6* expression levels in U87MG and A172 cell lines. Heterogeneity in the distribution of the five *TLRs* is observed between the cell lines, although both GBM cell lines present somatic mutation profiles of mesenchymal subtype. *TLR4* expression is high in both cell lines, whereas *TLR5* is undetectable in U87MG cell line.

4.5. *TLR1*, *TLR2*, *TLR4*, *TLR5*, and *TLR6* protein presence in GBM tissue

The five *TLRs* proteins were detected, by immunohistochemistry, in phenotypically GBM cells in all cases, including on the multinucleated GBM cells (Figure 7), corroborating the findings observed in the GBM cell lineages. The *TLR4* protein expression was the highest among the five studied *TLRs*. Interestingly, *TLR4* and *TLR5* protein expressions were also observed in tumor cell nuclei, as observed in the immunofluorescence preparations of the GBM cell lineages (Figure 5). The presence of few microglia in the GBM tumor sample were detected by IBA1 staining and few macrophages with CD68, which did not overlap with the *TLRs* positive tumor cells. The positive reaction for GFAP was consistent with the glial origin of the analyzed GBM tumor samples (Figure 8). Non-neoplastic brain samples were also stained for *TLRs*. As expected, the positivity for these receptors was observed in neurons and the

lowest expression was for TLR2. Similar to the staining pattern in tumor cells, TLR5 positivity was detected in neuron nuclei (Figure 8).

Figure 7. Immunohistochemistry for TLR1, TLR2, TLR4, TLR5, and TLR6 in GBM cases. Positive immunolabeling of GBM tumor cells for TLRs are demonstrated at 600x magnification of tumor tissues, and at 800x magnification in a multinucleated GBM tumor cell. The distribution of the Immunolabeling score (ILS) for these TLRs in five GBM cases was presented as a dispersion graph, where the black dots represent the mean ILS obtained by the two independent investigators for each individual tumor case, and the horizontal bar represent the mean ILS for each receptor. TLR4 and TLR5 positive staining were detected in tumor nuclei.

Figure 8. TLR1, TLR2 TLR4, TLR5, and TLR6 protein expression in non-neoplastic cases and microglia and macrophage detection in GBM sample. Immunohistochemistry for a representative non-neoplastic case stained for TLR1, TLR2, TLR4, TLR5, TLR6 and GFAP for glial cell identification. Presence of few microglia in the same GBM sample present previously was observed by IBA1 staining, and also few macrophages was detected by CD68 staining. GFAP positivity was shown in the GBM tumor sample confirming the glial origin of the tumor.

4.6. *In silico* exploration of TLR signaling pathway

A heatmap including selected target genes of TLR signaling pathways was built from the TCGA RNAseq dataset, identifying up- and down-regulated genes for each GBM subtype (Figure 9). The RPKM values (Attachment 1 – Supporting information Figure) were normalized by the z-score values for each gene to allow comparative analysis among them. *In silico* analysis highlighted the higher expression of TLRs in mesenchymal subtype relative to proneural and classical subtypes (Figure 4), and pointed out a downstream up-regulation through MYD88/TIRAP and NF- κ B, ending with increased expression levels of

genes that code for cytokines and genes related to cell proliferation. Particularly, expression levels of genes that code for IL-8, IL-6, IL-1 α , IL-1 β , and IL-18 were differentially increased in the mesenchymal GBM subtype compared to the other two subtypes (Attachment 1 - Supporting information Figure). Moreover, *RELA*, which codes for the nuclear subunit of NF- κ B, presented positive correlations ($p < 0.05$) by Spearman-rho test with expression levels of cell proliferation genes: *JUN* ($r = 0.422$ and $r = 0.536$) and *SRF* ($r = 0.462$ and $r = 0.577$) and in mesenchymal and classical subtypes, respectively. In contrast, the gene expressions related to interferon type I response presented low or undetermined z-score values (not shown). Interestingly, expression levels of genes involved in ripoptosome signaling, such as *RIPK3* and *MLKL*, were also comparatively increased in mesenchymal GBM subtype, and their values correlated to *TLR2* ($r = 0.795$ and $r = 0.661$, respectively), and to *TLR4* ($r = 0.575$ and $r = 0.360$, respectively). Of note, *PYCARD*, *NLRP3* and *CASP1*, which participate in the inflammasome pathway, presented higher expression levels in mesenchymal subtype of GBM compared to the other two subtypes (Attachment 1 – Supporting information Figure).

Figure 9. Heatmap with major genes of the TLR signaling pathways from the TCGA dataset. RPKM gene expression levels are normalized by z-scores, and comparatively up-regulated RNA expression values are presented in red and down-regulated values in blue. Mean values are in white. TLRs downstream signaling pathways: canonical, ripoptosome, and inflammasome

pathways are activated in mesenchymal GBM subtype. Genes of unrelated pathways were added to show their random expression levels, including a microglia marker.

4.7. TLR4 presence in human astrocytomas

4.7.1 TLR4 expression is the highest among the plasma membrane TLRs in human astrocytomas and U87MG cell line

The *TLR4* transcript expression was higher in AGII, AGIII and GBM compared to non-neoplastic brain tissue (NN)(Figure 10A). Particularly in GBM, a wide spread of *TLR4* transcription level was observed, and the highest expression was demonstrated in mesenchymal subtype, both in our GBM cohort (Figure 10B) and in the TCGA RNASeq dataset, with statistical significance in the latter (Figure 10C).

Figure 10. *TLR4* expression. Data for the *TLR4* mRNA levels observed by qRT-PCR indicating the importance of the target. (A) Comparison of *TLR4* expression levels in the astrocytoma grades (AGII-GBM) and NN, in our cohort. (B) The expression levels of *TLR4* in the GBM molecular subtypes: proneural (PN), classical (CS) and mesenchymal (MES) in our cohort, and in the TCGA RNAseq dataset (C). Mesenchymal subtype presented the highest expression level, being significant in the TCGA dataset compared to classical and proneural subtype ($p < 0.05$ Kruskal-Wallis and Dunn test).

4.8. The impact of inhibiting *TLR4* in U87MG cells

We analyze the viability by Presto blue of the U87MG cell line after *TLR4* activation. Proliferative profile after 24 hours of stimulation was observed when stimulated with high concentration of LPS (10 μ g/ml and 100 μ g/ml) (Figure 11A

and B). The presence of p65 in the cell nuclei compartment after 12 hrs of LPS stimulus was demonstrated by western blot (Figure 11C).

Figure 11. GBM U87MG cell line proliferative profile after LPS stimulation. (A) U87MG cells were treated with 3 different concentrations of LPS (1 $\mu\text{g/ml}$, 10 $\mu\text{g/ml}$ and 100 $\mu\text{g/ml}$) and cell proliferation was evaluated by Presto Blue reagent. The graphs represent levels of fluorescence at each time point and LPS concentration. (B) and the ratio with the non-treated cells. (C) Nuclear and cytoplasm protein extractions were performed after 2hs and 12hs of LPS treatment with 10 $\mu\text{g/ml}$. The p65 and Histone H3 proteins were demonstrated in cytoplasm and nucleus compartments by western blot, and the presence of p65 in nuclei was detected after 12hs of LPS stimulus.

4.8.1 TLR4 pathway pharmacological inhibition

Chemical blockage of TLR4 signaling pathway was tested by VGX-1027 treatment which resulted in a decrease of U87MG cell line proliferation rate, after LPS stimulus (Figure 12). These findings corroborate the participation of the TLR4 in a signaling pathway leading to a proliferative phenotype of U87MG cells.

Figure 12. TLR4 pathway inhibitor. TLR4 signaling pathway inhibition by VGX-1027 (1 μ M) diluted in ethanol (vehicle). The treatment analysis was performed after 4 different time points by Presto Blue. To analyze TLR4 inhibition, U87MG were treated with LPS (10 μ g/ml). The values were normalized by the U87MG non-treated cells, wherein * p <0.05 (Two-way ANOVAs and post hoc Bonferroni) and are present in relation to the non-treated cells **(A)** and the proliferation curve for each variable, the control (black), the cells treated only by the vehicle and LPS (10 μ g/ml) (light blue) and the cells treated by the VGX-1027 and LPS (10 μ g/ml) (dark blue) **(B)**.

4.8.2 TLR4 in tumor and microglia cells crosstalk

To analyze the distribution of TLR4 protein expression among specific cell compartments in GBM tumor, sequential GBM-TMA sections were stained for microglia IBA1 marker and TLR4. We highlighted different niches that were observed with distinct patterns of immunoreaction for both markers (Figure 13). Interestingly, microglia compartment showed increased in vascularized and necrotic areas with an activated profile. Furthermore, TLR4 was expressed in microglial cells on those areas. Phenotypically tumor cells showed increased TLR4 not only in cytoplasm but also in nuclei. However, the abundance of TLR4 protein expression varied further according to the density of microvessels, and to the proximity of necrotic area. Tumor areas with surveillant microglia phenotypes showed absent for TLR4 immunoreaction.

Figure 13. Immunohistochemistry of sequential TMA preparations of GBM cases for IBA1 microglia marker, and TLR4. A-B: positively IBA1 stained cells, microglia, were distributed surrounding vessels interspersed by TLR4 positive tumor cells. The small GBM cells in the bulk of tumor were negative for TLR4. **C-D:** quiescent microglia with narrow cytoplasmic ramifications were distributed randomly among tumor cells not immunoreacting for TLR4. **E-F:** another spot from an area of high vascular density of the same GBM case of C-D shows activated microglia with amoeboid morphology around the vessels and few slightly stained tumor cells for TLR4. **G-H:** GBM tumor cells completely surrounded by activated microglia, with intense positivity for TLR4. Tumor cell plasma membrane shows clear positivity for TLR4 as the endothelial cells. **I-J:** Activated microglia are distributed predominantly around vessels, and a gradient of positive staining for TLR4 is observed in tumor cells, with increase towards the center of vascularized tumor area. **K-L:** perinecrotic area of the same GBM case of I-J shows high density of microglia and intensely positive tumor cells for TLR4.

5. Discussion

In the present study, higher expression levels of *TLR1*, *TLR2*, *TLR4*, *TLR5*, and *TLR6* were demonstrated in human diffusely infiltrating astrocytomas (grades II to IV) in comparison to non-neoplastic brain tissue. Moreover, an associated expression of these receptors was observed in GBM cases, suggesting their role in tumor aggressiveness. The presence of TLR4 has been previously described in GBM, particularly in CD133⁺ tumor stem cells (79, 80), and also in GBM cell lineages A172, U87MG (41, 81, 82), and U251 (83). Such TLR4 positivity conferred a proliferative phenotype to these tumor cells (79, 83). Furthermore, the presence of TLR2 was also detected in murine GL261 glioma cell line, and the activation of this receptor leads to an invasive and migratory profile of the tumor cells (84). We further demonstrated herein the presence of TLR1, TLR2, TLR5, and TLR6 in U87MG and A172 cell lineages and in human GBM specimens, at gene and protein expression levels. TLR4 was the most expressed receptor in both cell lineages and tumor specimens. Interestingly, TLR4 and TLR5 positivity were detected in the tumor cell nuclei by immunofluorescence in cell lineages and by immunohistochemistry in tumor specimens. However, such observation needs confirmation in other cohorts before speculating their possible role in this localization. In spite of both cell lineages presenting somatic mutation profiles of mesenchymal molecular subtype of GBM, they present distinct expression profiles of these TLRs. U87MG cells showed higher expression of *TLR1*, *TLR2*, and *TLR4* compared to A172, in contrast to lower expression of *TLR5*. Such heterogeneity of these TLRs expression distributions was also observed in human astrocytoma samples from our cohort and in the TCGA dataset, particularly among GBMs, where higher expression levels were detected in mesenchymal subtype. To analyze the impact of these differential TLRs expressions among the molecular subtypes of GBM, we built a heatmap with the expression levels of the genes involved in pathways related to TLRs from the TCGA dataset (Figure 9). Interestingly, this approach showed clearly the downstream activation of the

canonical, ripoptosome, and inflammasome pathways, related to the up regulation of the TLRs, particularly in the mesenchymal subtype of GBM. The end targets of these pathways, including cytokines (*IL1A/B*, *IL6*, *IL8*, *IL18*) and genes related to cell proliferation (*JUN*, *SRF*), were up regulated, suggesting that the activation of these TLRs leads to tumor growth. In fact, activation of the TLR canonical pathway through TIRAP-MYD88 was related to NF- κ B (*RELA*) up regulated expression levels and positive correlation with *JUN*, and *SRF*, transcription factors implicated in cellular proliferation. The role of NF- κ B in tumor growth has been demonstrated in a mouse model of GBM (85) and in pulmonary tumor cells (86). Additionally, it has been reported that such a proliferative response may be time- and dose-dependent (87). Therefore, tumor inflammatory microenvironment may contribute to distinct NF- κ B pulsation-determining tumor cell behavior in each specific condition. The end targets of this pathway, IL-6, IL-1 α and IL-1 β , and IL-8 cytokines, presented increased expression in the mesenchymal subtype, and they have also been associated to tumor malignancy and tumor cell migration (60, 88-90). *IL1B* and *IL18* are also end targets of the inflammasome pathway, which were up regulated in the mesenchymal subtype. On the other hand, the increased expression of *RIPK3* and *MLKL* may suggest the up regulation of the ripoptosome pathway, and may allow the link with the presence of necrosis, one of the characteristics of GBM. In a rat glioma model, apoptosis was induced by activation of CASP8 and inhibition of RIPK1/RIPK3 complex (57, 91, 92).

The expression of *CASP8* was increased in the mesenchymal subtype of GBM; however, increased apoptosis is not expected and, therefore, avoidance of apoptosis should be under additional modulation. Moreover, according to the present *in silico* analysis, the interferon type I response by TLR4 endocytosis was unlikely.

Another hypothesis for the proliferative profile and TLR signaling in GBM is the pathway involving PTEN, which has been shown to regulate TIRAP and TLR4 internalization (93, 94), in addition to its classic suppressor role in the PI3K-AKT-mTOR pathway (95). Loss of function of PTEN may occur by its deletion or phosphorylation (65, 96). Our previous analysis of PTEN status in our GBM cohort demonstrated 24.32% and 26.66% of *PTEN* deletion, and

59.45% and 66.66% of PTEN phosphorylation in classical and mesenchymal subtypes, respectively (Figure 14) (77, 96), demonstrating more prevalent *PTEN* loss of function in the classical subtype. These *PTEN* alterations may lead to a decreased inhibition over TIRAP (Figure 15) and consequent TLR canonical pathway activation. In B cells, the absence of *PTEN* showed an increased in NF- κ B activity after TLR4 stimulation (97). Additionally NF- κ B negatively regulates *PTEN* (98, 99). Accordingly, in the classical subtype, loss of *PTEN* may also be implicated in tumor cell proliferation through TLR canonical activation (Figure 15). Actually, our analysis of the TCGA dataset showed significant correlation between *TIRAP* and *PTEN* expression levels among the classical GBM subtype ($r = 0.330$, $p < 0.05$, Spearman-rho test). Interestingly, a negative correlation was found between them in the proneural subtype ($r = -0.382$, $p < 0.05$), which presents better prognosis among the three analyzed GBM subtypes.

Figure 14. PTEN. The GBM cohort from our lab was previously analyzed for *PTEN* mutational and phosphorylation status. The number of cases for each status of *PTEN* and GBM subtype is presented. In green is the amount of cases presenting wild-type *PTEN*, in black the deleted *PTEN*, and in orange the Y240-phosphorylated *PTEN*.

Figure 15. Schematic proposition of the TLR canonical signaling pathway through TIRAP by PTEN regulation. PTEN alterations may lead to up regulation of TLR signaling pathway and may increase tumor cell proliferation. Loss of PTEN repressor role by its deletion or phosphorylation, as may occur more frequently in classical subtype, may activate downstream TLR canonical pathway through the decreased inhibition over TIRAP. This pathway through TIRAP-PTEN may not be the major mechanism in mesenchymal subtype, and the integrity of PTEN may inhibit TIRAP and then not activate this pathway in proneural subtype.

To further understand the TLRs role in tumor cells we chose to perform functional analysis targeting *TLR4*, whom expression level was higher compared to the others TLRs and also higher in the U87MG GBM tumor cell line. This cell line presents mesenchymal molecular subtype pattern, harboring Neurofibromin 1 (*NF1*) mutation (78). We designed functional assays employing LPS, a known agonist for TLR4 that activates pro-inflammatory pathways, in wild type U87MG cells and inhibited by a *TLR4* blocking pharmacological compound (VGX-1027). Interestingly, no proliferative response was observed in the wild-type U87MG cell treated with LPS, and a reasonable explanation for such observation was that the pro-proliferative signaling pathways might be already saturated in this mesenchymal subtype GBM cell line, and an activation of additional pro-proliferative pathway resulted in any phenotypic change. In fact, although no

significant change was detected, the NF- κ B nuclear translocation was demonstrated after 12 hours of LPS stimulation. The observed late phase of NF- κ B activation after 12 hours from the stimulation suggests that the activation did not occur through a canonical pathway, NF- κ B translocation to the nucleus is described early phases of the activation, ceasing after 100 minutes (87). The current finding might indicate a non-canonical TRIF dependent pathway activation (100)

The proliferative response of U87MG cells has been previously associated to NF- κ B activation (101, 102). Additionally, pro-inflammatory response of increased proliferation after TLR4 stimulation with cytokines production has been reported in tumor cells of different origins as hepatocarcinoma, esophagus, ovarian and melanoma after TLR4 stimulation (103-107). And, *in vivo* models of different tumors harboring up regulated TLR4 have shown increased tumor cell survival and the receptor inhibition has resulted in smaller tumors and prolonged animal survival (108). TLR4 has also been described related to drug resistance and metastasis (109). In fact, in the present study, the downregulation of TLR4 signaling pathway by chemical blockade with VGX-1027, resulted in a decrease of U87MG cell proliferation. VGX-1027 administration has been shown to decrease IL-1 β and TNF secretion and to downregulate NF- κ B and p38 MAP kinase (76). The VGX-1027 has been implicated in the attenuation LPS effect. Also, *in vitro*, *ex vivo* and *in vivo* models, the VGX-1027 has downregulated inflammation, mainly of macrophages (110). In two clinical studies, the VGX-1027 downregulated inflammation in rheumatoid disease (111).

However, TLR4 may also activate a pro-apoptotic pathway by CASP8 under inhibitory modulation of c-FLIP (CASP8 and FADD like apoptosis regulator - *CFLAR*) (112). The protein c-FLIP promotes cell survival preventing either apoptotic signaling by CASP8 or necroptotic signaling by RIPK1/RIPK3 (113).

The final response due to TLR4 activation will depend of tumor microenvironment, which is dynamically changing. TLR4 was present in tumor cells, but also in brain innate inflammatory cells, as microglia (Figure 15), and therefore both cells are theoretically prone to DAMPs, such as heat-shock

proteins (HSP), and extra-cellular matrix (ECM) molecules (43). GBM tumor cell migration as a process dependent of EGFR activated by TLR4 bound to HSP90, a DAMP signal, has been previously reported (83, 109, 114). Therefore, TLR4 activation has been associated to tumor promotion (115, 116). However, the complete range of PAMPs and DAMPs related to TLR4 as the precise mechanism of their activation pathways are still under investigation (117, 118).

Several evidences indicate the pro-tumorigenic effect of microglia in gliomas (66, 119-123). In the present study, we identified microglia expressing TLR4 protein around vessels and hypoxic areas. We speculated, based on these findings that TLR4 may lead GBM microglia to change to a pro-tumorigenic profile. Indeed, tumor microglia expressing TLR4 may be prone to tumor released activating factors. Among these factors, IL-6 secreted by the tumor cell may activate tumor microglia to this profile through TLR4. And, in response, microglia may produce IL-10 a cytokine capable to abrogate immune response (124, 125). Moreover, microglia also expresses TLR2, which can also be activated by DAMPs. TLR2 is also associated to glioma-immune system evasion and production of tumor growth factors (126, 127). Additionally, microglia has been described around vessels and hypoxic areas (128). Necrotic cells may stimulate cytokine production, angiogenic factors, as vascular endothelial growth factor (VEGF) (129-132), and ECM modulators (133) by tumor cells and microglia (102), a process that can be mediated by TLRs. Angiogenesis and pro-inflammatory response stimulation by necrotic cells are considered cancer hallmarks (1).

Thus, further studies of the TLR responses from each tumor compartment, which includes inflammatory cells in addition to tumor cells, will be essential to better understand the tumor behavior and to develop new strategies to try to control its aggressiveness.

6. Conclusions

- 1) Increased cell membrane *TLR1*, *TLR2*, *TLR4*, *TLR5*, and *TLR6* expressions were demonstrated in astrocytomas compared to non-neoplastic brain tissue, mostly in GBM group, and particularly in the mesenchymal subtype.
- 2) *In silico* exploration of the putative activation pathways by these up regulated TLRs, the canonical and inflammasome pathways in the mesenchymal subtype and the PTEN-TIRAP signaling in the classical subtype were demonstrated and their possible contribution to tumor growth.
- 3) According to the TCGA dataset the microglia compartment based on *AIF1* expression level did not parallel the *TLRs* up regulated expressions in mesenchymal subtype, suggesting that the tumor cells themselves may contribute to the increased expression of these receptors.
- 4) TLR4 activation by LPS stimulation lead to NF-kB late translocation to nucleus in U87MG cell and, the *TLR4* chemical blockage resulted in a U87MG cell proliferation decrease.
- 5) Microglia also present TLR4 expression and the final phenotype due to this receptor activation may be determined by the tumor microenvironment dynamics.

7. References

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8. Attachment

8.1. - Supporting information figure

Supporting information Figure. Expression levels of the genes participating in the TLR signaling pathways categorized by GBM molecular subtypes: proneural, classical, and mesenchymal from the TCGA RNASeq dataset. The dataset is composed of 37 proneural (PN), 40 classical (CS), and 55 mesenchymal (MES) subtype cases, wherein (*) $p < 0.05$ when compared to proneural cases and (†) $p < 0.05$ when compared to classical cases by Kruskal-Wallis and Dunn's test. The values were normalized in DEseq.

8.2. Attachment 2: Cep Feedback

APROVAÇÃO

O Comitê de Ética em Pesquisa da Faculdade de Medicina da Universidade de São Paulo, em sessão de 04/03/2015, APROVOU o Protocolo de Pesquisa nº 059/15 intitulado: “EXPRESSÕES DOS RECEPTORES DO TIPO TOLL EM ASTROCITOMAS HUMANOS DE DIFERENTES GRAUS DE MALIGNIDADE” apresentado pelo Departamento de NEUROLOGIA.

Cabe ao pesquisador elaborar e apresentar ao CEP-FMUSP, os relatórios parciais e final sobre a pesquisa (Resolução do Conselho Nacional de Saúde nº 466/12, inciso IX.2, letra "c").

Pesquisador (a) Responsável: Suely Kazue Nagahashi Marie

Pesquisador (a) Executante: Isabele Fattori Moretti

CEP-FMUSP, 06 de Março de 2015.

Prof. Dr. Paulo Eurípedes Marchiori
Vice-Coordenador
Comitê de Ética em Pesquisa

Comitê de Ética em Pesquisa da Faculdade de Medicina
e-mail: cep_fm@usp.br

APROVAÇÃO

O Comitê de Ética em Pesquisa da Faculdade de Medicina da Universidade de São Paulo, em sessão de 12/08/2015, APROVOU o Protocolo de Pesquisa nº 278/15 intitulado: "PAPEL DO FATOR DE TRANSCRIÇÃO MITOCONDRIAL A COMO AGONISTA DE RECEPTORES TIPO TOLL EM LINHAGENS CELULARES DE GLIOBLASTOMAS" apresentado pelo Departamento de NEUROLOGIA

Cabe ao pesquisador elaborar e apresentar ao CEP-FMUSP, os relatórios parciais e final sobre a pesquisa (Resolução do Conselho Nacional de Saúde nº 466/12, inciso IX.2, letra "c").

Pesquisador (a) Responsável: Suely Kazue Nagahashi Marie

Pesquisador (a) Executante: Katia Guimarães Benigno

CEP-FMUSP, 12 de Agosto de 2015.

Prof. Dr. Roger Chammas
Coordenador
Comitê de Ética em Pesquisa

CIÊNCIA

O Comitê de Ética em Pesquisa da Faculdade de Medicina da Universidade de São Paulo, em sessão de 09 de Março de 2016, TOMOU CIÊNCIA do(s) documento(s) abaixo mencionado(s) no Protocolo de Pesquisa nº 278/15, intitulado: "Papel do Fator de Transcrição Mitochondrial A como Agonista de Receptores Tipo Toll em Linhagens Celulares de Glioblastomas" apresentado pelo NEUROLOGIA.

• Mudança de Pesquisador Executante para Isabele Fattori Moretti

Pesquisador (a) Responsável: Suely Kazue Nagahashi Marie

CEP-FMUSP, 09 de Março de 2016

Profa. Dra. Maria Aparecida Azevedo Koike Folgueira
Coordenador
Comitê de Ética em Pesquisa

8.3. Attachment 3: qRT-PCR data

Diagnosis	Cases	Ct TLR1	mean Ct TLR1	Ct TLR2	mean Ct TLR2	Ct TLR4	mean Ct TLR4	Ct TLR5	mean Ct TLR5	Ct TLR6	mean Ct TLR6	Ct TBP	mean Ct TBP	Ct HPRT	mean Ct HPRT	Ct GUS-b	mean Ct GUS-b	Geometric mean Housekeeping	TLR1 ΔCt	TLR1 2 ^{-ΔCt}	TLR2 ΔCt	TLR2 2 ^{-ΔCt}	TLR4 ΔCt	TLR4 2 ^{-ΔCt}	TLR5 ΔCt	TLR5 2 ^{-ΔCt}	TLR6 ΔCt	TLR6 2 ^{-ΔCt}
NN	108	29.72	29.71	30.61	30.55	28.99	28.79	30.81	30.61	29.90	30.05	28.36	28.42	22.68	22.58	24.48	24.30	24.98	4.73	0.04	5.57	0.02	3.81	0.07	5.63	0.02	5.07	0.03
NN	108	29.7		30.49		28.58		30.41		30.20		28.48		22.47		24.11												
NN	132	29.28	29.25	28.17	28.11	27.40	27.52	29.24	29.20	32.66	32.80	27.66	27.69	23.93	23.99	25.02	25.17	25.57	3.68	0.08	2.54	0.17	1.95	0.26	3.63	0.08	7.23	0.01
NN	132	29.21		28.05		27.63		29.15		32.94		27.71		24.05		25.31												
NN	167	32.33	32.28	Ind.	Ind.	28.90	29.05	31.00	31.14	Ind.	Ind.	25.37	25.58	21.75	21.84	24.39	24.59	23.95	8.33	0.00	Ind.	Ind.	5.10	0.03	7.19	0.01	Ind.	Ind.
NN	167	32.23		Ind.		29.19		31.27		Ind.		25.79		21.93		24.78												
NN	173	Ind.	Ind.	31.01	30.935	29.49	29.24	32.49	32.42	Ind.	Ind.	28.15	28.2	24.35	24.51	26.16	26.33	26.30	Ind.	Ind.	4.63	0.04	2.94	0.13	6.12	0.01	Ind.	Ind.
NN	173	Ind.		30.86		28.99		32.35		Ind.		28.25		24.67		26.49												
NN	179	28.04	28.01	28.88	28.895	26.01	26.01	28.58	28.80	28.40	28.23	25.97	26.12	21.44	21.55	22.39	22.65	23.36	4.65	0.04	5.54	0.02	2.65	0.16	5.44	0.02	4.87	0.03
NN	179	27.97		28.91		26.00		29.02		28.06		26.27		21.65		22.9												
NN	189	28.85	29	30.39	30.66	27.42	27.54	30.87	30.68	30.23	30.39	28.07	28.08	22.96	23.15	25.39	25.23	25.40	3.59	0.08	5.26	0.03	2.13	0.23	5.27	0.03	4.98	0.03
NN	189	29.14		30.93		27.65		30.48		30.54		28.08		23.33		25.07												
NN	196	32.17	32.12	30.75	30.665	28.65	28.60	31.88	31.38	Ind.	Ind.	27.91	27.78	24.45	24.49	25.61	25.74	25.96	6.16	0.01	4.70	0.04	2.63	0.16	5.42	0.02	Ind.	Ind.
NN	196	32.07		30.58		28.54		30.88		Ind.		27.64		24.52		25.87												
NN	226	Ind.	Ind.	30.31	30.105	27.16	27.11	30.72	30.37	30.32	30.24	25.78	26.07	21.99	22.06	24.35	24.46	24.14	Ind.	Ind.	5.97	0.02	2.97	0.13	6.23	0.01	6.10	0.01
NN	226	Ind.		29.9		27.06		30.02		30.16		26.36		22.13		24.56												
NN	232	30.69	30.96	29.81	30.065	28.42	28.44	30.01	30.08	31.20	31.24	27.11	27.07	22.57	22.80	24.73	24.81	24.83	6.13	0.01	5.24	0.03	3.61	0.08	5.25	0.03	6.41	0.01
NN	232	31.22		30.32		28.46		30.15		31.28		27.02		23.02		24.88												
NN	237	30.8	30.82	29.23	29.145	28.38	28.59	31.23	31.15	31.74	31.69	26.76	26.61	22.51	22.61	22.45	22.53	23.84	6.98	0.01	5.31	0.03	4.75	0.04	7.31	0.01	7.85	0.00
NN	237	30.83		29.06		28.79		31.07		31.63		26.45		22.7		22.6												
NN	245	31.22	31.31	30.87	30.845	29.20	28.97	31.55	31.35	Ind.	Ind.	27.23	27.13	23.54	23.73	25.25	25.38	25.37	5.94	0.02	5.47	0.02	3.60	0.08	5.98	0.02	Ind.	Ind.
NN	245	31.4		30.82		28.74		31.15		Ind.		27.03		23.91		25.51												
NN	263	Ind.	Ind.	33.89	33.78	30.53	30.50	34.76	35.60	Ind.	Ind.	29.08	29.32	27.11	27.10	28.08	28.28	28.22	Ind.	Ind.	5.56	0.02	2.28	0.21	7.38	0.01	Ind.	Ind.
NN	263	Ind.		33.67		30.47		36.44		Ind.		29.55		27.09		28.47												
NN	312	Ind.	Ind.	30.67	30.61	28.98	28.79	31.81	32.20	Ind.	Ind.	28.45	28.52	25.92	26.09	27.54	27.36	27.31	Ind.	Ind.	3.30	0.10	1.48	0.36	4.89	0.03	Ind.	Ind.
NN	312	Ind.		30.55		28.59		32.58		Ind.		28.59		26.26		27.18												
NN	330	Ind.	Ind.	30	29.745	27.72	27.67	30.59	30.58	Ind.	Ind.	27.22	27.33	24.11	24.30	24.66	24.67	25.40	Ind.	Ind.	4.35	0.05	2.27	0.21	5.18	0.03	Ind.	Ind.
NN	330	Ind.		29.49		27.62		30.57		Ind.		27.43		24.49		24.67												
NN	332	34.88	35.15	31.01	30.76	30.21	30.34	33.07	33.15	Ind.	Ind.	28.63	28.64	27.95	28.01	27.54	27.50	28.04	7.11	0.01	2.72	0.15	2.29	0.20	5.10	0.03	Ind.	Ind.
NN	332	35.42		30.51		30.46		33.22		Ind.		28.65		28.06		27.46												
NN	349	32.18	32.13	29.26	29.235	28.46	28.33	Ind.	Ind.	Ind.	Ind.	29.85	29.64	26.89	26.84	26.17	26.40	27.59	4.54	0.04	1.65	0.32	0.74	0.60	Ind.	Ind.	Ind.	Ind.
NN	349	32.08		29.21		28.20		Ind.		Ind.		29.43		26.79		26.62												
NN	714	31.43	30.96	29.39	29.47	28.17	28.19	Ind.	Ind.	Ind.	Ind.	27.42	27.56	25.01	24.84	25.34	25.46	25.93	5.03	0.03	3.54	0.09	2.26	0.21	Ind.	Ind.	Ind.	Ind.
NN	714	30.49		29.55		28.21		Ind.		Ind.		27.7		24.67		25.58												
NN	755	30.69	30.36	27.9	27.945	27.74	27.54	Ind.	Ind.	Ind.	Ind.	26.99	27.11	24.5	24.41	24.92	25.13	25.52	4.83	0.04	2.42	0.19	2.01	0.25	Ind.	Ind.	Ind.	Ind.
NN	755	30.02		27.99		27.33		Ind.		Ind.		27.22		24.31		25.34												
NN	805	Ind.	Ind.	28.4	28.22	28.15	28.08	29.89	30.22	Ind.	Ind.	26.62	26.88	24.21	24.32	24.48	24.66	25.26	Ind.	Ind.	2.96	0.13	2.82	0.14	4.96	0.03	Ind.	Ind.
NN	805	Ind.		28.04		28.01		30.54		Ind.		27.13		24.42		24.83												
NN	861	Ind.	Ind.	28.79	28.56	28.03	28.05	26.37	26.28	Ind.	Ind.	26.43	26.53	24.74	24.66	25.35	25.24	25.46	Ind.	Ind.	3.10	0.12	2.58	0.17	0.81	0.57	Ind.	Ind.
NN	861	Ind.		28.33		28.06		26.18		Ind.		26.62		24.57		25.13												

Diagnosis	Cases	Ct TLR1	mean Ct TLR1	Ct TLR2	mean Ct TLR2	Ct TLR4	mean Ct TLR4	Ct TLR5	mean Ct TLR5	Ct TLR6	mean Ct TLR6	Ct TBP	mean Ct TBP	Ct HPRT	mean Ct HPRT	Ct GUS-b	mean Ct GUS-b	Geometric mean Housekeeping	TLR1 Δ Ct	TLR1 2 ^{-ΔCT}	TLR2 Δ Ct	TLR2 2 ^{-ΔCT}	TLR4 Δ Ct	TLR4 2 ^{-ΔCT}	TLR5 Δ Ct	TLR5 2 ^{-ΔCT}	TLR6 Δ Ct	TLR6 2 ^{-ΔCT}
NN	929	Ind.	Ind.	28.73	28.825	27.47	27.53	Ind.	Ind.	Ind.	Ind.	27.48	27.55	25.54	25.69	25.36	25.32	26.16	Ind.	Ind.	2.66	0.16	1.36	0.39			Ind.	Ind.
NN	929	Ind.		28.92		27.58		Ind.		Ind.		27.61		25.83		25.27												
NN	969	Ind.	Ind.	29.01	28.96	29.04	28.94	25.93	26.02	Ind.	Ind.	27.25	27.25	24.77	24.89	25.27	25.40	25.83	Ind.	Ind.	3.13	0.11	3.11	0.12	0.19	0.87	Ind.	Ind.
NN	969	Ind.		28.91		28.84		26.11		Ind.		27.25		25.01		25.53			Ind.									
AGII	55	28.49	28.51	28.2	28.29	26.12	26.10	29.10	29.27	29.52	29.67	25.94	26.14	25.43	25.59	23.97	24.16	25.28	3.23	0.11	3.01	0.12	0.82	0.57	3.98	0.06	4.39	0.05
AGII	55	28.53		28.38		26.08		29.43		29.82		26.33		25.75		24.35												
AGII	76	29.54	29.36	29.48	29.285	27.12	27.29	30.51	30.42	31.64	31.50	26.25	26.4	26.87	26.87	25.27	25.39	26.21	3.15	0.11	3.08	0.12	1.08	0.47	4.21	0.05	5.29	0.03
AGII	76	29.17		29.09		27.46		30.32		31.35		26.55		26.86		25.5												
AGII	101	29.26	29.37	27.91	28.015	28.17	28.27	28.80	28.73	Ind.	Ind.	26.33	26.33	27.18	27.39	24.46	24.62	26.09	3.28	0.10	1.93	0.26	2.18	0.22	2.64	0.16	Ind.	Ind.
AGII	101	29.47		28.12		28.36		28.66		Ind.		26.33		27.6		24.77												
AGII	118	Ind.	Ind.	32.57	32.65	30.58	30.68	31.93	31.94	Ind.	Ind.	29.56	29.83	28.99	29.01	27.83	27.95	28.92	Ind.	Ind.	3.73	0.08	1.76	0.30	3.02	0.12	Ind.	Ind.
AGII	118	Ind.		32.73		30.77		31.94		Ind.		30.1		29.02		28.07												
AGII	239	Ind.	Ind.	29.42	29.6	26.84	26.83	30.70	30.53	Ind.	Ind.	26.2	26.31	26.97	27.12	24.91	24.85	26.07	Ind.	Ind.	3.53	0.09	0.75	0.59	4.46	0.05	Ind.	Ind.
AGII	239	Ind.		29.78		26.81		30.36		Ind.		26.41		27.27		24.79												
AGII	250	32.56	32.49	31.98	32.195	29.36	29.23	33.11	33.27	Ind.	Ind.	28.21	27.92	25.32	25.50	26.36	26.45	26.60	5.88	0.02	5.59	0.02	2.63	0.16	6.66	0.01	Ind.	Ind.
AGII	250	32.41		32.41		29.10		33.42		Ind.		27.63		25.67		26.54												
AGII	254	30.13	30.14	Ind.	Ind.	26.24	26.31	29.06	29.20	32.29	32.04	27.35	27.34	27.18	27.36	25.67	26.10	26.93	3.21	0.11			-0.62	1.54	2.27	0.21	5.11	0.03
AGII	254	30.15		Ind.		26.37		29.33		31.78		27.33		27.54		26.52												
AGII	267	30.16	30.29	30.89	30.61	26.74	26.97	29.95	29.73	Ind.	Ind.	26.11	26.25	25.3	25.35	25.79	25.78	25.79	4.50	0.04	4.82	0.04	1.18	0.44	3.94	0.07	Ind.	Ind.
AGII	267	30.42		30.33		27.19		29.50		Ind.		26.39		25.39		25.76											Ind.	
AGII	328	31.72	31.45	30.49	30.295	29.57	29.44	31.25	31.40	Ind.	Ind.	27.49	27.38	24.09	23.99	26.78	26.70	25.98	5.47	0.02	4.32	0.05	3.46	0.09	5.42	0.02	Ind.	Ind.
AGII	328	31.18		30.1		29.30		31.54		Ind.		27.27		23.89		26.61											Ind.	
AGII	341	30.37	30.66	31.57	31.42	26.78	26.83	29.55	29.67	30.58	30.64	26.62	26.55	26.51	26.60	25.32	25.26	26.13	4.53	0.04	5.29	0.03	0.70	0.62	3.54	0.09	4.51	0.04
AGII	341	30.95		31.27		26.87		29.79		30.69		26.47		26.69		25.19												
AGII	346	28.07	27.98	27.59	27.65	27.36	27.53	28.04	28.13	29.86	29.85	27.18	27.12	25.57	25.55	24.75	24.73	25.78	2.20	0.22	1.87	0.27	1.75	0.30	2.35	0.20	4.07	0.06
AGII	346	27.89		27.71		27.69		28.22		29.84		27.05		25.52		24.7												
AGII	392	31.68	31.8	31.2	31.065	28.97	28.68	29.29	29.65	Ind.	Ind.	26.76	26.92	25.53	25.48	26	26.15	26.17	5.62	0.02	4.89	0.03	2.51	0.18	3.48	0.09	Ind.	Ind.
AGII	392	31.91		30.93		28.39		30.01		Ind.		27.07		25.43		26.29											Ind.	
AGII	412	30.76	31.01	29.84	30.09	29.10	29.10	30.02	30.30	32.08	31.96	32.23	32.43	29.02	29.10	26.64	26.62	29.28	1.73	0.30	0.81	0.57	-0.18	1.13	1.01	0.50	2.68	0.16
AGII	412	31.26		30.34		29.10		30.57		31.84		32.62		29.17		26.59												
AGII	452	28.25	28.37	26.85	26.885	25.17	25.18	28.13	28.18	29.08	28.87	26.64	26.68	26.33	26.54	24.67	24.95	26.04	2.32	0.20	0.84	0.56	-0.87	1.82	2.14	0.23	2.82	0.14
AGII	452	28.48		26.92		25.18		28.23		28.65		26.71		26.75		25.22												
AGII	453	30.13	30.22	29.83	29.665	28.82	29.01	28.72	28.75	28.59	28.58	27.77	27.86	28.75	28.95	27.42	27.37	28.05	2.16	0.22	1.61	0.33	0.95	0.52	0.69	0.62	0.53	0.69
AGII	453	30.3		29.5		29.19		28.77		28.57		27.95		29.14		27.32												
AGII	467	30.09	30.21	30.11	30.345	27.19	27.17	30.08	30.06	30.11	30.14	24.88	25.06	24.62	24.59	24.96	24.85	24.83	5.38	0.02	5.51	0.02	2.33	0.20	5.22	0.03	5.30	0.03
AGII	467	30.33		30.58		27.14		30.03		30.16		25.24		24.56		24.73												
AGII	490	28.61	28.43	29.21	29.315	25.76	25.67	28.55	28.59	29.26	29.26	27.51	27.54	26.45	26.50	24.47	24.49	26.14	2.29	0.20	3.17	0.11	-0.48	1.39	2.44	0.18	3.12	0.12
AGII	490	28.25		29.42		25.57		28.62		29.26		27.56		26.54		24.5												
AGII	577	31.29	31.03	30.19	29.995	27.34	27.28	30.16	30.12	31.25	31.46	27.06	27.15	26.73	26.94	24.9	24.90	26.31	4.72	0.04	3.69	0.08	0.97	0.51	3.81	0.07	5.15	0.03
AGII	577	30.77		29.8		27.21		30.08		31.67		27.23		27.14		24.89												

Diagnosis	Cases	Ct TLR1	mean Ct TLR1	Ct TLR2	mean Ct TLR2	Ct TLR4	mean Ct TLR4	Ct TLR5	mean Ct TLR5	Ct TLR6	mean Ct TLR6	Ct TBP	mean Ct TBP	Ct HPRT	mean Ct HPRT	Ct GUS-b	mean Ct GUS-b	Geometric mean Housekeeping	TLR1 ΔCt	TLR1 2 ^{-ΔCt}	TLR2 ΔCt	TLR2 2 ^{-ΔCt}	TLR4 ΔCt	TLR4 2 ^{-ΔCt}	TLR5 ΔCt	TLR5 2 ^{-ΔCt}	TLR6 ΔCt	TLR6 2 ^{-ΔCt}
AGII	715	Ind.	Ind.	32.17	32.37	Ind.	Ind.	33.49	33.34	Ind.	Ind.	31.42	31.37	27.88	27.78	27.36	27.34	28.77	Ind.	Ind.	3.60	0.08			4.57	0.04	Ind.	Ind.
AGII	715	Ind.		32.57		Ind.		33.19		Ind.		31.31		27.67		27.31			Ind.								Ind.	
AGII	806	34.5	34.24	31.5	31.35	29.72	29.56	33.81	34.05	Ind.	Ind.	29.43	29.19	28.56	28.62	28.01	28.24	28.68	5.56	0.02	2.67	0.16	0.88	0.54	5.37	0.02	Ind.	Ind.
AGII	806	33.97		31.2		29.40		34.29		Ind.		28.95		28.68		28.46											Ind.	
AGII	999	29.81	29.99	30.03	29.925	27.39	27.39	30.09	30.05	31.14	30.88	25.74	25.86	25.46	25.38	24.89	24.98	25.40	4.58	0.04	4.52	0.04	1.98	0.25	4.65	0.04	5.48	0.02
AGII	999	30.16		29.82		27.38		30.01		30.62		25.97		25.3		25.06												
AGII	1016	31.97	32.05	31.91	31.85	29.47	29.44	31.07	31.07	32.76	32.95	26.89	26.77	27.1	27.08	26.14	26.20	26.68	5.37	0.02	5.17	0.03	2.76	0.15	4.39	0.05	6.27	0.01
AGII	1016	32.13		31.79		29.41		31.07		33.14		26.64		27.06		26.26												
AGII	1095	28.31	28.59	28.23	28.3	26.35	26.62	28.38	28.33	28.91	28.78	25.75	25.71	26.94	27.13	25.48	25.66	26.15	2.44	0.18	2.15	0.23	0.47	0.72	2.18	0.22	2.62	0.16
AGII	1095	28.87		28.37		26.89		28.28		28.64		25.67		27.31		25.83												
AGII	1099	30.55	30.55	29.51	29.435	26.07	26.02	29.43	29.60	30.66	30.63	26.69	26.84	26.03	26.20	26.55	26.42	26.49	4.06	0.06	2.95	0.13	-0.47	1.39	3.11	0.12	4.14	0.06
AGII	1099	30.55		29.36		25.96		29.77		30.59		26.99		26.37		26.29												
AGII	1113	28.61	28.68	28.12	28.07	25.18	25.14	27.26	27.42	29.61	29.57	26.06	26.26	26.01	26.13	24.53	24.64	25.66	3.02	0.12	2.41	0.19	-0.53	1.44	1.76	0.30	3.91	0.07
AGII	1113	28.75		28.02		25.09		27.58		29.53		26.45		26.25		24.75												
AGII	1155	29.71	29.7	27.72	27.88	27.24	27.20	28.45	28.61	30.63	30.53	26.67	26.84	24.45	24.45	24.41	24.65	25.29	4.41	0.05	2.59	0.17	1.91	0.27	3.32	0.10	5.24	0.03
AGII	1155	29.68		28.04		27.16		28.76		30.43		27		24.44		24.89												
AGIII	28	29.84	29.68	27.66	27.8	26.03	26.03	29.49	29.40	31.48	31.45	28.32	28.45	27.37	27.22	26.04	26.23	27.28	2.39	0.19	0.52	0.70	-1.26	2.39	2.12	0.23	4.16	0.06
AGIII	28	29.51		27.94		26.02		29.31		31.41		28.58		27.06		26.42												
AGIII	34	31.89	32.15	30.31	30.41	28.21	28.25	32.09	31.93	33.25	33.33	26.46	26.6	26.51	26.58	24.77	26.75	26.64	5.51	0.02	3.77	0.07	1.61	0.33	5.29	0.03	6.69	0.01
AGIII	34	32.4		30.51		28.29		31.77		33.40		26.73		26.64		28.73												
AGIII	73	31.07	30.93	28.91	28.945	29.66	29.86	30.19	30.26	32.70	32.42	30.32	30.45	27.44	27.31	25.51	25.44	27.66	3.27	0.10	1.29	0.41	2.20	0.22	2.60	0.16	4.76	0.04
AGIII	73	30.79		28.98		30.05		30.33		32.14		30.58		27.18		25.36												
AGIII	233	Ind.	Ind.	31.26	31.05	30.35	30.27	29.95	29.84	Ind.	Ind.	29.37	29.3	26.16	26.33	25.2	25.71	27.07	Ind.	Ind.	3.98	0.06	3.20	0.11	2.77	0.15	Ind.	Ind.
AGIII	233	Ind.		30.84		30.18		29.73		Ind.		29.22		26.49		26.22												
AGIII	249	29.38	29.66	29.18	29.24	27.29	27.57	29.01	29.04	31.80	31.75	26.55	26.75	24.74	24.81	23.56	23.61	25.02	4.63	0.04	4.22	0.05	2.55	0.17	4.01	0.06	6.72	0.01
AGIII	249	29.93		29.3		27.85		29.06		31.69		26.94		24.88		23.66												
AGIII	338	31.09	31.31	29.95	29.965	28.51	28.51	30.62	30.88	Ind.	Ind.	31.28	31.17	28.38	28.29	26.95	26.65	28.64	2.67	0.16	1.33	0.40	-0.13	1.10	2.24	0.21	Ind.	Ind.
AGIII	338	31.52		29.98		28.50		31.14		Ind.		31.06		28.19		26.34												
AGIII	347	34.11	34.06	30.55	30.525	32.54	32.74	33.80	33.01	Ind.	Ind.	31.42	31.37	30.5	30.37	28.03	28.20	29.95	4.11	0.06	0.58	0.67	2.79	0.14	3.06	0.12	Ind.	Ind.
AGIII	347	34.01		30.5		32.93		32.22		Ind.		31.31		30.24		28.37												
AGIII	360	30.04	30.06	29.97	29.795	27.20	27.24	29.72	29.76	31.06	31.04	27.58	27.46	26.03	26.07	28.83	27.14	26.88	3.17	0.11	2.91	0.13	0.36	0.78	2.87	0.14	4.15	0.06
AGIII	360	30.07		29.62		27.28		29.79		31.01		27.34		26.1		25.44												
AGIII	366	32.14	32.17	31.31	31.125	30.87	30.71	32.48	33.05	Ind.	Ind.	28.93	28.86	27.21	27.30	24.6	24.64	26.87	5.30	0.03	4.25	0.05	3.84	0.07	6.18	0.01	Ind.	Ind.
AGIII	366	32.2		30.94		30.55		33.62		Ind.		28.78		27.38		24.67												
AGIII	410	Ind.	Ind.	30.22	30.375	26.04	25.88	28.57	28.45	30.05	30.10	29.12	29.24	25.99	26.17	25.2	25.22	26.82	Ind.	Ind.	3.56	0.09	-0.94	1.92	1.63	0.32	3.28	0.10
AGIII	410	Ind.		30.53		25.72		28.33		30.15		29.36		26.34		25.23												
AGIII	428	31.47	31.42	29.37	29.38	30.21	30.20	31.21	31.26	Ind.	Ind.	28.25	28.5	27.3	27.20	25.31	25.41	27.01	4.41	0.05	2.37	0.19	3.19	0.11	4.25	0.05	Ind.	Ind.
AGIII	428	31.36		29.39		30.19		31.31		Ind.		28.75		27.1		25.5												
AGIII	478	27.22	27.2	26.77	26.875	25.69	25.69	28.34	28.27	27.56	27.53	27.12	27.12	23.86	23.83	23.22	23.16	24.64	2.56	0.17	2.23	0.21	1.05	0.48	3.63	0.08	2.89	0.14
AGIII	478	27.18		26.98		25.69		28.20		27.50		27.12		23.8		23.09												

Diagnosis	Cases	Ct TLR1	mean Ct TLR1	Ct TLR2	mean Ct TLR2	Ct TLR4	mean Ct TLR4	Ct TLR5	mean Ct TLR5	Ct TLR6	mean Ct TLR6	Ct TBP	mean Ct TBP	Ct HPRT	mean Ct HPRT	Ct GUS- b	mean Ct GUS-b	Geometric mean Housekeeping	TLR1 Δ Ct	TLR1 2 ^{-ΔCt}	TLR2 Δ Ct	TLR2 2 ^{-ΔCt}	TLR4 Δ Ct	TLR4 2 ^{-ΔCt}	TLR5 Δ Ct	TLR5 2 ^{-ΔCt}	TLR6 Δ Ct	TLR6 2 ^{-ΔCt}
AGIII	514	26.26	26.16	26.29	26.345	24.74	24.76	27.57	27.67	28.48	28.62	27.32	27.15	25.89	25.88	23.73	23.92	25.62	0.54	0.69	0.73	0.60	-0.86	1.81	2.05	0.24	3.00	0.12
AGIII	514	26.06		26.4		24.78		27.76		28.76		26.98		25.87		24.11												
AGIII	734	29.05	29.11	27.84	27.795	30.36	30.10	31.54	31.81	33.17	33.03	30.17	30.19	28.1	28.20	26.02	25.99	28.07	1.04	0.49	-0.28	1.21	2.03	0.24	3.73	0.08	4.95	0.03
AGIII	734	29.17		27.75		29.84		32.07		32.88		30.21		28.29		25.95												
AGIII	905	Ind.	Ind.	30.98	31.085	30.20	30.05	31.55	32.05	Ind.	Ind.	30.56	30.41	29.36	29.24	26.64	26.81	28.78	Ind.	Ind.	2.31	0.20	1.27	0.41	3.27	0.10	Ind.	Ind.
AGIII	905	Ind.		31.19		29.90		32.55		Ind.		30.25		29.12		26.97												
AGIII	981	29.6	29.53	29.06	29.005	28.21	28.18	30.01	29.84	31.05	31.25	26.75	26.9	25.35	25.42	24.66	24.85	25.71	3.82	0.07	3.30	0.10	2.47	0.18	4.13	0.06	5.54	0.02
AGIII	981	29.46		28.95		28.14		29.66		31.45		27.05		25.48		25.04												
AGIII	1033	30.15	30.3	28.9	28.94	28.85	28.95	30.03	29.97	Ind.	Ind.	27.33	27.48	27	27.09	26.02	26.15	26.90	3.40	0.09	2.04	0.24	2.05	0.24	3.07	0.12	Ind.	Ind.
AGIII	1033	30.45		28.98		29.04		29.91		Ind.		27.63		27.17		26.28												
AGIII	1036	33.16	33.05	32.8	32.915	31.32	31.23	31.89	31.80	Ind.	Ind.	31.73	31.84	29.03	29.22	27.26	27.34	29.41	3.64	0.08	3.51	0.09	1.82	0.28	2.39	0.19	Ind.	Ind.
AGIII	1036	32.93		33.03		31.13		31.71		Ind.		31.94		29.4		27.42												
GBM	35	26.17	26.26	25.18	25.16	26.28	26.29	26.67	26.78	28.71	28.76	28.12	28.13	24.61	24.39	22.58	22.82	25.01	1.24	0.42	0.15	0.90	1.28	0.41	1.77	0.29	3.75	0.07
GBM	35	26.34		25.14		26.30		26.89		28.81		28.14		24.16		23.05												
GBM	74	28.73	28.93	26.04	26.045	27.09	27.09	28.79	28.93	29.45	29.48	27.05	27.13	26.19	26.43	20.43	20.52	24.50	4.42	0.05	1.54	0.34	2.58	0.17	4.43	0.05	4.98	0.03
GBM	74	29.12		26.05		27.08		29.07		29.51		27.21		26.66		20.6												
GBM	175	27.23	27.38	27.06	26.925	26.71	26.85	27.59	27.45	29.61	29.80	28.34	28.42	26.51	26.48	24.17	23.86	26.18	1.19	0.44	0.74	0.60	0.67	0.63	1.27	0.42	3.61	0.08
GBM	175	27.52		26.79		26.99		27.31		29.98		28.5		26.45		23.54												
GBM	194	27.6	27.52	27.79	27.595	26.52	26.61	27.42	27.46	29.49	29.69	27.92	27.9	25.28	25.36	22.76	22.72	25.24	2.28	0.21	2.36	0.20	1.37	0.39	2.22	0.22	4.45	0.05
GBM	194	27.44		27.4		26.69		27.49		29.88		27.88		25.44		22.68												
GBM	204	28.42	28.45	27.44	27.35	27.74	27.61	27.74	27.83	31.13	31.15	27.49	27.72	24.93	24.90	22.87	23.10	25.16	3.29	0.10	2.19	0.22	2.45	0.18	2.66	0.16	5.99	0.02
GBM	204	28.48		27.26		27.48		27.91		31.17		27.94		24.86		23.32												
GBM	208	27.73	27.76	26	26.1	27.05	27.05	27.72	27.72	30.34	30.47	29.15	29.23	25.97	26.07	23.86	23.93	26.32	1.43	0.37	-0.22	1.17	0.72	0.61	1.40	0.38	4.14	0.06
GBM	208	27.78		26.2		27.04		27.72		30.59		29.31		26.17		24												
GBM	256	26.91	26.82	25.87	25.875	27.73	27.92	28.72	28.83	30.03	30.03	28.51	28.52	26.03	26.01	24.1	24.09	26.14	0.68	0.63	-0.27	1.21	1.77	0.29	2.69	0.16	3.88	0.07
GBM	256	26.73		25.88		28.10		28.94		30.02		28.53		25.99		24.08												
GBM	269	31.04	31.33	31.62	31.625	29.63	29.63	29.76	29.79	Ind.	Ind.	27.05	27.17	26.35	26.19	23	23.23	25.47	5.85	0.02	6.15	0.01	4.15	0.06	4.31	0.05	Ind.	Ind.
GBM	269	31.61		31.63		29.62		29.81		Ind.		27.28		26.03		23.45												
GBM	274	27.98	27.98	26.97	26.875	28.83	28.96	29.43	29.38	Ind.	Ind.	27.12	27.26	25.39	25.57	23.47	23.46	25.38	2.60	0.16	1.50	0.35	3.58	0.08	4.00	0.06	Ind.	Ind.
GBM	274	27.98		26.78		29.08		29.33		Ind.		27.39		25.74		23.44												
GBM	297	26.31	26.39	26.15	26.24	27.27	27.12	28.90	28.91	30.40	30.36	26.03	26.09	24.62	24.74	22.11	22.15	24.27	2.12	0.23	1.97	0.26	2.85	0.14	4.64	0.04	6.09	0.01
GBM	297	26.46		26.33		26.97		28.91		30.32		26.15		24.86		22.18												
GBM	317	30.29	30.06	28.71	28.565	27.82	27.77	30.00	29.89	Ind.	Ind.	29.63	29.86	23.77	23.76	24.63	24.64	25.95	4.10	0.06	2.61	0.16	1.82	0.28	3.93	0.07	Ind.	Ind.
GBM	317	29.82		28.42		27.72		29.77		Ind.		30.09		23.75		24.64												
GBM	356	28.07	28.04	27.27	27.245	27.77	27.71	31.51	31.46	30.19	30.35	28.27	28.16	25.53	25.46	23.81	23.98	25.81	2.23	0.21	1.44	0.37	1.90	0.27	5.65	0.02	4.54	0.04
GBM	356	28.01		27.22		27.64		31.41		30.50		28.04		25.39		24.14												
GBM	370	28.98	29.07	28.14	28.085	27.18	27.16	28.65	28.74	30.57	30.57	28.51	28.55	25.61	25.78	22.77	22.90	25.64	3.43	0.09	2.45	0.18	1.52	0.35	3.10	0.12	4.93	0.03
GBM	370	29.15		28.03		27.14		28.83		30.56		28.59		25.95		23.02												
GBM	384	30.53	30.41	28.29	28.315	29.06	28.98	29.22	29.39	Ind.	Ind.	27.15	27.17	25.05	25.04	23.04	23.10	25.04	5.37	0.02	3.27	0.10	3.94	0.07	4.34	0.05	Ind.	Ind.
GBM	384	30.29		28.34		28.90		29.55		Ind.		27.19		25.02		23.15											Ind.	

Diagnosis	Cases	Ct TLR1	mean Ct TLR1	Ct TLR2	mean Ct TLR2	Ct TLR4	mean Ct TLR4	Ct TLR5	mean Ct TLR5	Ct TLR6	mean Ct TLR6	Ct TBP	mean Ct TBP	Ct HPRT	mean Ct HPRT	Ct GUS- b	mean Ct GUS-b	Geometric mean Housekeeping	TLR1 Δ Ct	TLR1 2 ^{-ΔCT}	TLR2 Δ Ct	TLR2 2 ^{-ΔCT}	TLR4 Δ Ct	TLR4 2 ^{-ΔCT}	TLR5 Δ Ct	TLR5 2 ^{-ΔCT}	TLR6 Δ Ct	TLR6 2 ^{-ΔCT}
GBM	391	30.26	30.3	28.75	28.845	27.54	27.53	31.29	31.40	29.15	29.15	30.27	30.23	25.86	25.94	24.11	23.94	26.58	3.72	0.08	2.27	0.21	0.95	0.52	4.82	0.04	2.57	0.17
GBM	391	30.34		28.94		27.52		31.50		29.15		30.19		26.02		23.76												
GBM	397	29.41	29.46	29.55	29.825	29.90	30.17	31.19	31.40	32.42	32.47	28.01	28.18	25.09	24.97	23.61	23.64	25.53	3.93	0.07	4.30	0.05	4.64	0.04	5.87	0.02	6.94	0.01
GBM	397	29.51		30.1		30.43		31.60		32.51		28.35		24.84		23.67												
GBM	405	29.29	29.22	28.64	28.815	27.08	27.14	29.16	29.17	29.42	29.53	25.55	25.29	23.75	23.68	22.9	22.83	23.91	5.31	0.03	4.91	0.03	3.23	0.11	5.26	0.03	5.62	0.02
GBM	405	29.15		28.99		27.20		29.17		29.64		25.03		23.6		22.75												
GBM	427	28.38	28.21	27.23	27.145	27.25	27.36	27.71	27.64	28.30	28.25	25.82	25.85	25.37	25.33	24.19	24.28	25.14	3.06	0.12	2.00	0.25	2.22	0.22	2.49	0.18	3.10	0.12
GBM	427	28.03		27.06		27.47		27.56		28.19		25.88		25.29		24.36												
GBM	442	28.49	28.62	27.12	27.125	28.69	28.91	27.83	27.70	29.26	29.29	27.08	27.19	24.88	24.83	22.12	22.22	24.66	3.96	0.06	2.47	0.18	4.25	0.05	3.04	0.12	4.63	0.04
GBM	442	28.75		27.13		29.13		27.57		29.31		27.3		24.77		22.31												
GBM	450	30.77	30.65	27.85	27.79	29.33	29.52	29.30	29.21	29.39	29.27	27.6	27.8	25.25	25.29	23.24	23.43	25.44	5.21	0.03	2.35	0.20	4.08	0.06	3.77	0.07	3.83	0.07
GBM	450	30.52		27.73		29.70		29.11		29.14		27.99		25.32		23.61												
GBM	458	29.06	29.04	28.24	28.26	29.06	29.06	28.00	27.88	29.57	29.81	28.32	28.31	25.3	25.36	23.25	23.36	25.60	3.44	0.09	2.66	0.16	3.46	0.09	2.28	0.21	4.21	0.05
GBM	458	29.02		28.28		29.05		27.75		30.04		28.3		25.41		23.47												
GBM	485	26.33	26.54	27.09	27.11	26.67	26.90	28.02	27.87	28.00	27.90	28.89	28.96	24.45	24.49	23.46	23.64	25.59	0.95	0.52	1.52	0.35	1.31	0.40	2.28	0.21	2.31	0.20
GBM	485	26.74		27.13		27.12		27.72		27.79		29.02		24.52		23.81												
GBM	496	28.47	28.6	28.58	28.42	28.20	28.14	27.07	27.08	29.56	29.84	29.1	29.25	24.98	25.24	25.92	25.74	26.68	1.92	0.26	1.74	0.30	1.46	0.36	0.40	0.76	3.16	0.11
GBM	496	28.73		28.26		28.08		27.09		30.12		29.39		25.49		25.56												
GBM	498	27.83	27.65	27.18	27.18	26.68	26.97	27.09	27.08	28.14	28.21	26.97	27.19	22.98	23.04	22.14	22.35	24.10	3.55	0.09	3.08	0.12	2.86	0.14	2.98	0.13	4.10	0.06
GBM	498	27.47		27.18		27.25		27.07		28.27		27.4		23.1		22.56												
GBM	503	28.12	28.18	27.49	27.47	27.19	27.45	28.27	28.28	Ind.	Ind.	28.56	28.39	25	25.15	23.66	23.61	25.64	2.54	0.17	1.83	0.28	1.81	0.29	2.64	0.16	Ind.	Ind.
GBM	503	28.24		27.45		27.70		28.28		Ind.		28.22		25.29		23.55											Ind.	
GBM	510	27.45	27.39	26.24	26.105	27.08	27.18	27.96	27.94	27.79	27.66	27.14	27.15	24.55	24.30	22.32	22.35	24.52	2.87	0.14	1.58	0.33	2.65	0.16	3.41	0.09	3.14	0.11
GBM	510	27.33		25.97		27.27		27.91		27.53		27.16		24.04		22.38												
GBM	522	28.01	28.04	25.36	25.31	25.90	25.78	26.74	26.81	27.15	26.98	28.57	28.65	25.52	25.36	22.33	21.94	25.16	2.87	0.14	0.15	0.90	0.61	0.65	1.64	0.32	1.82	0.28
GBM	522	28.06		25.26		25.65		26.87		26.81		28.72		25.19		21.55												
GBM	524	27.76	27.91	25.29	25.295	26.27	26.56	27.36	27.16	27.75	27.77	27.59	27.77	24.67	24.69	23	22.80	25.00	2.90	0.13	0.29	0.82	1.55	0.34	2.16	0.22	2.77	0.15
GBM	524	28.05		25.3		26.84		26.96		27.79		27.94		24.7		22.6												
GBM	547	30.41	30.19	28.24	28.305	29.25	29.30	33.28	32.74	31.11	30.91	28.11	28.08	26.3	26.21	24.93	25.11	26.44	3.75	0.07	1.87	0.27	2.86	0.14	6.30	0.01	4.47	0.05
GBM	547	29.97		28.37		29.34		32.19		30.70		28.05		26.11		25.28												
GBM	555	27.6	27.53	26.72	26.785	27.24	27.21	27.00	27.06	28.10	28.35	27.89	28.07	25.32	25.35	22.87	22.81	25.31	2.21	0.22	1.47	0.36	1.90	0.27	1.74	0.30	3.04	0.12
GBM	555	27.45		26.85		27.18		27.11		28.60		28.24		25.37		22.74												
GBM	573	31.31	31.1	30.5	30.455	31.19	31.31	32.01	31.66	Ind.	Ind.	33.46	33.67	29.58	29.43	27.61	27.40	30.05	1.04	0.49	0.40	0.76	1.25	0.42	1.61	0.33	Ind.	Ind.
GBM	573	30.88		30.41		31.42		31.31		Ind.		33.88		29.27		27.18											Ind.	
GBM	592	Ind.	Ind.	31.32	31.45	Ind.	Ind.	34.12	33.92	Ind.	Ind.	31	31.11	30	29.69	27.09	27.32	29.33	Ind.	Ind.	2.12	0.23			4.59	0.04	Ind.	Ind.
GBM	592	Ind.		31.58		Ind.		33.72		Ind.		31.22		29.37		27.55			Ind.								Ind.	
GBM	629	28.79	29.04	27.58	27.655	27.87	28.04	29.78	29.67	30.00	30.09	30.02	30.21	26.39	26.62	24.02	24.22	26.90	2.13	0.23	0.75	0.59	1.13	0.46	2.77	0.15	3.18	0.11
GBM	629	29.28		27.73		28.20		29.56		30.17		30.39		26.85		24.42												
GBM	632	26.96	26.98	26.58	26.59	28.05	28.07	26.70	26.75	27.46	27.52	26.45	26.67	24.19	24.28	23.1	23.14	24.65	2.33	0.20	1.94	0.26	3.42	0.09	2.10	0.23	2.87	0.14
GBM	632	27		26.6		28.09		26.80		27.58		26.88		24.37		23.17												

Diagnosis	Cases	Ct TLR1	mean Ct TLR1	Ct TLR2	mean Ct TLR2	Ct TLR4	mean Ct TLR4	Ct TLR5	mean Ct TLR5	Ct TLR6	mean Ct TLR6	Ct TBP	mean Ct TBP	Ct HPRT	mean Ct HPRT	Ct GUS-b	mean Ct GUS-b	Geometric mean Housekeeping	TLR1 ΔCt	TLR1 2 ^{-ΔCt}	TLR2 ΔCt	TLR2 2 ^{-ΔCt}	TLR4 ΔCt	TLR4 2 ^{-ΔCt}	TLR5 ΔCt	TLR5 2 ^{-ΔCt}	TLR6 ΔCt	TLR6 2 ^{-ΔCt}
GBM	638	28.5	28.78	28	28.055	28.81	28.56	28.93	28.94	Ind.	Ind.	30.69	30.92	26.16	26.38	23.32	23.42	26.73	2.04	0.24	1.32	0.40	1.83	0.28	2.21	0.22	Ind.	Ind.
GBM	638	29.05		28.11		28.31		28.95		Ind.		31.15		26.6		23.52											Ind.	
GBM	640	28.87	28.63	28.02	27.84	27.09	27.06	27.29	27.29	29.04	29.00	31.53	31.39	26.62	26.47	26.08	25.84	27.79	0.83	0.56	0.05	0.97	-0.73	1.66	-0.51	1.42	1.21	0.43
GBM	640	28.38		27.66		27.03		27.28		28.96		31.25		26.32		25.6												
GBM	642	29.25	29.27	29.42	29.195	27.48	27.53	30.45	30.41	30.21	30.30	28.37	28.37	25.65	25.49	23.47	23.39	25.67	3.60	0.08	3.53	0.09	1.86	0.28	4.74	0.04	4.63	0.04
GBM	642	29.28		28.97		27.57		30.37		30.38		28.37		25.33		23.3												
GBM	663	Ind.	Ind.	29.32	29.37	30.71	30.59	35.47	34.61	Ind.	Ind.	32.03	32.07	30.83	31.16	28.44	28.25	30.45	Ind.	Ind.	-1.08	2.11	0.14	0.91	4.16	0.06	Ind.	Ind.
GBM	663	Ind.		29.42		30.46		33.75		Ind.		32.11		31.48		28.06			Ind.								Ind.	
GBM	684	29.42	29.41	28.43	28.405	27.96	27.85	28.23	28.46	29.06	28.94	29.68	29.89	24.92	24.74	22.43	22.69	25.60	3.81	0.07	2.81	0.14	2.25	0.21	2.86	0.14	3.34	0.10
GBM	684	29.4		28.38		27.74		28.69		28.82		30.09		24.55		22.95												
GBM	687	29.03	29.08	29.01	28.93	28.55	28.46	29.20	29.27	Ind.	Ind.	27.78	27.69	25.56	25.59	23.82	23.91	25.68	3.40	0.09	3.25	0.11	2.77	0.15	3.58	0.08	Ind.	Ind.
GBM	687	29.13		28.85		28.36		29.33		Ind.		27.6		25.61		23.99											Ind.	
GBM	698	28.82	28.84	28.11	27.99	28.50	28.77	29.26	29.17	Ind.	Ind.	28.86	28.86	23.51	23.59	22.43	22.33	24.77	4.07	0.06	3.22	0.11	4.00	0.06	4.40	0.05	Ind.	Ind.
GBM	698	28.85		27.87		29.03		29.07		Ind.		28.85		23.66		22.22											Ind.	
GBM	724	Ind.	Ind.	28.11	27.84	28.32	28.28	28.34	28.22	29.76	29.85	29.17	29.12	25.64	25.77	23.54	23.50	26.03	Ind.	Ind.	1.81	0.28	2.25	0.21	2.19	0.22	3.82	0.07
GBM	724	Ind.		27.57		28.23		28.10		29.94		29.07		25.9		23.45			Ind.									
GBM	743	29.04	29.25	26.73	26.77	27.66	27.65	28.93	29.00	29.85	29.78	29.63	29.63	27.68	27.65	25.21	25.39	27.50	1.75	0.30	-0.73	1.66	0.15	0.90	1.50	0.35	2.28	0.21
GBM	743	29.46		26.81		27.64		29.06		29.70		29.63		27.61		25.56												
GBM	750	27.38	27.32	26.08	26.07	27.04	26.84	28.09	28.26	29.06	28.84	29.14	29.14	25.12	25.30	22.35	22.38	25.45	1.87	0.27	0.62	0.65	1.38	0.38	2.80	0.14	3.39	0.10
GBM	750	27.26		26.06		26.63		28.42		28.62		29.13		25.47		22.4												
GBM	792	26.57	26.8	26.88	26.935	29.32	29.23	31.14	31.44	31.65	31.68	28.52	28.46	25.56	25.78	24.2	24.25	26.10	0.70	0.62	0.83	0.56	3.12	0.11	5.34	0.02	5.57	0.02
GBM	792	27.03		26.99		29.13		31.74		31.70		28.4		25.99		24.29												
GBM	795	29.99	30.14	28.5	28.485	28.31	28.37	27.72	27.89	31.09	31.13	30.55	30.82	26.88	27.12	24.2	24.22	27.25	2.89	0.14	1.23	0.43	1.11	0.46	0.63	0.65	3.87	0.07
GBM	795	30.29		28.47		28.42		28.05		31.16		31.09		27.35		24.24												
GBM	852	27.61	27.66	27.23	27.2	27.41	27.67	27.89	27.79	29.70	29.82	29.05	28.91	26.59	26.62	23.66	23.71	26.32	1.34	0.40	0.88	0.54	1.35	0.39	1.47	0.36	3.49	0.09
GBM	852	27.71		27.17		27.93		27.69		29.93		28.76		26.64		23.76												
GBM	854	27.54	27.65	26.67	26.62	27.89	27.83	29.19	29.20	29.42	29.32	29.2	29.36	26.35	26.60	24.14	24.24	26.65	1.00	0.50	-0.03	1.02	1.18	0.44	2.55	0.17	2.67	0.16
GBM	854	27.76		26.57		27.76		29.21		29.21		29.52		26.84		24.33												
GBM	875	29.51	29.55	29.3	29.48	28.97	28.71	31.69	31.52	Ind.	Ind.	29.37	29.55	26.17	26.30	23.86	24.06	26.54	3.01	0.12	2.94	0.13	2.17	0.22	4.97	0.03	Ind.	Ind.
GBM	875	29.59		29.66		28.45		31.34		Ind.		29.73		26.42		24.26											Ind.	
GBM	879	27.22	27.33	26.41	26.405	27.44	27.48	27.61	27.68	27.65	27.77	25.53	25.37	22.86	23.13	21.54	21.64	23.33	4.00	0.06	3.08	0.12	4.15	0.06	4.35	0.05	4.44	0.05
GBM	879	27.44		26.4		27.51		27.74		27.89		25.21		23.4		21.73												
GBM	881	31.48	31.49	31.63	31.45	31.99	31.89	33.81	33.87	Ind.	Ind.	29.39	29.36	26.99	27.07	25.43	25.47	27.25	4.24	0.05	4.20	0.05	4.64	0.04	6.62	0.01	Ind.	Ind.
GBM	881	31.49		31.27		31.78		33.93		Ind.		29.32		27.14		25.5											Ind.	
GBM	884	28.7	28.52	26.82	26.915	28.46	28.18	30.51	30.40	28.92	28.78	27.42	27.57	25.4	25.51	23.09	23.24	25.37	3.14	0.11	1.54	0.34	2.81	0.14	5.03	0.03	3.40	0.09
GBM	884	28.33		27.01		27.90		30.29		28.63		27.72		25.61		23.38												
GBM	885	28.51	28.37	26.97	26.985	25.80	25.90	27.51	27.55	29.37	29.38	27.54	27.58	25.27	25.42	22.11	22.21	24.97	3.40	0.09	2.02	0.25	0.93	0.52	2.58	0.17	4.41	0.05
GBM	885	28.23		27		26.00		27.58		29.38		27.62		25.56		22.31												
GBM	891	28.09	28.3	28.99	28.895	27.66	27.61	29.81	29.93	30.48	30.37	26.63	26.71	24.14	24.22	23.05	23.16	24.65	3.64	0.08	4.24	0.05	2.96	0.13	5.27	0.03	5.71	0.02
GBM	891	28.5		28.8		27.56		30.04		30.25		26.79		24.3		23.26												

Diagnosis	Cases	Ct TLR1	mean Ct TLR1	Ct TLR2	mean Ct TLR2	Ct TLR4	mean Ct TLR4	Ct TLR5	mean Ct TLR5	Ct TLR6	mean Ct TLR6	Ct TBP	mean Ct TBP	Ct HPRT	mean Ct HPRT	Ct GUS-b	mean Ct GUS-b	Geometric mean Housekeeping	TLR1 ΔCt	TLR1 2 ^{-ΔCt}	TLR2 ΔCt	TLR2 2 ^{-ΔCt}	TLR4 ΔCt	TLR4 2 ^{-ΔCt}	TLR5 ΔCt	TLR5 2 ^{-ΔCt}	TLR6 ΔCt	TLR6 2 ^{-ΔCt}
GBM	901	Ind.	Ind.	27.62	27.54	28.82	28.75	29.83	29.67	31.40	31.23	27.09	27.2	26.27	26.36	24.84	24.72	26.07	Ind.	Ind.	1.47	0.36	2.68	0.16	3.59	0.08	5.16	0.03
GBM	901	Ind.		27.46		28.68		29.50		31.06		27.31		26.45		24.6			Ind.									
GBM	903	27.1	27.07	26.18	26.09	27.11	26.90	29.92	29.99	28.33	28.22	26.96	27.21	26.71	26.72	24.01	24.22	26.01	1.05	0.48	0.08	0.95	0.88	0.54	3.98	0.06	2.20	0.22
GBM	903	27.03		26		26.68		30.06		28.10		27.45		26.73		24.43												
GBM	925	27.36	27.24	26.91	26.91	27.40	27.57	28.21	28.36	28.58	28.61	28.87	28.91	27.13	27.39	25.1	25.06	27.07	0.17	0.89	-0.16	1.12	0.50	0.71	1.29	0.41	1.54	0.34
GBM	925	27.12		26.91		27.73		28.50		28.63		28.94		27.64		25.01												
GBM	930	28.82	28.91	27.51	27.535	28.40	28.26	29.71	29.52	31.74	31.65	28.61	28.58	26.95	26.92	26.2	26.08	27.17	1.74	0.30	0.36	0.78	1.08	0.47	2.34	0.20	4.47	0.05
GBM	930	29		27.56		28.11		29.32		31.55		28.55		26.89		25.96												
GBM	1002	27.62	27.35	26.63	26.84	26.04	25.78	29.71	29.50	26.21	26.35	29.53	29.59	26.05	26.18	23.05	23.13	26.17	1.18	0.44	0.67	0.63	-0.39	1.31	3.33	0.10	0.18	0.88
GBM	1002	27.07		27.05		25.52		29.28		26.48		29.65		26.31		23.2												
GBM	1003	28.66	28.7	27.75	27.475	27.57	27.65	28.25	28.29	29.71	29.53	28.18	28.19	25.77	25.65	24.5	24.51	26.07	2.63	0.16	1.40	0.38	1.57	0.34	2.22	0.21	3.46	0.09
GBM	1003	28.74		27.2		27.72		28.33		29.35		28.2		25.53		24.52												
GBM	1007	29.96	30	29.14	29.105	27.23	27.17	30.38	30.80	31.07	31.28	25.51	25.48	22.81	22.86	23.08	23.27	23.84	6.16	0.01	5.26	0.03	3.32	0.10	6.95	0.01	7.44	0.01
GBM	1007	30.04		29.07		27.10		31.21		31.49		25.45		22.91		23.46												
GBM	1009	30.3	30.22	27.8	27.695	27.83	27.87	29.55	29.27	31.19	31.30	27.39	27.42	24.01	24.03	22.08	22.23	24.47	5.75	0.02	3.23	0.11	3.40	0.09	4.80	0.04	6.83	0.01
GBM	1009	30.13		27.59		27.91		28.99		31.41		27.45		24.05		22.37												
GBM	1018	Ind.	Ind.	31.68	31.78	33.64	33.56	32.18	32.10	33.11	33.15	31.38	31.39	30.49	30.71	29.37	29.36	30.47	Ind.	Ind.	1.31	0.40	3.09	0.12	1.63	0.32	2.68	0.16
GBM	1018	Ind.		31.88		33.47		32.01		33.18		31.39		30.92		29.34			Ind.									
GBM	1070	29.52	29.37	27.81	27.925	27.26	27.18	28.17	28.16	30.94	31.18	26.09	26.13	25.05	25.17	22.49	22.66	24.60	4.77	0.04	3.32	0.10	2.58	0.17	3.55	0.09	6.58	0.01
GBM	1070	29.22		28.04		27.10		28.14		31.42		26.16		25.28		22.82												
GBM	1074	28.85	28.75	28.09	27.845	26.17	26.44	28.03	28.14	28.16	28.05	28.78	28.71	25.26	25.25	23.35	23.32	25.66	3.08	0.12	2.18	0.22	0.78	0.58	2.47	0.18	2.38	0.19
GBM	1074	28.64		27.6		26.71		28.24		27.93		28.64		25.24		23.28												
GBM	1077	28.93	28.75	27.26	27.155	27.29	27.01	26.63	26.59	29.13	29.13	27.51	27.4	25.46	25.69	21.51	21.55	24.75	3.99	0.06	2.40	0.19	2.26	0.21	1.84	0.28	4.38	0.05
GBM	1077	28.56		27.05		26.73		26.55		29.13		27.29		25.92		21.59												
GBM	1084	29.11	29.31	27.41	27.43	28.23	28.32	30.46	30.26	Ind.	Ind.	29.77	29.85	26.94	26.89	25.27	25.32	27.29	2.02	0.25	0.14	0.91	1.03	0.49	2.97	0.13	Ind.	Ind.
GBM	1084	29.51		27.45		28.41		30.06		Ind.		29.92		26.83		25.37											Ind.	
GBM	1091	29.77	29.53	28.51	28.48	25.98	26.07	27.71	27.66	29.29	29.34	28.42	28.63	24.14	24.16	20.67	20.68	24.27	5.26	0.03	4.21	0.05	1.80	0.29	3.39	0.10	5.07	0.03
GBM	1091	29.29		28.45		26.16		27.60		29.39		28.83		24.17		20.68												
GBM	1103	27.93	27.83	27.15	27.175	26.85	27.02	27.39	27.32	28.69	28.70	28.11	28.14	25.24	25.45	23.29	23.49	25.62	2.20	0.22	1.55	0.34	1.40	0.38	1.69	0.31	3.07	0.12
GBM	1103	27.72		27.2		27.19		27.24		28.70		28.16		25.66		23.69												
GBM	1118	Ind.	Ind.	30.35	30.47	29.73	29.89	31.85	31.88	31.79	31.96	31.58	31.3	28.3	28.41	26.41	26.35	28.62	Ind.	Ind.	1.85	0.28	1.27	0.41	3.26	0.10	3.34	0.10
GBM	1118	Ind.		30.59		30.04		31.91		32.12		31.02		28.52		26.29			Ind.									
GBM	1122	27.07	27.04	26.35	26.31	25.61	25.67	26.60	26.49	27.24	27.30	27.62	27.46	24.44	24.48	22.91	22.90	24.87	2.16	0.22	1.44	0.37	0.80	0.58	1.62	0.33	2.42	0.19
GBM	1122	27		26.27		25.73		26.38		27.35		27.29		24.52		22.89												
GBM	1123	28.99	29.06	28.52	28.6	29.39	29.32	29.68	29.41	32.00	31.82	27.59	27.35	25.69	25.86	23.36	23.37	25.47	3.59	0.08	3.13	0.11	3.85	0.07	3.94	0.07	6.35	0.01
GBM	1123	29.13		28.68		29.24		29.14		31.63		27.1		26.02		23.37												
GBM	1124	29.02	29.04	30.47	30.365	26.78	26.94	28.47	28.31	28.67	28.84	27.4	27.23	24.66	24.75	24.65	24.51	25.47	3.57	0.08	4.90	0.03	1.47	0.36	2.84	0.14	3.37	0.10
GBM	1124	29.05		30.26		27.09		28.14		29.01		27.05		24.84		24.37												
GBM	1133	Ind.	Ind.	28.89	29.02	30.51	30.82	30.06	30.26	Ind.	Ind.	26.27	26.48	25.78	25.90	23.26	23.41	25.23	Ind.	Ind.	3.79	0.07	5.59	0.02	5.03	0.03	Ind.	Ind.
GBM	1133	Ind.		29.15		31.12		30.46		Ind.		26.69		26.02		23.56			Ind.								Ind.	

Diagnosis	Cases	Ct TLR1	mean Ct TLR1	Ct TLR2	mean Ct TLR2	Ct TLR4	mean Ct TLR4	Ct TLR5	mean Ct TLR5	Ct TLR6	mean Ct TLR6	Ct TBP	mean Ct TBP	Ct HPRT	mean Ct HPRT	Ct GUS-b	mean Ct GUS-b	Geometric mean Housekeeping	TLR1 ΔCt	TLR1 2 ^{-ΔCt}	TLR2 ΔCt	TLR2 2 ^{-ΔCt}	TLR4 ΔCt	TLR4 2 ^{-ΔCt}	TLR5 ΔCt	TLR5 2 ^{-ΔCt}	TLR6 ΔCt	TLR6 2 ^{-ΔCt}
GBM	1144	27.57	27.58	27.91	27.9	25.88	25.84	28.81	28.72	28.15	28.23	26.46	26.47	23.38	23.49	24.05	24.15	24.67	2.91	0.13	3.23	0.11	1.17	0.44	4.05	0.06	3.56	0.08
GBM	1144	27.58		27.89		25.80		28.63		28.30		26.47		23.59		24.25												
GBM	1161	27.63	27.79	26.66	26.625	25.25	25.34	27.15	27.20	29.45	29.40	27.15	27.16	24.58	24.64	22.54	22.82	24.81	2.98	0.13	1.82	0.28	0.53	0.69	2.39	0.19	4.59	0.04
GBM	1161	27.94		26.59		25.42		27.24		29.35		27.17		24.69		23.1												
GBM	1162	27.58	27.56	28.4	28.29	26.16	26.12	27.54	27.47	28.84	28.71	27.33	27.15	24.57	24.80	24.22	24.27	25.37	2.18	0.22	2.92	0.13	0.74	0.60	2.09	0.23	3.33	0.10
GBM	1162	27.53		28.18		26.07		27.39		28.57		26.96		25.03		24.31												
GBM	1169	27.51	27.49	26.94	26.91	24.43	24.62	28.54	28.64	28.21	28.12	27.04	27.2	24.26	24.37	22.61	22.50	24.61	2.88	0.14	2.30	0.20	0.00	1.00	4.03	0.06	3.50	0.09
GBM	1169	27.47		26.88		24.80		28.74		28.02		27.35		24.47		22.39												
GBM	1190	27.05	27.12	26.61	26.555	27.38	27.22	27.22	27.25	28.13	28.15	25.52	25.46	23.82	23.87	22.08	22.26	23.82	3.29	0.10	2.73	0.15	3.39	0.10	3.42	0.09	4.33	0.05
GBM	1190	27.18		26.5		27.05		27.27		28.17		25.39		23.91		22.43												
GBM	1194	Ind.	Ind.	32.55	32.34	31.73	31.59	32.09	32.68	Ind.	Ind.	30.46	30.43	28.7	28.72	25.49	25.60	28.18	Ind.	Ind.	4.16	0.06	3.41	0.09	4.50	0.04	Ind.	Ind.
GBM	1194	Ind.		32.13		31.44		33.27		Ind.		30.39		28.74		25.71			Ind.								Ind.	
GBM	1199	Ind.	Ind.	33.54	33.043	31.34	31.41	33.11	32.96	Ind.	Ind.	30.07	30.09	30.06	30.16	27.5	27.56	29.24	Ind.	Ind.	3.80	0.07	2.17	0.22	3.72	0.08	Ind.	Ind.
GBM	1199	Ind.		33.95		31.48		32.81		Ind.		30.11		30.26		27.61			Ind.								Ind.	
GBM	1205	35.22	35.16	33.14	33.28	Ind.	Ind.	34.34	34.28	Ind.	Ind.	30.8	30.89	29.84	29.91	27.33	27.31	29.33	5.83	0.02	3.95	0.06			4.95	0.03	Ind.	Ind.
GBM	1205	35.1		33.42		Ind.		34.22		Ind.		30.97		29.98		27.28											Ind.	
GBM	1212	28.8	28.67	28.52	28.475	27.10	26.92	29.04	28.79	29.82	29.64	26.68	26.85	24.89	25.08	23.27	23.38	25.06	3.61	0.08	3.42	0.09	1.86	0.28	3.73	0.08	4.58	0.04
GBM	1212	28.53		28.43		26.73		28.53		29.46		27.02		25.26		23.48												
GBM	1232	26.25	26.14	25.07	25	24.83	24.86	26.20	26.15	27.43	27.29	26.12	26.18	23.38	23.43	21.66	21.75	23.72	2.42	0.19	1.28	0.41	1.14	0.45	2.43	0.19	3.57	0.08
GBM	1232	26.03		24.93		24.89		26.09		27.15		26.24		23.48		21.84												
GBM	1237	29.3	29.21	28.24	28.28	28.17	28.03	29.09	29.17	31.10	31.36	26.22	26.19	23.78	23.90	22.95	23.13	24.37	4.84	0.04	3.91	0.07	3.66	0.08	4.80	0.04	6.99	0.01
GBM	1237	29.11		28.32		27.89		29.24		31.62		26.15		24.02		23.3												
GBM	1243	28.07	28.11	26.2	26.275	26.91	26.76	28.30	28.14	29.61	29.48	26.35	26.42	24.93	25.03	23.24	23.50	24.95	3.15	0.11	1.32	0.40	1.80	0.29	3.18	0.11	4.52	0.04
GBM	1243	28.14		26.35		26.60		27.97		29.34		26.49		25.12		23.76												
GBM	1250	26.57	26.6	25.41	25.47	26.30	26.27	27.49	27.47	28.36	28.34	27.36	27.56	23.87	24.01	22.74	22.93	24.75	1.84	0.28	0.72	0.61	1.52	0.35	2.72	0.15	3.58	0.08
GBM	1250	26.62		25.53		26.24		27.45		28.31		27.76		24.15		23.11												

8.4. Attachment 4: Paper formal acceptance

13/07/2018

Gmail - Notification of Formal Acceptance for PONE-D-18-04477R2 - [EMID:57adf6bd174b5b92]

Isabele Fattori Moretti <isabelemoretti@gmail.com>

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7 de junho de 2018 16:59

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PONE-D-18-04477R2
Plasmatic membrane toll-like receptor expressions in human astrocytomas

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8.5. Attachement 5: Published paper in PlosOne - Plasmatic membrane toll-like receptor expressions in human astrocytomas

RESEARCH ARTICLE

Plasmatic membrane toll-like receptor expressions in human astrocytomas

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Abstract

Toll-like receptors (TLRs) are the first to identify disturbances in the immune system, recognizing pathogens such as bacteria, fungi, and viruses. Since the inflammation process plays an important role in several diseases, TLRs have been considered potential therapeutic targets, including treatment for cancer. However, TLRs' role in cancer remains ambiguous. This study aims to analyze the expression levels of plasmatic cell membrane TLRs (TLR1, TLR2, TLR4, TLR5, and TLR6) in human astrocytomas the most prevalent tumors of CNS different grades (II-IV). We demonstrated that TLR expressions were higher in astrocytoma samples compared to non-neoplastic brain tissue. The gene and protein expressions were observed in GBM cell lines U87MG and A172, proving their presence in the tumor cells. Associated expressions between the known heterodimers TLR1-TLR2 were found in all astrocytoma grades. In GBMs, the mesenchymal subtype showed higher levels of TLR expressions in relation to classical and proneural subtypes. A strong association of TLRs with the activation of cell cycle process and signaling through canonical, inflammasome and ripoptosome pathways was observed by *in silico* analysis, further highlighting TLRs as interesting targets for cancer treatment.

Introduction

For the past decade, the inflammatory response in the tumor microenvironment has been in the spotlight due to the controversy regarding its ultimate effect. Despite the expectation that inflammation would wield a positive effect against tumorigenesis, relevant studies about the tumor-promoting effects of immune cells have been published. The resulting bioactive molecules from inflammation can provide the tumor microenvironment with growth factors that sustain proliferative signaling, with survival factors that impair apoptosis, along with proangiogenic and extra-cellular matrix-remodeling factors that facilitate invasion, metastasis, and the formation of new blood vessels [1].

Toll-like receptors (TLRs) act as the first line of defense, recognizing nonself-molecules and activating inflammatory processes [2]. Therefore, TLRs have been considered as potential targets for tumor therapeutics [3, 4].

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Competing interests: The authors have declared that no competing interests exist.

There are two subgroups of TLRs, based on the subcellular location of these proteins and their respective pathogen-associated molecular pattern (PAMP) ligands. In humans, cell surface TLR1, TLR2, TLR4, TLR5, and TLR6 comprise one group, recognizing mainly microbial membrane components such as lipids, lipoproteins, and proteins. The other group consists of TLR3, TLR7, TLR8, and TLR9, which are expressed exclusively in intracellular vesicles, including endoplasmic reticulum (ER), endosomes, lysosomes, and endolysosomes, and recognizing microbial nucleic acids. TLR2 and TLR4 also identify endogenous molecules rising upon tissue injury, the damage-associated molecular patterns (DAMPs), such as heat-shock proteins, including HSP70, HSP60 Gp96, HSP22, and HSP72, and high-mobility group box-1, protein, as well as extra-cellular matrix (ECM) molecules such as biglycan, tenascin-C, versican, and fragments of ECM molecules (oligosaccharides of hyaluronic acid and heparan sulfate) [5].

Structurally, TLRs are integral membrane receptors, presenting a N-terminal ligand recognition domain, with a leucine-rich repeat motif, a single transmembrane helix, and a C-terminal cytoplasmic signaling domain—known as Toll IL-1 receptor (TIR) domain due to the homology of signaling domains of IL-1R family members [6].

TLR1, TLR2, TLR4, TLR5, and TLR6 are involved in a diversity of cellular responses, ranging from cell proliferation to cell death, and as such they are the targets of the current study in astrocytomas. Cell surface TLRs may present distinct signaling pathways. Below is a brief summary of their signaling pathways:

Canonical signaling pathway of plasmatic membrane TLRs involves myeloid differentiation primary response 88 (MYD88), which activates nuclear factor-kappa B (NF- κ B) by a protein complex with kinases that recruit tumor necrosis factor receptor associated factor 6 (TRAF6) and leads to proinflammatory cytokines production, such as interleukin-6 (IL-6), interleukin-1 β (IL-1 β) and α , tumor necrosis factor (TNF), interleukin-8 (IL-8) and interleukin-18 (IL-18). The final result of this pathway is a cell survival profile [7]. NF- κ B canonical activation after TLR signaling is constituted by the heterodimerization of p65 and p50 subunits. After activation of the I κ B kinase, I κ B is phosphorylated, ubiquitinated, and degraded by the proteasome that enables NF- κ B translocation to nucleus, triggering transcriptional activity [8, 9]. Several of such activated transcripts lead to the upregulation of positive regulators for cell cycle as cyclin D1, with a fundamental role in cellular division and DNA synthesis [10], c-Myc, responsible for enhancing expression of genes related to proliferation [11, 12], c-Jun [13], and serum response factor (SRF) [14, 15].

Pathway through TLR2 dimerization with TLR1 or TLR6. TLR2 can form a heterodimer complex with both TLR1 and TLR6 when activated. The heterodimerization of TLR2 with TLR1 or TLR6 expands the ligand spectrum, enabling the innate immune system to recognize different structures of pathogens associated molecular patterns. The downstream signal pathways remain the same for both heterodimers [16].

Pathway through TLR4 endocytosis, activating TIR-domain-containing adapter-inducing interferon- β (TRIF), with interferon type 1 response [17].

Pathway through TLR4 activating ripoptosome complex formation, composed of receptor-interacting serine/threonine kinase 1 and 3 (RIPK1/3) and mixed lineage domain kinase like pseudokinase (MLKL), tumor necrosis factor receptor type 1-associated death domain protein (TRADD), and fas-associated protein with death domain (FADD). Ripoptosome activation can lead to apoptosis when caspase 8 (CASP8) is present, or to necroptosis when it is absent. TLR5 and TLR2 are indirectly involved in ripoptosome activation by TNF secretion [18–20].

Pathway through TLR4, TLR2, and TLR5 forming inflammasome complex, with the production of Caspase-1 and the proinflammatory cytokines IL-1 β and IL-18. The inflammasome complex is constituted by NLRP3, PYCARD, and procaspase 1 [21, 22].

The present study was performed in astrocytoma, a brain tumor with an astrocytic phenotype. The 2007 World Health Organization (WHO) Classification of Tumors of the Central Nervous System (CNS) divided astrocytomas in four malignant grades, I to IV. Glioblastoma (GBM; grade IV astrocytoma) is the most frequent malignant CNS tumor, with a median overall survival of 15 months under current standard of care treatment, which consists of total macroscopic surgical resection combined with radiotherapy and chemotherapy with the alkylating agent, temozolomide [23, 24].

With the onset of next generation sequencing, it was possible to assess the molecular landscape characterizing GBMs. In an attempt to better elucidate the involved molecular tumorigenic processes, and to better guide the adjuvant therapeutic strategies, three main molecular patterns, associated to somewhat distinct clinical outcomes, were identified: 1) the proneural subtype with molecular markers related to progenitor neuronal cells; 2) the classical subtype with markers of proliferative cells; and 3) the mesenchymal subtype with markers of epithelial-mesenchymal transition. Patients exhibiting molecular markers for the mesenchymal subtype presented poor prognosis and the worst response to standard of care, and proneural markers showed better prognosis [25–28]. The result of this initiative prompted a new WHO classification, in 2016, which partially includes molecular aspects of GBMs as segment of its official characterization [29].

This study aims to analyze: 1) TLR (1, 2, 4, 5, and 6) expressions in WHO grades II to IV (diffusely infiltrative) human astrocytomas and in GBM molecular subtypes, 2) correlation of TLRs expression levels, and 3) the presence of these receptors in GBM cell lines.

Materials and methods

Tumor samples and ethical statement

The studied cases were composed of 140 astrocytoma grade II-IV samples, collected during therapeutic surgical intervention in the Neurology Department of the Hospital das Clinicas of University of Sao Paulo School of Medicine, by the institutional neurosurgery group. The cases were stratified according to the WHO classification as: 22 non-neoplastic (NN) cases from epilepsy surgery, 26 astrocytoma grade II (AGII) cases, 18 astrocytoma grade III (AGIII) cases, and 96 astrocytoma grade IV (GBM) cases, this cohort has been previously published [30, 31]. The procedures were performed with informed and approved consent according to the Institutional Ethical Committee guidelines at the Hospital das Clinicas of University of Sao Paulo School of Medicine (691/05). The present study was approved by the same institution (059/15) to use the biorepository.

Sample preparation and RNA extraction

The samples were macrodissected and frozen in liquid nitrogen right after the surgical removal, then cryosectioned for RNA extraction. For the tumor tissue analysis, a 6 µm thick section and hematoxylin-eosin staining was made to guarantee the absence of necrotic, gliosis, non-neoplastic areas, and more than 80% of tumor cells in all tumor tissue specimens [32, 33].

The RNA extraction was accomplished by the RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer instructions. The RNA concentration and purity were evaluated by NanoDrop, and 1.8–2.0 values for 260 nm and 280 nm absorbance ratios were considered satisfactory. RNA quality was checked by electrophoresis in agarose gel. Reverse transcription was performed with 1 µg of RNA treated with DNase I (FPLC-puro, GE Healthcare, Uppsala, Sweden), and amplified with random primers and oligodT oligonucleotides, RNase inhibitor, and the SuperScript III reverse transcriptase (Thermo Fisher Scientific, Carlsbad, CA), following the manufacturer instructions. Finally, the cDNA was treated with RNase H (GE

Healthcare, Uppsala, Sweden), diluted in TE (Tris/EDTA) buffer and stored at -20°C for posterior use by quantitative real-time PCR (qRT-PCR) analysis.

Quantitative real-time PCR

TLR1, *TLR2*, *TLR4*, *TLR5*, and *TLR6* mRNA levels were evaluated by qRT-PCR, using Power SYBR Green. The results were normalized with the geometrical mean of three reference genes for each sample, as previously described [34]: hypoxanthine phosphoribosyltransferase (*HPRT*), glucuronidase beta (*GUSB*), and TATA box-binding protein (*TBP*). The primers were designed to amplify 80–120pb, with melting temperature around 60°C and synthesized by Exxtend (Campanas, Brazil) and IDT (Coralville, IA). The primers were designed as described in Table 1.

Primers were optimized to the minimum concentration for the minor cycle threshold (Ct), the maximum amplification efficiency, and minimum nonspecific amplifications. The mix was composed by cDNA (3 µl), Power SYBR Green PCR Master Mix (Thermo Fisher Scientific, Carlsbad, CA) (6 µl), and the reverse and forward primers (3 µl of each). The qRT-PCR was done in duplicate using the ABI Prism 7500 (Thermo Fisher Scientific, Carlsbad, CA) following the protocol: 2 minutes at 50°C, 10 minutes in 95°C, and 40 cycles of 15 seconds at 95°C, and 1 minute at 60°C. The expression values were assessed by the formula $2^{-\Delta Ct}$ as ΔCt is: the Ct of analyzed gene–geometric mean Ct of the reference genes.

Immunofluorescence

The presence of TLR proteins in tumor cells was analyzed by immunofluorescence. A172 and U87MG human GBM cell lineages were acquired from ATCC and authenticated by short tandem repeats (STR) analysis using GenePrint 10 System (Promega, Madison, WI). Cells were cultured in monolayer with DMEM medium (Dulbecco's Modified Eagle's Medium, (Thermo Fisher Scientific, Carlsbad, CA), 10% fetal bovine serum and 100 µg/ml streptomycin and 100 IU/ml penicillin.

Cells were fixed with methanol and acetone (1:1), the membrane was permeabilized with Triton-X-100 (0.1%), and, to avoid unspecific reactions, the cells were treated with 2% bovine serum albumin. The primary antibodies anti-TLR1 (ab180798, rabbit polyclonal, 1:400 diluted), anti-TLR2 FITC-conjugated (AB_945852, ab59711, mouse monoclonal, 1:50 diluted), anti-TLR4 (AB_446735, ab22048, mouse monoclonal 1:200 diluted), anti-TLR5 (AB_793183, sc-57461, mouse monoclonal, 1:50 diluted, Santa Cruz, CA), and anti-TLR6 (AB_2205406, ab37072, rabbit polyclonal, 1:50 diluted) (Abcam, Cambridge, UK) were incubated overnight at 4°C. The secondary antibodies (Thermo Fisher Scientific, Carlsbad, CA) goat anti-Rabbit IgG H&L (Alexa Fluor 568) and goat anti-Mouse IgG H&L (Alexa Fluor 568 and 488) were

Table 1. Used primer sequences.

Gene	forward primer (5'-3')	reverse primer (5'-3')
<i>TLR1</i>	GGCACCCTACAAAAGGAATC	GATAATGGCAAAATGGAAGATGCT
<i>TLR2</i>	TGTGGGTGAAGCACTGGAC	TTGCGGTCACAAGACAGAGAAG
<i>TLR4</i>	TTTATCCAGGTGTGAAATCCAGAC	TCCAGAAAAGGCTCCCAGG
<i>TLR5</i>	CATACTCCTGATGCTACTGACAACG	GCAGATGAGAGTAGGGAAGTCCA
<i>TLR6</i>	AAACGGCACATTCTTCCACAA	TTTGTCGTTGTTGTACTGTGGTTG
Reference gene		
<i>HPRT</i>	TGAGGATTTGGAAAGGGTGT	AGCACACAGAGGGCTACAA
<i>GUSB</i>	GAAAATACGTGGTTGGAGAGCTCATT	CGAGTGAAGATCCCCTTTTTA
<i>TBP</i>	AGGATAAGAGAGCCACGAACCA	CTTGCTGCCAGTCTGGACTGT

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incubated for one hour, and nuclei were stained with DAPI (Thermo Fisher Scientific, Carlsbad, CA). The preparations were analyzed in confocal microscopic Zeiss 510 LSM META and Zeiss 780-NLO (Thornwood, NY). The retrieved images were analyzed by Image J/Fiji [35].

Immunohistochemistry

Paraffin embedded tissue of five representatives cases of GBM, and five non-neoplastic brain tissue from surgical epilepsy cases were stained for the five TLRs, Iba1, CD68 and GFAP by immunohistochemistry using the Novolink kit (Novolink; Novocastra, Newcastle-upon-Tyne, UK), following the manufacture guide. The sections were processed to antigen retrieval, by citrate buffer (10mM, pH6.0) for 3 minutes at 122°C, using an electric cooker (BioCare Medical, Walnut Creek, USA). After protein blocking, the tissue was incubated with antibodies: anti-TLR1(ab180798, rabbit polyclonal, 1:800 diluted), anti-TLR2 (AB_307008, ab9100, mouse monoclonal, 1:50 diluted), anti-TLR4 (AB_446735, ab22048, mouse monoclonal 1:800 diluted), anti-TLR5 (AB_793183, sc-57461, mouse monoclonal, 1:500 diluted, Santa Cruz, CA), anti-TLR6 (AB_2205406, ab37072, rabbit polyclonal, 1:200 diluted) (Abcam, Cambridge, UK), anti-CD68 (AB_2687454, M0718, mouse monoclonal, 1:1600 diluted, DAKO, CA), anti-IBA1 (AB_2224403, ab15690, mouse monoclonal, 1:700 diluted, Abcam, Cambridge, UK), anti-GFAP (AB_10013382, Z0334, rabbit polyclonal, 1:600, DAKO, CA) at 20° for 16 hours. The reaction performed by the kit uses diaminobenzidine, and for nuclear staining Harris hematoxylin was used. To obtained optimal dilution tonsil sections was used. The immunoreactions for the five TLRs were analyzed according to a semi-quantitative score system considering the intensity of staining (0: negative, 1: weak, 2: moderate and 3: strong), and percentage of immune-positive cells (0: no cells stained, 0,5: 1–10%, 1: 10–25%, 2: 26–50% 3: 51–75% and 4: 76–100%). An immunolabeling score (ILS) was obtained by the product of the intensity of staining and the percentage of stained cells, by two independent investigators (IFM, SKNM), and simultaneous revision were performed to obtain the final score in case the concordance was not achieved. Digital photomicrographs of representative fields were captured and processed using Adobe Illustrator CS6 (Adobe System, San Jose, CA).

Statistical analysis

The statistical analysis was performed in the program SPSS version 23.0 (IBM, Armonk, NY). The data distribution was evaluated by Kolmogorov-Smirnov test. The non-parametric results were evaluated by Kruskal-Wallis test, followed by post hoc Dunn's test. The correlations between each two receptors gene expression levels were assessed by Spearman-rho. The associations are considered strong when $r \geq 0.7$, moderate when $0.4 < r < 0.7$, and low when $r \leq 0.4$. Differences were considered significant for $p < 0.05$.

The GBM RNAseq dataset from *The Cancer Genome Atlas* (TCGA - <http://cancergenome.nih.gov/>) was downloaded from *.rsem.genes.normalized_results address. The data was analyzed by DE-seq and the RPKM values were normalized by the z-score.

Results

TLR1, TLR2, TLR4, TLR5, and TLR6 mRNAs were up-regulated in diffuse astrocytomas

Genes coding the five receptors, *TLR1*, 2, 4, 5, and 6, presented higher expression levels in astrocytoma samples compared to non-neoplastic brain tissues, with statistical significance ($p < 0.05$) (Fig 1A). Only *TLR2* expression in AGII did not present any statistical difference compared to NN tissue, in contrast to the statistical difference observed in AGIII and GBM

Fig 1. TLR1, TLR2, TLR4, TLR5, and TLR6 expression levels in astrocytomas of different malignant grades. (A) The analyzed samples consisted of 22 non-neoplastic (NN) cases, 26 astrocytoma grade II (AGII) cases, 18 astrocytoma grade III (AGIII) cases, and 96 glioblastoma (GBM) cases. Data are represented by box and whisker plots, with the median represented by the line in the middle of the boxes, and top and bottom boxes represent the first and third quartiles. qRT-PCR values are normalized by three housekeeping genes (*HPRT*, *GUSB*, *TBP*). For statistical analysis, Kruskal-Wallis and Dunn's tests were applied, wherein (*) $p < 0.05$ when compared to NN cases and (†) $p < 0.05$ when compared to AGII (Dunn test), all the genes present $p < 0.01$ (Kruskal-Wallis). (B) Correlation between TLR2-TLR1, TLR2-TLR6, TLR2-TLR4, TLR2-TLR5, TLR4-TLR1, TLR4-TLR5, and TLR4-TLR6 are demonstrated in GBM cases. Statistical analysis was made by the Spearman-rho correlation, and $p < 0.05$ were considered significant.

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($p < 0.05$). TLR1, TLR2, and TLR6 mRNA expression median values showed an increase in parallel to the increase of malignancy. On the other hand, the median TLR4 expression in GBM cases was lower than in the other astrocytoma grades (AGII-AGIII).

Associated TLRs expressions were observed in GBM

All TLRs expressions were correlated between themselves in GBM, with statistical significance of $p < 0.05$ (Fig 1B). Expression levels of TLR2 and TLR1 presented the strongest correlation ($r = 0.8448$, $p < 0.0001$ by Spearman-rho test).

Higher *TLR4* and *TLR6* median expression levels were observed in mesenchymal GBM compared to other molecular subtypes

Mesenchymal GBM subtype presented higher *TLR4* and *TLR6* mRNA median levels than other molecular subtypes in our cohort (S1 Fig.) [36]. However, given the reduced sample number, particularly for the proneural and mesenchymal GBM cases in this cohort, statistical significance was not achieved. Therefore, we performed a similar analysis in molecular GBM subtypes of TCGA public dataset. In this larger cohort, all five TLRs presented higher expression in mesenchymal subtype with statistical significance compared to proneural and classical subtypes (Fig 2).

TLR1, *TLR2*, *TLR4*, *TLR5*, and *TLR6* were present in tumor cell lines

The five TLR proteins were identified in tumor cells, in both GBM cell lineages with mutational status consistent for the mesenchymal subtype (U87MG and A172): RB transcriptional corepressor (*RB1*) missense mutation in A172, and neurofibromin (*NF1*) missense and deletion mutation in U87MG [37]. For the immunofluorescence observation (Fig 3), *TLR5* presented lower protein expression in U87MG compared to A172. The evaluation of these *TLR* mRNA levels by qRT-PCR corroborated their presence in both cell lineages; *TLR5* mRNA expression level was not detected in U87MG cells by qRT-PCR (S2 Fig.).

Fig 2. *TLR1*, *TLR2*, *TLR4*, *TLR5*, and *TLR6* expression levels in GBM molecular subtypes from TCGA dataset. Data are represented by box and whisker plots, with the median represented by the line in the middle of the boxes, and top and bottom boxes represent the first and third quartiles. The dataset was divided into 37 proneural (PN) cases, 40 classical (CS) subtype cases, and 55 mesenchymal (MES) subtype cases, in which (*) and (†) are $p < 0.05$ when mesenchymal group was compared to proneural cases and to classical cases, respectively (Dunn's test) and $p < 0.01$ (Kruskal-Wallis).

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Fig 3. Immunofluorescence of TLR1, TLR2, TLR4, TLR5, and TLR6 in GBM cell lines. A172 (A) and U87MG (B). TLR1, TLR4, and TLR6 are stained in red, TLR2 and TLR5 in green, and nuclei in blue by DAPI. The presence of all five TLRs was detected in both cell lines. Expression of TLR5 was more intense in A172 compared to U87MG. TLR4 and TLR5 positivity were detected in both tumor lineage cells nuclei. Magnification of 400x.

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TLR1, TLR2, TLR4, TLR5, and TLR6 protein presence in GBM tissue

The five TLRs proteins were detected in phenotypically GBM cells in all cases, including on the multinucleated GBM cells, corroborating the findings observed in the GBM cell lineages. The TLR4 protein expression was the highest among the five studied TLRs. Interestingly, TLR4 and TLR5 protein expressions were also observed in tumor cell nuclei, as observed in the immunofluorescence preparations of the GBM cell lineages (Fig 4). The presence of few microglia in the GBM tumor sample were detected by IBA1 staining and few macrophages with CD68, which did not overlap with the TLRs positive tumor cells. The positive reaction for GFAP was consistent with the glial origin of the analyzed GBM tumor samples (S3 Fig). Non-neoplastic brain samples were also stained for TLRs. As expected, the positivity for these receptors were observed in neurons and the lowest expression was for TLR2. Similar to the staining pattern in tumor cells, TLR5 positivity was detected in neuron nuclei (S3 Fig).

In silico exploration of TLR signaling pathway

A heatmap including selected target genes of TLR signaling pathways was built from the TCGA RNAseq dataset. The RPKM values (S4 Fig.) were normalized by the z-score values for

Fig 4. Immunohistochemistry for TLR1, TLR2, TLR4, TLR5, and TLR6 in GBM cases. Positive immunolabelling of GBM tumor cells for TLRs are demonstrated at 600x magnification of tumor tissues, and at 800x magnification in a multinucleated GBM tumor cell. The distribution of the Immunolabeling score (ILS) for these TLRs in five GBM cases was presented as a dispersion graph, where the black dots represent the mean ILS obtained by the two independent investigators for each individual tumor case, and the horizontal bar represent the mean ILS for each receptor. TLR4 and TLR5 positive staining were detected in tumor nuclei.

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each gene to allow comparative analysis among them (Fig 5). *In silico* analysis highlighted the higher expression of TLRs in mesenchymal subtype relative to proneural and classical subtypes (S4 Fig.), and pointed out a downstream upregulation through MYD88/TIRAP and NF- κ B, ending with increased expression levels of genes that code for cytokines and genes related to cell proliferation. Particularly, expression levels of genes that code for IL-8, IL-6, IL-1 α , IL-1 β , and IL-18 were differentially increased in the mesenchymal GBM subtype compared to the other two subtypes (S4 Fig.). Moreover, *RELA*, which codes for the nuclear subunit of NF- κ B, presented positive correlations ($p < 0.05$) by Spearman-rho test with expression levels of cell proliferation genes: *JUN* ($r = 0.422$ and $r = 0.536$) and *SRF* ($r = 0.462$ and $r = 0.577$) and in mesenchymal and classical subtypes, respectively. In contrast, the gene expressions related to interferon type I response presented low or undetermined z-score values (not shown). Interestingly, expression levels of genes involved in ripoptosome signaling, such as *RIPK3* and *MLKL*, were also comparatively increased in mesenchymal GBM subtype, and their values correlated to *TLR2* ($r = 0.795$ and $r = 0.661$, respectively), and to *TLR4* ($r = 0.575$ and $r = 0.360$, respectively). Of note, *PYCARD*, *NLRP3* and *CASP1*, which participate in the inflammasome pathway, presented higher expression levels in mesenchymal subtype of GBM compared to the other two subtypes (S4 Fig.).

Fig 5. Heatmap with major genes of the TLR signaling pathways from the TCGA dataset. RPKM gene expression levels are normalized by z-scores, and comparatively up-regulated RNA expression values are presented in red and down-regulated values in blue. Mean values are in white. TLRs downstream signaling pathways: canonical, ripoptosome, and inflammasome pathways are activated in mesenchymal GBM subtype. Genes of unrelated pathways were added to show their randomic expression levels, including a microglia marker.

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Discussion

In the present study, higher expression levels of *TLR1*, *TLR2*, *TLR4*, *TLR5*, and *TLR6* were demonstrated in human diffusely infiltrating astrocytomas (grades II to IV) in comparison to non-neoplastic brain tissue. Moreover, an associated expression of these receptors were observed in GBM cases, suggesting their role in tumor aggressiveness. The presence of *TLR4* has been previously described in GBM, particularly in CD133⁺ tumor stem cells [38, 39], and also in GBM cell lineages A172, U87MG [3, 40, 41], and U251 [42]. Such *TLR4* positivity conferred a proliferative phenotype to these tumor cells [38, 42]. Furthermore, the presence of *TLR2* was also detected in murine GL261 glioma cell line, and the activation of this receptor leads to an invasive and migratory profile of the tumor cells [43]. We further demonstrated herein the presence of *TLR1*, *TLR2*, *TLR5*, and *TLR6* in U87MG and A172 cell lineages and in human GBM specimens, at gene and protein expression levels. *TLR4* was the most expressed receptor in both cell lineages and tumor specimens. Interestingly, *TLR4* and *TLR5* positivity were detected in the tumor cell nuclei by immunofluorescence in cell lineages and by immunohistochemistry in tumor specimens. However, such observation need confirmation in other cohorts before speculating their possible role in this localization (Figs 3, 4 and S2). In spite of both cell lineages presenting somatic mutation profiles of mesenchymal molecular subtype of GBM, they present distinct expression profiles of these TLRs. U87MG cells showed higher expression of *TLR1*, *TLR2*, and *TLR4* compared to A172, in contrast to lower expression of *TLR5* (Figs 3 and S2). Such heterogeneity of these TLRs expression distributions was also observed in human astrocytoma samples from our cohort and in the TCGA dataset, particularly among GBMs, where higher expression levels were detected in mesenchymal subtype (Figs 1, 2, 5 and S1). To analyze the impact of these differential TLRs expressions among the molecular subtypes of GBM, we built a heatmap with the expression levels of the genes involved in pathways related to TLRs from the TCGA dataset (Fig 5). Interestingly, this approach showed clearly the downstream activation of the canonical, ripoptosome, and inflammasome pathways, related to the upregulation of the TLRs, particularly in the mesenchymal subtype of GBM. The end targets of these pathways, including cytokines (*IL1A/B*, *IL6*, *IL8*, *IL18*) and genes related to cell proliferation (*JUN*, *SRF*), were upregulated, suggesting that the activation of these TLRs leads to tumor growth. In fact, activation of the TLR canonical pathway through TIRAP-MYD88 was related to NF- κ B (*RELA*) upregulated expression levels and positive correlation with *JUN*, and *SRF*, transcription factors implicated in cellular proliferation. The role of NF- κ B in tumor growth has been demonstrated in a mouse model of GBM [44] and in pulmonary tumor cells [45]. Additionally, it has been reported that such a proliferative response may be time- and dose-dependent [46]. Therefore, tumor inflammatory micro-environment may contribute to distinct NF- κ B pulsation-determining tumor cell behavior in each specific condition. The end targets of this pathway, IL-6, IL-1 α and IL-1 β , and IL-8 cytokines, presented increased expression in the mesenchymal subtype, and they have also been associated to tumor malignancy and tumor cell migration [22, 47–49]. *IL1B* and *IL18* are also end targets of the inflammasome pathway, which were upregulated in the mesenchymal subtype. On the other hand, the increased expression of *RIPK3* and *MLKL* may suggest the upregulation of the ripoptosome pathway, and may allow the link with the presence of necrosis, one of the characteristics of GBM. In a rat glioma model, apoptosis was induced by activation of *CASP8* and inhibition of *RIPK1/RIPK3* complex [19, 50, 51].

The expression of *CASP8* was increased in the mesenchymal subtype of GBM; however, increased apoptosis is not expected and, therefore, avoidance of apoptosis should be under additional modulation. Moreover, according to the present *in silico* analysis, the interferon type I response by *TLR4* endocytosis was unlikely.

Another hypothesis for the proliferative profile and TLR signaling in GBM is the pathway involving PTEN, which has been shown to regulate TIRAP and TLR4 internalization [52, 53], in addition to its classic suppressor role in the PI3K-AKT-mTOR pathway [54]. Loss of function of PTEN may occur by its deletion or phosphorylation [27, 55]. Our previous analysis of PTEN status in our GBM cohort demonstrated 24.32% and 26.66% of *PTEN* deletion, and 59.45% and 66.66% of PTEN phosphorylation in classical and mesenchymal subtypes, respectively (S5 Fig.) [36, 55], demonstrating more prevalent *PTEN* loss of function in the classical subtype. These PTEN alterations may lead to a decreased inhibition over TIRAP (Fig 6) and consequent TLR canonical pathway activation. In B cells, the absence of PTEN showed an increased in NF- κ B activity after TLR4 stimulation [56]. Additionally NF- κ B negatively regulate PTEN [57, 58]. Accordingly, in the classical subtype, loss of PTEN may also be implicated in tumor cell proliferation through TLR canonical activation (Fig 5). Actually, our analysis of the TCGA dataset showed significant correlation between *TIRAP* and *PTEN* expression levels among the classical GBM subtype ($r = 0.330$, $p < 0.05$, Spearman-rho test). Interestingly, a negative correlation was found between them in the proneural subtype ($r = -0.382$, $p < 0.05$), which presents better prognosis among the three analyzed GBM subtypes.

Fig 6. Schematic proposition of the TLR canonical signaling pathway through TIRAP by PTEN regulation. PTEN alterations may lead to upregulation of TLR signaling pathway and may increase tumor cell proliferation. Loss of PTEN repressor role by its deletion or phosphorylation, as may occur more frequently in classical subtype, may activate downstream TLR canonical pathway through the decreased inhibition over TIRAP. This pathway through TIRAP-PTEN may not be the major mechanism in mesenchymal subtype, and the integrity of PTEN may inhibit TIRAP and then not activate this pathway in proneural subtype.

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In summary, increased cell membrane *TLR1*, *TLR2*, *TLR4*, *TLR5*, and *TLR6* expressions were demonstrated in astrocytomas compared to non-neoplastic brain tissue, mostly in GBM group, and particularly in the mesenchymal subtype. *In silico* exploration of the putative activation pathways by these upregulated TLRs, the canonical and inflammasome pathways in the mesenchymal subtype and the PTEN-TIRAP signaling in the classical subtype demonstrated their increase and their possible contribution to tumor growth. According to the TCGA dataset the microglia compartment based on *AIF1* expression level did not parallel the *TLRs* upregulated expressions in mesenchymal subtype, suggesting that the tumor cells themselves may contribute to the increased expression of these receptors.

Further understanding of the particular TLR responses from each tumor compartment, which includes inflammatory cells in addition to tumor cells, will be essential to designing new therapeutic strategies involving TLRs.

Supporting information

S1 Fig. TLRs expression levels in the GBM subtypes of our cohort composed of 14 proneural (PN), 38 classical (CS) and 16 mesenchymal (MES) subtype cases. Horizontal bars indicate the mean value of each group.
(TIF)

S2 Fig. TLRs expression levels in U87MG and A172 cell lines. A heterogeneity in the distribution of the five *TLRs* is observed between the cell lines, although both GBM cell lines present somatic mutation profiles of mesenchymal subtype. *TLR4* expression is high in both cell lines, whereas *TLR5* is undetectable in U87MG cell line.
(TIF)

S3 Fig. TLRs protein expression in non-neoplastic cases and microglia and macrophage detection in GBM sample. Immunohistochemistry for a representative non-neoplastic case stained for *TLR1*, *TLR2*, *TLR4*, *TLR5*, *TLR6* and GFAP for glial cell identification. Presence of few microglia in the same GBM sample of Fig 4 was observed by IBA1 staining, and also few macrophages was detected by CD68 staining. GFAP positivity was shown in the GBM tumor sample confirming the glial origin of the tumor.
(TIF)

S4 Fig. Expression levels of the genes participating in the TLR signaling pathways divided by GBM molecular subtypes: Proneural, classical, and mesenchymal from the TCGA RNA-Seq dataset. The dataset is composed of 37 proneural (PN), 40 classical (CS), and 55 mesenchymal (MES) subtype cases, wherein (*) $p < 0.05$ when compared to proneural cases and (†) $p < 0.05$ when compared to classical cases by Kruskal-Wallis and Dunn's test. The values were normalized in DEseq.
(TIF)

S5 Fig. PTEN. The GBM cohort from our lab was previously analyzed for PTEN mutational and phosphorylation status. The number of cases for each status of PTEN and GBM subtype are presented. In green is the amount of cases presenting wild-type PTEN, in black the deleted *PTEN*, and in orange the Y240-phosphorylated PTEN.
(TIF)

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