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RelB acts as a molecular switch to drive chronic inflammation in glioblastoma multiforme (GBM).

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RelB acts as a Molecular Switch to Drive Chronic Inflammation in Glioblastoma Multiforme (GBM).

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By

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1.4 List of Abbreviations

BAFF	B-cell activating factor
CCL2	CC-chemokine ligand 2
CD	Cell Differentiation [marker]
COX-2	cyclooxygenase 2
CSFs	colony-stimulating factors
DC	Dendritic Cell
ERK	Extracellular signal related kinase
GAM	Glioma associated myeloid cell
GBM	Glioblastoma Multiforme
IDH1	Isocitrate Dehydrogenase 1
IκB	Inhibitor of NF-κB
IKK	IκB Kinase
IL-1R1	Interluekin-1 type Receptor 1
IL-1R1AcP	Interluekin-1 Receptor 1 Associated Protein
IL-1B	interleukin 1β
IL-23	Interleukin-23

IL-6	interleukin 6
IL-8	interleukin-8
JAK	Janus Kinase
JNK	c-Jun N-Terminal Protein Kinase
LIF	Leukemia Inhibitory Factor
LTb	Lymphotoxin B
MAPK	Mitogen Activated Protein Kinase
MCP	Macrophage chemokine protein
MIP	Macrophage Inflammatory protein
NF1	Neurofibromin 1
NIK	NF-κB Inducing Kinase
Oct2	Octamer Binding Protein 2
OSM	Oncostatin M
PDGFRA	Platelet Derived Growth Factor Receptor A
PRC	Polycomb Repressor Complex
Rb	Retinoblastoma protein
RHD	Rel Homology Domain
SH2	Src-homology-2

SIRT1	Sirtuin 1
STAT	Signal Transducer and Activator of Transcription
TLR	Toll Like Receptor
TNFa	Tumor Necrosis Factor alpha
TRAF	TNF Receptor Associated Factor
VEGF	Vasculoendothelial Growth Factor
YY1	Ying Yang 1

1.5 Abstract

Abstract

RelB acts as a Molecular Switch toDrive Chronic Inflammation in Glioblastoma Multiforme (GBM).

By Michael Waters, B.S.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2017.

Major Director: Tomasz Kordula and Department of Biochemistry and Molecular Biology

Inflammation is a homeostatic response to tissue injury or infection, which is normally short- lived and quickly resolves to limit tissue damage. In contrast, chronic inflammation has been linked to a variety of human diseases, including cancers such as glioblastoma multiforme (GBM). GBMs are very aggressive tumors with very low patient survival rates [1, 2], which have not improved in several decades. GBM tumors are characterized by necrosis and profound inflammation [3, 4,80,82-83,92]; with cytokines secreted by both GBM cells and the tumor microenvironment. The mechanisms by which chronic inflammation develops and persists in GBM regardless of multiple anti-inflammatory feedback loops remain elusive. We found that expression of the NF-κB family member RelB is similarly induced in primary human astrocytes and GBM cells. However, while RelB suppresses expression of cytokines in astrocytes, it unexpectedly enhances expression of cytokines in GBM cells. Thus, the anti-inflammatory RelB-driven feedback loop found in astrocytes is converted into a feed-forward loop fueling chronic inflammation in GBM. Additionally, we established the molecular mechanism for the lack of RelB dependent silencing in GBM. A previous RelB-dependent epigenetic silencing mechanism, shown in macrophages during sepsis, requires histone deacetylase Sirtuin 1 (SIRT1).

We found that SIRT1 is needed to repress cytokines in astrocytes in a RelB dependent manner to establish “sterile” inflammatory tolerance. Significantly, we discovered that one allele of the SIRT1 gene is deleted in 80% of GBM tumors, and that human GBM tumors display drastically diminished SIRT1 expression and protein levels. Additionally, we found that the ubiquitous transcription factor Ying Yang 1 (YY1) interacts with RelB and forms RelB/YY1/p50 complexes which transactivate cytokine expression specifically in GBM cells and not primary astrocytes. The specificity of this interaction is due to YY1 sequestration in the cytoplasm of astrocytes and constitutive nuclear presence in GBM cells. Thus, our data supports the premise that SIRT1 deletion and GBM-specific YY1 nuclear translocation turns RelB into a molecular switch that plays a critical role in chronic inflammation associated with GBM, and supports a mechanistic model which explains the development and persistence of this chronic inflammation.

2 Chapter 1: Introduction

2.1 Cancer and Chronic Inflammation

Inflammation is a fundamental response to tissue injury or infection which leads to the elimination of its initial cause, clearance of necrotic cells, and subsequent tissue repair. Although inflammation is normally short- lived and quickly resolves to limit tissue injury, persistent chronic inflammation has been linked to many human diseases including a variety of cancers [5,6].

Secreted inflammatory mediators are vital constituents in the tumor microenvironment. In some cancers, such as hepatocellular carcinoma, chronic inflammation directly leads to oncogenic chances and tumor initiation [5,6]. In other cancers, *de novo* oncogenic changes induce a chronic inflammatory microenvironment which promotes tumor progression and spread. In either case, the inflammatory microenvironment of tumors yields many tumor-promoting effects [3-6,9]. Proinflammatory signals lead to the proliferation and survival of malignant cells, promotes angiogenesis, and alters responses to chemotherapeutics [5-6,9,84].

The hallmarks of cancer-related inflammation include the infiltration of inflammatory cells, the presence of inflammatory mediators such as cytokines, chemokines and prostaglandins, and molecules promoting tissue remodeling and angiogenesis. Inflammatory genes which have been implicated in the development and progression of a wide variety of human cancers include (i) interleukin-1B (IL-1B) and interleukin-6 (IL-6), two major proinflammatory cytokines (ii) cyclooxygenase 2 (COX2), which controls the production of prostaglandins, (iii) chemokines which are tactic for myeloid cells such as CC-chemokine ligand 2 (CCL2), (iv) colony-

stimulating factors (CSFs), which promote the survival and recruitment of immune cells, (v) molecules promoting angiogenesis such as interleukin-8 (IL-8) and vasculoendothelial growth factor (VEGF), and (vii) extracellular-matrix-degrading enzymes [5].

In addition to inflammatory mediators a variety of downstream inflammatory signaling pathways display constitutive activation in variety of human cancers. Notably, Nuclear Factor-kappa B (NF-κB) family member signaling and Signal Transducer and Activator of Transcription 3 (STAT3) are downstream of inflammatory mediators, display constitutive activation in many cancers, and serve as a convergence point for many tumor promoting actions [9,84].

2.2 NF-κB-STAT3 Signaling

The NF-κB pathway is composed of five members p65 (RelA), RelB, c-Rel, p50/p105 (NFKB1), and p52/p100(NFKB2). Each protein contains a conserved ~300 amino acid Rel homology domain (RHD) which is responsible for dimerization, nuclear translocation, and binding to DNA [7,48].

Within the NF-κB family is the 'Rel' subfamily composed of c-Rel, RelB and RelA. These proteins have in common a C-terminal transcriptional transactivation domain. The other two NF-kB family members, p105/p50 and p100/p52 do not possess a transcriptional transactivation domain but instead, contain multiple Ankyrin repeats in their C-terminal domains, acting to inhibit the nuclear translocation of NF-κB dimers. p105/p50 and p100/p52 proteins are processed by proteolysis which removes this C-terminal domain, and thus allows nuclear translocation and transcriptional activation of NF-κB dimers [7,84].

Inhibitor of NF-κB (IκB) proteins tightly regulate the activity of NF-κB pathway. There are several IκB proteins (IκBα, IκBbβ IκBγ, IκBε) each of which displays different affinities for NF-κB dimers. Phosphorylation and subsequent proteolysis of IκB proteins due to NF-κB activating stimuli relieves IκB protein inhibition [7,50].

There are three pathways leading to the nuclear translocation and transcriptional activation of NF-κB dimers: (i) the canonical (or classical) pathway, (ii) the non-canonical (or alternative) pathway, and (iii) the RelB-canonical pathway. The canonical (or classical) NF-κB pathway is the best studied, and the proinflammatory arm of NF-κB signaling. In the canonical pathway, binding of a wide variety of ligands (ex. IL-1, TNF, LPS) to their associated receptors recruits adaptors such as TNF Receptor Associated Factors (TRAFs) to the cytoplasmic domain of the receptor. These adaptors recruit the IκB Kinase (IKK) complex which is then activated to phosphorylate IκB, at two serine residues, marking it for K48 ubiquitination and degradation by the proteasome. IκB degradation leads to the nuclear translocation and activity of p65/p50 dimers. A predominant function of the canonical NF-κB pathway is stimulating the production of secondary mediators of the proinflammatory response such as cytokines and chemokines [7,46,50,84].

The non-canonical pathway relies on the activation and processing of p100/RelB complexes. The non-canonical pathway is activated by different extracellular mediators such as Lymphotoxin B (LTb) and B-cell activating factor (BAFF). Ligand/receptor interaction leads to the stabilization of NF-κB Inducing Kinase (NIK). NIK phosphorylates a separate IKK complex containing two IKKa subunits, which phosphorylates p100 on two serine residues adjacent to the ankrin repeat domain. This serine phosphorylation leads to p100 processing, and nuclear

translocation of p52/RelB dimers to stimulate transcription. The targets of the non-canonical pathway are most notably involved in lymphogenesis [7,46,50].

Lastly, a third pathway, the RelB-canonical pathway, has been described. The RelB canonical pathway utilizes RelB from the non-canonical pathway and p50 from the canonical pathway. These dimes are bound by IκBa and stimulated to translocate to the nucleus by canonical NF-κB stimuli. Importantly RelB/p50 dimers only form in cells producing large amounts of RelB; RelB has a higher affinity for p100/p52 than p50. To date, little is known about the RelB/p50 dependent signaling program; multiple reports indicate that its main function is as a repressor of transcription, and a negative feedback loop limiting p65/p50 induced inflammation [46,48].

As stated previously, The NF-κB pathway is a key coordinator of the inflammatory microenvironment and has emerged as an important endogenous tumor promoter. Numerous studies in multiple cancers have shown that NF-κB signaling is necessary for cancer cell survival, proliferation, invasion, maintenance of stem like characteristics, genomic instability and recruitment of tumor associated immune cells [7-9,84]

In addition to constitutive NF-κB activation, constitutive STAT3 signaling has been demonstrated in most human cancers. There are seven known STAT proteins: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6. STAT proteins homo/heterodimerize to form functional signaling complexes in response to inducing stimuli. Additionally, several STATs can form tetramers or higher order structures to exert their signaling effects. In the active receptor complex, STAT proteins are tyrosine phosphorylated in response to activating stimuli. Tyrosine phosphorylation of STAT proteins leads to their dimerization and nuclear translocation. Finally,

these STAT dimers recognize specific DNA elements in the promoter regions of target genes to activate transcription [5,8,9].

STAT3 signaling has been shown to increase tumor cell proliferation, survival and metastatic invasion [5-6,9]. The best characterized inducers of the STAT3 signaling axis are the IL-6 family of cytokines including IL-6, OSM, LIF, and IL-23 [8,9]. Given the concordant and constitutive activation of NF-κB and STAT3 in many cancers, and their overlapping functions, it is not surprising that the pathways interact at multiple levels to enhance cancer aggressiveness. Both factors induce the expression of a highly overlapping program of proliferative, anti-apoptotic, and angiogenic genes. Interestingly STAT3 is involved in p300 acetylation, which is needed to activate many NF-κB target genes. Conversely, NF-κB dependent products, such as IL-6, activate STAT3 [9,87].

2.3 Glioblastoma

Gliomas are tumors of the central nervous system arising from supporting glial cells, and are the most common primary tumor of the central nervous system (CNS) in adults [10]. Traditionally, gliomas are classified into three subgroups: (i) oligodendroglioma, (ii) astrocytoma, or (iii) oligoastrocytoma based on the histologic appearance of the resected tumor. Histologic grade IV astrocytoma, known as glioblastoma multiforme (GBM), is characterized by necrosis, hypercellularity, and *de novo* microvascular proliferation. Patients with GBM demonstrate an extremely poor median survival of 15 months [1,2,10]. GBMs represent 55% of the adult diffuse glioma patient population, making them the most common and most deadly primary brain tumor [10].

Most GBM tumors have genetic defects in three major pathways: (i) p53 tumor suppression, (ii) Retinoblastoma protein (Rb)/cell cycle control, and (iii) receptor tyrosine kinase/Ras/phosphoinositide 3-kinase signal transduction [11]. While these pathways have been well established and known for some time, recent studies utilizing high-throughput gene expression data revealed extensive inter-tumor heterogeneity which could not be explained by mutational and genome copy number analysis [12].

Subsequent unsupervised clustering analysis of GBMs identified four gene expression based groups, labeled proneural, neural, classical, and mesenchymal; the subtypes were named based on pathway enrichment analysis of subtype gene classifiers [12-14]. Further annotation of GBM subtypes with genomic abnormalities revealed statistically significant associations of somatic alterations with transcriptional subtypes: Platelet Derived Growth Factor Receptor A (PDGFRA) amplifications, and Isocitrate Dehydrogenase 1 (IDH1) and TP53 mutations were most frequently found in the proneural group, EGFR alterations were found in the classical group, and NF1 abnormalities were most common in mesenchymalGBM [12].

Subsequent discoveries using GBM subtyping have solidified the importance of classification of GBM tumors through transcriptional classification [13]. Importantly, the mesenchymal subtype displays the worst prognosis [14], and is notable for the most robust expression of cytokines, chemokines and inflammatory markers [15].

While increased expression of many proinflammatory cytokines indicates a poor prognosis in GBM, chronic inflammation is present in all GBM tumors; GBMs are surrounded by a pool of pro-inflammatory cytokines, chemokines and growth factors. Thus, a growing body of literature suggests that the inflammatory microenvironment acts as driving force for the progression of GBM into highly malignant and invasive tumors. Consistent with its

role in other malignancies, inflammatory cytokines greatly enhance the proliferation, invasiveness and ‘stemness’ of GBM cells, and thus actively contribute to the global tumor phenotype. Unsurprisingly therefore, a host *in vivo* studies have demonstrated that proinflammatory signaling within GBM cells drives tumor bulk, invasion, angiogenesis, and drug resistance [3-4,14-15,83,87,92].

2.4 Infiltrating Myeloid Cells in GBM

Glioma associated myeloid cells (GAMs) make up 5-30% of the bulk tumor mass in GBM [16]. Given the prolific infiltration of these cells, their activation and behavior drastically influences the aggressiveness of GBM tumors. A bulk of literature beginning in the 1990s has demonstrated a positive correlation between the number of GAMs, the grade of glioma, and the prognosis of patients GBM [17,18]. Based on histologic appearance, GAMs are activated, assuming and amoeboid or spherical appearance [19]. While the secretome of GAMs has not been fully agreed upon, likely due to intertumoral heterogeneity, it is widely accepted that GAMs actively support tumor growth, by secreting matrix-degrading enzymes, cytokines, and immunosuppressive factors [20].

While the literature is constantly evolving, classically, activated macrophages assume either an M1 or an M2 phenotype. The M1 phenotype is characterized by the expression of STAT1 and the production of anti-tumor immune responses by presenting antigens to adaptive immune cells, and phagocytosing tumor cells [21]. In comparison, the M2 pathway, is characterized by expression of the scavenger receptors CD163, CD204, mannose receptor C type 1 (CD206), intracellular STAT3, and the production of immunosuppressive cytokines [22]. Based on several high-impact studies describing the M1 versus M2 dichotomy during *in vitro* culture in GBM, investigators have presumed that the M2 type of polarization occurs within the

in vivo tumor microenvironment. However, comprehensive transcriptomic profiling of ex vivo human macrophages indicates that this is an over simplification and that the majority of GAMs resembled an M0 or undifferentiated phenotype [23].

As stated previously however, despite the transcriptomic similarity of GAMs with M0 macrophages, GAMs are still histologically activated, express matrix modifying enzymes, and perform immunological functions. GAMs rely on globally attracting and activating signaling molecules for their recruitment and acquisition of phenotype in GBM. Among chemokine pathways involved in GAM chemoattraction, the macrophage chemokine protein (MCP) family was the first and most comprehensively described [24-26, 33]. Other families of proteins which have been shown to act as GAM attractants and activators include the colony stimulating factor (CSF) family of proteins [27-31, 33], and the macrophage inflammatory protein (MIP) family [32, 33].

2.5 IL-1B Signaling

IL-1B has been described as the predominant proinflammatory cytokine associated with the host response to injury and infection in the CNS [34-36]. Two of isoforms of IL-1 exist, IL-1B and IL-1A. IL1B and IL1A both bind the IL-1 type I receptor (IL-1R1), which subsequently dimerizes with an accessory protein: Interluekin-1 Receptor 1 Associated Protein (IL-1R1AcP) to activate the downstream signaling cascades [35, 36]. Within glial cells, IL-1 can activate multiple signaling cascades, including the NF-KB pathway and the mitogen-activated protein kinase (MAPK) pathways p38 MAPK, c-Jun N-Terminal Protein Kinase (JNK) and Extracellular signal related kinase 1 and 2 (ERK1/2) [37]. Importantly, this IL-1 signaling cascade initiates the

synthesis of a wide variety of secondary proinflammatory mediators such as cytokines, chemokines and prostaglandins, and thus plays a major role in the establishment of a proinflammatory microenvironment.

Additionally, IL-1 signaling drives astrocytes to change their morphology and secretome in a process called "reactive astroglosis." 'Reactive' astrocytes undergo hypertrophic changes and increase their proliferation. Importantly, IL-1B activated astrocytes produce a host of factors necessary for angiogenesis, vessel plasticity and astrocyte migration which are critical for recovery and repair [38].

The level of IL-1B expression in normal healthy individuals is low. However, its expression dramatically increases in response to injury or infection [34]. While IL-1B has a homeostatic role in normal tissue, *in vitro* IL-1B signaling has been shown to increase the pathogenic behavior of GBM cells. Specifically, IL-1B has been shown to drive the migration, proliferation and invasion of GBM cell lines, alter the GBM secretion profile, and has been suggested to be an important mediator of angiogenesis [40-42]. Interestingly, GBM cells themselves secrete large amounts of IL-1B [39].

2.6 OSM and IL-6 Family Signaling

OSM belongs to the IL-6 family of cytokines and is expressed mainly in monocytes, macrophages and microglia, neutrophils, T-cells, and dendritic cells [43]. As previously mentioned, the IL-6 family of cytokines include IL-6, OSM, LIF, and IL-23. These cytokines perform numerous biological functions in a wide variety of tissues including cell survival, proliferation, migration, control of apoptosis, and cell development and differentiation [43-45].

Similar to other cytokines, the IL-6 family signals by inducing the heterodimerization of its receptors. The IL-6 Receptor family include semi-specific receptors such as IL-6Rβ, LIFRβ, or OSMRβ. Ligand binding to a semi-specific receptor triggers heterodimerization with a common gp130 subunit, and subsequent activation of Janus kinases (JAKs). JAKs phosphorylate tyrosine residues within the cytoplasmic domain of gp130 subunit which creates a docking site for Src-homology-2 (SH2) domain containing proteins, including STATs [8].

OSM signals through gp130/LIFR, and gp130/OSMRβ receptor complexes. Downstream, OSM activates STAT1, STAT3, STAT5, ERK1/2 and p38 but not JNK. In the CNS, OSM is detected only in patients with neuroinflammatory disease such as MS, and it localized to activated microglia and infiltrating leukocytes. OSM has been shown to stimulate the expression of matrix metalloproteinases in astrocytes and IL-6, MCP-1 in astrocytes and brain endothelial cells [43-44].

Upregulation of OSM has also been detected in GBM when compared to the non-neoplastic brain biopsy samples [45]. Currently, there is disagreement in the literature about the role played by OSM in GBM; it has been shown *in vitro* to exert both, growth promoting and inhibitory effects [44].

2.7 RelB

RelB is a gene belonging the NF-κB transcription factor family. It is located on chromosome 19q13.32 and encodes a protein of 579 amino acids [46]. RelB, as a member of the NF-κB family, contains an ~300 amino acid Rel homology domain (RHD) which, as stated previously, is necessary for its DNA binding, dimerization, and nuclear localization [47,50].

However, RelB is unique among NF-κB family members with respect to its leucine zipper motif [46-47]. Leucine zippers are domains that facilitate pleiotropic interactions with other proteins containing leucine zippers [49].

RelB is often constitutively expressed in lymphocytes and dendritic cells (DCs) [54,55]. However, in many non-immune cell types, RelB requires stimulation to be produced [56,100]. The best-studied transactivator of induced RelB mRNA in immune cells is RelA, which can bind proximal RelB promoter NF-κB sites [56]; additionally, RelB can autoregulate its own mRNA levels by binding NF-κB sites in its own promoter [57].

RelB is best known for its roles in lymphoid development, DC biology, and non-canonical NF-κB signaling [50]. Additionally, RelB has recently been shown to be major contributor to chromatin biology, and acts to silence gene families through chromatin condensation. Specifically, RelB is required to repress proinflammatory genes during endotoxin tolerance [51] and couples with the nicotinamide adenine dinucleotide (NAD+)-dependent histone deacetylase SIRT1 to integrate signals from the inflammatory microenvironment with changes in the epigenetic landscape of cells [52].

Previous studies have implicated RelB in GBM behavior. RelB is a silhouette marker for the most inflammatory subtype of GBM, mesenchymal GBM [12]. Additionally, RelB has been implicated in maintaining GBM stem-like characteristics, mesenchymal transition, and to directly affect GBM migration, and invasion. Each of these actions has been attributed to the role of RelB in non-canonical NF-κB signaling [61-64].

2.8 SIRT1 and Endotoxin Tolerance

Sirtuins are a highly-conserved family of proteins, and belong to the family of class III histone deacetylases. Importantly, the family requires NAD+ as a cofactor for deacetylase activity. This finding has implicated the sirtuin family as a fundametnal link between energy, the cellular redox state, and the expression profile of cells [67]. Namely, the sirtuin family has been shown to respond to changes in NAD+ levels by remodeling the chromatin structure of genes annotated to immunological and metabolic pathways [65-70].

SIRT1, putatively known as a major metabolic regulator, has been shown to function in many biological settings such as aging, insulin resistance, cellular metabolism and sepsis. Importantly, in the context of acute inflammation, SIRT1 epigenetically reprograms proinflammatory genes to establish 'immunologic tolerance' and prevent chronic inflammatory signaling [51,52,65].

Perhaps the best elucidated mechanism demonstrating SIRT1 negative feedback of proinflammatory signals is in the macrophage model of sepsis. Specifically, Toll Like Receptor 4 (TLR4) stimulation due to endotoxin, in collaboration with increases in nuclear NAD+ concentration, activates SIRT1, which promotes facultative heterochromatin formation at the promotors of proinflammatory genes such as IL-1B [51,52]. Mechanistically, SIRT1 first directly binds and deactivates p65 through deacetylation [51]. SIRT1 also induces RelB transcription and promotes RelB replacement of p65 on cytokine promoters. RelB and SIRT1 then physically interact to form a mature repressor complex [52,72].

Interestingly, in a variety of biological settings, SIRT1 transcription is reduced in states of chronic inflammation. For instance, SIRT1 transcription is decreased in fat deposits contributing

to obesity related inflammation [68], the brain in Alzheimer's disease [69], and arterial inflammation in atherosclerosis [68].

2.9 YY1

The Transcription factor Ying Yang 1 (YY1) is a ubiquitous and multifunctional zinc-finger transcription factor which controls many diverse functions including transcriptional control, cell growth control, large scale chromosomal dynamics, X-chromosome inactivation, and DNA repair [73,76]. Transcriptionally, YY1 can function as either as an activator or a repressor depending on its DNA binding site, associated transcription factors, and cell context. While full knockout of YY1 was shown to be embryonic lethal, studies using YY1 +/- mice and Xenopus have demonstrated the importance of YY1 in development, neuronal differentiation and neurotransmitter transport in the nervous system [74,75]. Given the role of YY1 in development and growth, it comes as little surprise that YY1 has been implicated in cancer biology. Increased YY1 levels have been observed in lung, liver, bladder, cervix, skin, bone, breast, ovary, prostate, and colon cancers compared to normal control tissue [76]. Additionally, YY1 levels have been shown to correlate with a poor prognosis in prostate, breast and bone cancers. Independent studies using siRNA knockdown of YY1 on cancer cell lines have demonstrated that YY1 controls the proliferation, motility, and invasion through soft agar of both ovarian cancer cell lines and osteogenic sarcoma cells in two independent studies [76].

The signaling mechanism by which YY1 exerts its effects is controversial, and most likely variable between cell types. One well studied mechanism of action of YY1 is as a member of the Polycomb Repressor 2 complex (PRC2)[77]. YY1 contains a Recruitment of Polycomb (REPO)

domain which allows YY1 to direct PRC2 to near genomic areas, and to deacetylate and methylate histones [77].

It has previously been demonstrated that NF-κB signaling can control YY1 expression [78]. Additionally, one study has shown that YY1 directly interacts with NF-κB family members to form a transcriptional activating complex. Namely, it has been shown to form a direct transcriptional activating complex with RelB, p50, and Octamer Binding Protein 2 (Oct2) in the promoters of the IgG heavy chain in Leukemic cells. Interestingly, YY1 was shown to interact only with RelB and not p65 or p52[79].

2.10 Hypothesis/Aim

Our group hypothesized that within GBM cells, there are inflammatory signaling cascades which evade negative feedback and promote chronic inflammatory signaling. Thus, we aimed to (i) identify cytokines drivers which initiate such GBM-specific inflammatory pathways, (ii) identify changes in inflammatory signaling between GBM cells and astrocytes which lead to a loss of negative feedback, and (iii) potentially identify new players which could be targeted to limit aberrant chronic inflammation in GBM.

3 Chapter 2: RelB-canonical signaling plays opposite roles in GBM and normal glial cells

3.1 Abstract

Robust angiogenesis, radioresistance, and invasion make glioblastoma multiforme (GBM) one of the most difficult to treat, and lethal cancers [2,81-82]. A driving factor in the profound aggressiveness of GBM is the establishment of a chronic inflammatory microenvironment [3-4,83-84]. Physiologically proinflammatory signals initiate a cascade of negative feedback mechanisms which prevent chronic inflammation. To date, it is unclear how GBM tumor cells evade negative feedback mechanisms to establish a chronic proinflammatory microenvironment. In preliminary studies, we have identified two 'driver' proinflammatory cytokines, IL-1B and OSM, that are specifically overexpressed in patients with the most common and deadly mesenchymal subtype of GBM, which displays extensive necrosis and inflammation [14-15]. The link between inflammation, activation of NF-κB, and cancer has now been firmly established [85]. Classically, canonical or non-canonical NF-κB pathways lead to the activation of p65/p50 or RelB/p52 heterodimers, respectively. Surprisingly, we found that RelB drives GBM progression by RelB/p50 heterodimers, which are believed to function only in cells expressing high levels of RelB [85,86]. We found that, in untransformed astrocytes, RelB/p50 signaling functions to repress transcription, and act as a potent negative feedback loop for IL-1/OSM induced inflammation. RelB has previously been shown to be a master regulator of

epigenetic inflammatory tolerance in sepsis [51,52]. Novelly, we demonstrate that the molecular mechanism underlying RelB negative feedback in astrocytes is one of "sterile" inflammatory tolerance. Strikingly, we show that in GBM cells RelB switches roles, and acts as a global transcriptional activator, promoting a feedforward proinflammatory loop to establish chronic inflammation. Lastly, we established a molecular mechanism for the lack of RelB dependent silencing in GBM. The RelB-dependent epigenetic silencing shown in macrophages requires histone deacetylase SIRT1, and we found that SIRT1 is needed to repress cytokines in astrocytes in a RelB dependent manner. Significantly, we discovered that one allele of the SIRT1 gene is deleted in 80% of GBM tumors, and that human GBM tumors display drastically diminished SIRT1 expression and protein levels. Thus, we show that that cytokine-induced RelB/p50 heterodimers act as a molecular switch to establish chronic inflammation in GBM and promote GBM aggressiveness.

3.2 Introduction

GBM is a major clinical problem and patients with these aggressive tumors have very low survival rates [1-3]. Despite extensive research and the use of multimodal clinical approaches, including surgical resection, radio- and chemo-therapy, little improvement has been made in patient survival in the past decade. Therefore, there is a desperate need for novel research approaches and clinical therapies.

GBM tumors are characterized by necrosis and profound inflammation [4,14-15,82-83]. Cytokines and growth factors secreted by both GBM cells and the tumor microenvironment initiate signaling cascades, which support GBM progression [82,83,87]. Chronic inflammation promotes GBM cell proliferation, migration, invasion, resistance to apoptosis, and the maintenance of stem cell like properties [4,64,82-83,87-92]. Physiologically, inflammation is short lived, with a host of negative feedback loops to initiate an adaptive phase of inflammation and shut off proinflammatory signals. Currently, it is unclear how tumors evade negative feedback loops, and drive chronic inflammation.

For the past decade, inflammation and aberrant NF-κB signaling have been linked to oncogenesis [84]; however, it has been attributed to either p65/p50 or RelB/p52. Classically, canonical or non-canonical NF-κB pathways lead to the activation of p65/p50 or RelB/p52 heterodimers, respectively. RelB is a silhouette marker of mesenchymal GBM [12], the most proinflammatory GBM subtype. Interestingly however, cytokine-induced RelB expression is

regarded as one of the critical negative feedback loops limiting acute inflammation driven by p65/p50 [51-52,93-94].

To date, three mechanisms have been proposed to explain the anti-inflammatory actions of RelB. First proposed was that RelB binds p65 to form a transcriptionally inactive complex [93]. Additionally, Saccani et al. proposed that RelB/p50 dimers displace active p65/p50 dimers off cytokine promoters [94]. Most recently however McCall et al. elegantly demonstrated that RelB acts as an epigenetic modifier of the chromatin structure of cytokine promoters in collaboration with SIRT1, in response to TLR4 stimulation and NF-κB activation [51-52].

Our preliminary studies indicated that both IL-1B and OSM are potential drivers of chronic inflammation of GBM. Additionally, we observed that IL-1B and OSM are potent activators of the RelB-canonical pathway, and that RelB is an independent marker of severe disease and a proinflammatory phenotype in GBM. Given the current need to understand mechanisms by which chronic inflammation develops in GBM, we investigated the role of RelB in limiting the spread of chronic inflammation in GBM.

3.3 Materials and Methods

Cell Culture Human cortical astrocyte cultures were established using dissociated human cerebral tissue. Cortical tissue was provided by Advanced Bioscience Resources (Alameda, CA), and the protocol for obtaining postmortem fetal neural tissue complied with the federal guidelines for fetal research and with the Uniformed Anatomical Gift Act. Human glioblastoma U373-MG cells were obtained from American Type Culture Collection (Rockville, MD), whereas human glioma U87 cells were obtained from Dr. Jaharul Haque (Cleveland Clinic Foundation, Cleveland, OH). Primary GBM 12 cells were obtained from Dr. Paul Dent (Virginia Commonwealth University, Richmond, VA). Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, antibiotics, sodium pyruvate, and non-essential amino acids.

Down-regulation of target genes Expression of RelB and SIRT1 was down-regulated using SmartPool siRNAs (siRelB and siSIRT1) from Dharmacon (Dharmacon, Int., Lafayette, CO). siRNA was transfected into astrocytes and GBM cells using Dharmafect 1, according to the manufacturer's instructions.

RNA isolation and quantitative qPCR Total cellular RNA was prepared by Trizol (Invitrogen, Carlsbad, CA), following the manufacturer's protocol. Subsequently, 1 ug of total RNA was reverse-transcribed using the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). IL1B, IL6, IL8, COX-2 and GAPDH mRNA levels were measured using pre-mixed primer-probe sets, and TaqMan Universal PCR Master Mix according to the supplier's

instructions (Applied Biosystems, Foster City, CA). The cDNAs were diluted 10-fold (for the target genes) or 100-fold (for GAPDH), and amplified using the ABI 7900HT cycler. Gene expression levels were normalized to GAPDH mRNA levels, and presented as a fold induction with mean values +/- standard deviation.

Cytokines and Cell Stimulation Cells were stimulated with 25 ng/ml OSM (R&D, Systems, Inc., Minneapolis, MN), or 10 ng/ml IL-1 (a gift from Immunex Corp., Seattle, WA).

Western Blot The cells were lysed in 10 mM Tris (pH 7.4), 150 mM sodium chloride, 1 mM EDTA, 0.5% Nonidet P-40, 1% Triton X-100, 1 mM sodium orthovanadate, 0.2 mM PMSF, and protease inhibitor mixture (Roche Applied Science, Indianapolis, IN). Samples were separated using SDS-PAGE and transferred onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). The anti-RelB, anti-p65, anti-p50, anti–β-tubulin, and anti-GAPDH antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antigen-Antibody complexes were visualized by ECL using Immobilon Western blotting kit (Millipore, Temecula, CA).

Generation of SIRT1 Overexpressing Cells To generate stable clones, 4 ug SIRT1 expression plasmids were transfected into a 10 cm dish of U373 cells. Clones selected in DMEM containing 75 ug/ml puromycin were subsequently pooled.

Unbiased Cytokine KM Analysis Cytokine and Cytokine Receptor expression data was downloaded from Nature, 2008 GBM TCGA cohort. For each gene the mean Z-score normalized expression value was calculated. Patients were divided into high and low expressing groups based on the mean value of gene expression. Kaplan Meier analysis was performed for each gene based on high and low expression groups. Cytokines and cytokine receptors were

ranked based on the statistical significance of the individual Kaplan Meier analysis. Rank of cytokines and cytokine receptors were combined to create a combined rank score to indicate the prognostic impact of each cytokine signaling program.

Nuclear and Cytosolic Fraction Assays Cells were washed with cold PBS and re-suspended in buffer containing 10 mM Hepes (pH 7.8), 10 mM KCl, 0.1 mM EDTA, 1 mM Na3VO4, 1 mM DTT, 1:500 protease inhibitors (Sigma) and 0.2 mM PMSF, and incubated on ice for 15 min. NP-40 was added (0.75%) and cells were vortexed for 10 sec. Nuclei and supernatant cytoplasm were separated by centrifugation at 3000 rpm for 3 min at 4˚C. Nuclei were re- suspended in buffer containing 20 mM Hepes (pH 7.8), 0.4 M NaCl, 1 mM EDTA, 1 mM Na3VO4, 1 mM DTT and 1:500 protease inhibitors and incubated on ice for 15 min. Nuclear extracts were cleared by centrifugation at 14,000 x g for 5 min at 4˚C.

Lentiviral CRIPSR/Cas9 Guide RNAs were constructed using the MIT CRISPR guide design tool (http://crispr.mit.edu/). Lentivirus cloning, and packaging was conducted using GeCKO lentiviral crispr toolbox according to manufacturers instructions (http://genome-engineering.org/gecko/). After viral transduction, cells were selected with puromycin, and screened for homozygous knockout single colonies.

Migration Cells were cultures in 6 well plates and serum starved overnight. Cytokines were added at time 0hrs. Pictures were taken of scratched wells at time 0hrs and 24 hours. Cells migrating past original scratch boundary were enumerated and reported.

SIRT1 Activity Assay Cells were removed from media and incubated in lysis buffer without PMSF or peptidase added. Lysates were sonicated on ice. 200ug of cell lysate was incubated with anti-SIRT1 antibody overnight at +4 degrees C. 20ul of 50% Protein A beads were added to

lysate and incubated with gentle rocking for 3 hours at +4 degrees C. Lysate was centrifuged and bead pellet was washed in lysis buffer. Next, beads were washed in SIRT1 assay buffer [50mM Tris-Hcl ph 8.8, 0.5 mM DTT). Beads were added to Flouro-Substrate mixture according to AbCam instructions (ab156065), and absorbance at 455 nm was tabulated.

Clinical Samples Patient RNA samples and tumor slices were provided by the VCU Tissue Acquisition and Analysis core (TDAAC).

Immunoflouresence Cells cultured on sterile coverslips in a 6 well dish. Cells were then washed with PBS and fixed for 10 minutes in 2.5% paraformaldehyde at room temperature. Cells were then washed with 0.3M glycine and permeabilized with 0.1% triton-X. Cells were blocked with 5% BSA/1% NGS for 1 hr and then incubated with primary antibody overnight at +4 degrees C. Cells were then washed and incubated with 1:500 dilution of secondary antibody for 1 hour protected from light. Cells were washed again, counterstained with Hoescht [1:20,000] and mounted on slides using VectaShield.

Immunohistochemistry Slides were fixed in ice cold acetone for 20 minutes, then incubated in 1% hydrogen peroxide. Next, sliders were washed in PBS and blocked in 5%BSA/1%Normal Goat Serum for 1 hour. The slides were then incubated with primary antibody and blocking buffer overnight at +4 degrees C. The next day slides were washed in PBS and incubated with EnVision+ secondary reagent (Agilent Pathology Solutions) for 20 minutes, washed in PBS, and then exposed using DAB+ chromogen for 10 minutes.

Microarray processing and differential expression analysis Microarrays were processed using the R statistical package requiring the libraries ‘affy’, ‘affyPLM’. Quality control was performed using analysis of 3’/5’ ratios, generating representative array images, and creating RNA

degradation plots after generating affybatch objects using the 'affy' and 'affPLM' libraries. Expression summaries of arrays were generated after background correction, and normalization using robust multichip averaging. Statistical significance was assessed using S-testing.

RNAseq processing and differential expression analysis RNA was isolated using the 'mirVana RNA isolation kit' according to manufacturer's instructions (ThermoFisher Scientific, Waltham MA). RNA was sent to the University of Cinncinnati Genomics Core for quality control and RNA-sequencing analysis. Data was analyzed using the tuxedo pathway, . fastq read files were aligned using BowTie2, transcript assembly and FPKM estimates achieved using CuffLinks, and testing for differential gene expression and promoter usage will be accomplished using CuffDff.

Statistical Analysis All experiments were repeated at least three times with consistent results. Data are presented as mean ± SD. GraphPad Prism software was used for statistical analyses unless otherwise indicated. A Bonferroni posthoc test was used for one-way ANOVA comparisons, with $p < 0.05$ being considered statistically significant. Independent sample Student *t* test was used for unpaired observations.

3.4 Results

3.4.1 IL-1B and OSM signaling are hallmark cytokines of a poor prognosis and potential drivers of chronic inflammation in GBM

Although proinflammatory cytokines are secreted transiently, during acute inflammation, chronic inflammation develops in GBM and promotes GBM cell proliferation, migration, invasion, resistance to apoptosis, and the maintenance of stem cell like properties [4,64,82-83,87-92]. To identify specific cytokines which support GBM aggressiveness and potentially drive the establishment of chronic inflammation in GBM we utilized an unbiased approach and correlated cytokine expression with clinical outcome data from The Cancer Genome Atlas (TCGA). Specifically, we built an R function, 'CytoAnalysis', which uses Kaplan Meier analysis to identify cytokine genes and receptors which are statistically significant markers of a poor prognosis. Next the program uses a rank based method to combine prognostic scores for cytokines and their associated receptors to eliminate bystander effects and identify driver cytokine signaling pathways (figure 3.1). We identified IL-1B and OSM as two cytokine signaling pathways whose expression is negatively correlated with patient survival (Figure 3.2).

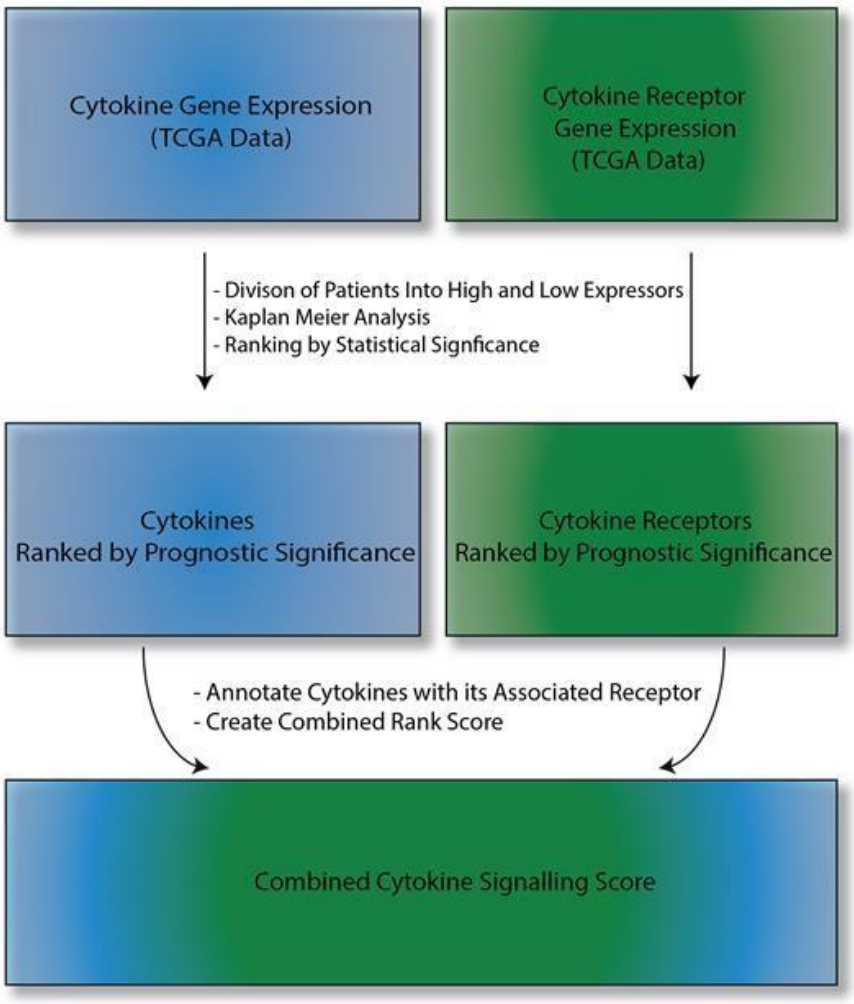
Given that members of the IL-1B and OSM signaling pathway are predictive markers of severe disease, we investigated whether IL-1B and OSM expression is correlated. Thus, our group could delineate if IL-1B and OSM signaling pathways are engaged in similar or disparate microenvironments. To address this we correlated the expression of IL-1B and OSM using GBM patient expression data from TCGA. We obtained a strong, statistically significant, positive regressive relationship between IL-1B and OSM; with a Pearson correlation of 0.769 (Figure 3.3). Additionally, we sought to characterize the biological significance the IL-1B/OSM Pearson

correlation. To do this, we again utilized the TCGA GBM patient cohort, and ranked every expressed gene in the genome according to their Pearson correlation with IL-1B. Interestingly, we found that OSM is the second most correlated gene in the genome with IL-1B according to this patient group (Figure 3.3). These data suggest that IL-1B and OSM expression and signaling occur together within the brain tumor microenvironment. This finding is agreement with previous studies which suggest that IL-1B secreted from GBM tumor cells stimulates the production of OSM from resident microglia [95].

Lastly, we queried whether these genes were highest in most aggressive and most inflammatory mesenchymal subtype. Using GBM patient samples whose subtype had been annotated by TCGA, we found that both IL-1B and OSM expression is statistically enriched in mesenchymal GBM in comparison to all other subtypes (Figure 3.4). In conclusion, using our unbiased approach we identified two hallmark cytokines which are markers of a poor prognosis, expressed in the same microenvironment, and most prominent in the most inflammatory subtype of GBM. Thus, when modelling a pathological inflammatory microenvironment *in vitro* we performed IL-1B and OSM cotreatment.

Figure 3.1 Using an unbiased approach to identify potential drivers of chronic inflammation.

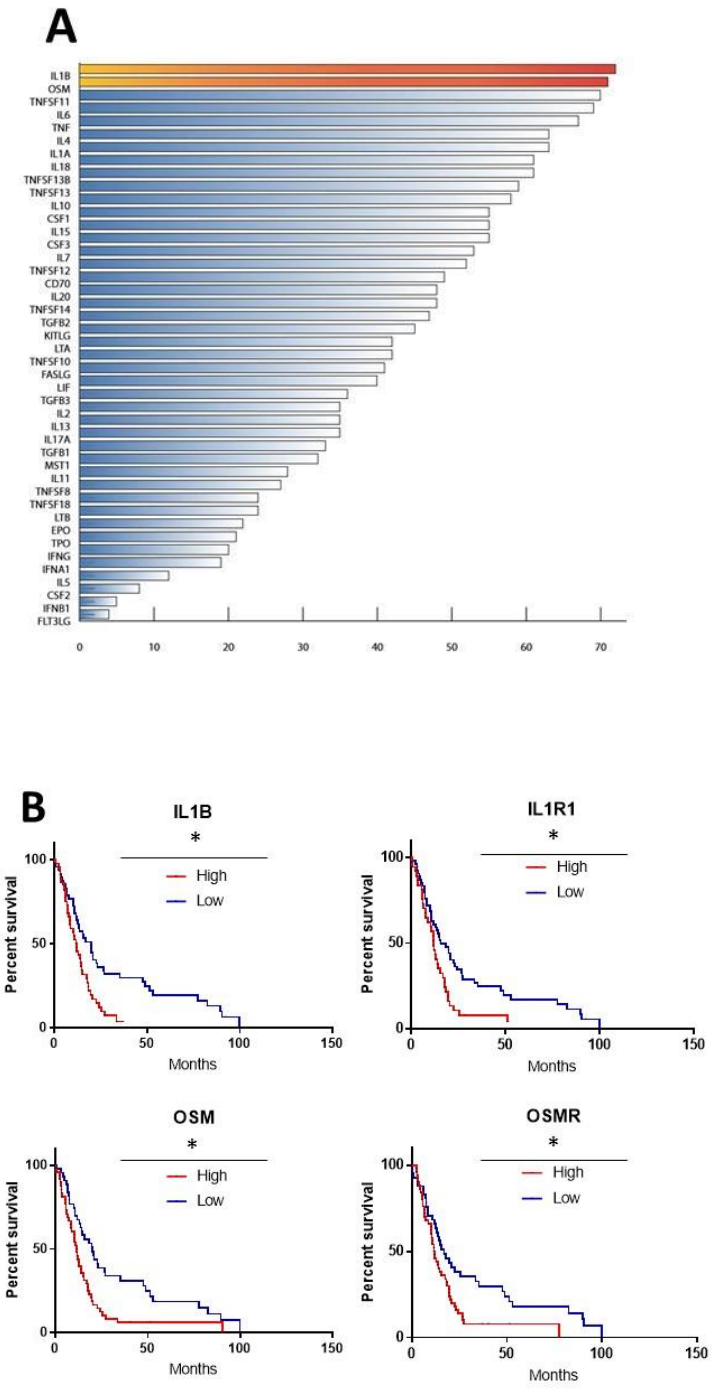
Workflow of CytoAnalysis. CytoAnalysis independently interfaces with TCGA to download cytokine gene expression data and cytokine receptor expression data from the GBM patient cohort (n=208). For each gene, CytoAnalysis calculates the mean Z-score normalized expression value, and uses the mean Z-score expression value to group patients into high and low expressors. Next, CytoAnalysis performs iterative Kaplan Meier analysis for all cytokine and cytokine receptor genes and returns a p-value indicating the strength of the prognostic relationship between each gene and GBM patient survival. This p-value is used to rank cytokines and cytokine receptors according to the best indicators of a poor prognosis. Lastly CytoAnalysis combines the rank of cytokines and their associated receptors to generate a combined cytokine signaling score.


Cytokine Gene Expression
(TCGA Data)
Cytokine Receptor
Gene Expression
(TCGA Data)
- Divison of Patients Into High and Low Expressors
- Kaplan Meier Analysis
- Ranking by Statistical Signficance
Cytokines
Ranked by Prognostic Significance
Cytokine Receptors
Ranked by Prognostic Significance
- Annotate Cytokines with its Associated Receptor
- Create Combined Rank Score
Combined Cytokine Signalling Score

3-1

Figure 3.2 Kaplan Meier Analysis of Cytokines and Receptors.

A) Combined rank score of cytokine signaling programs as determined by CytoAnalysis. Scores were calculated as described in the materials and methods. B) Individual Kaplan Meier analysis of the IL-1B and OSM signaling programs. Patients were annotated as high and low expressors of each gene using the mean Z-score expression level as a cutoff. Statistical significance was assessed using the Cox-Proportional Hazards model.

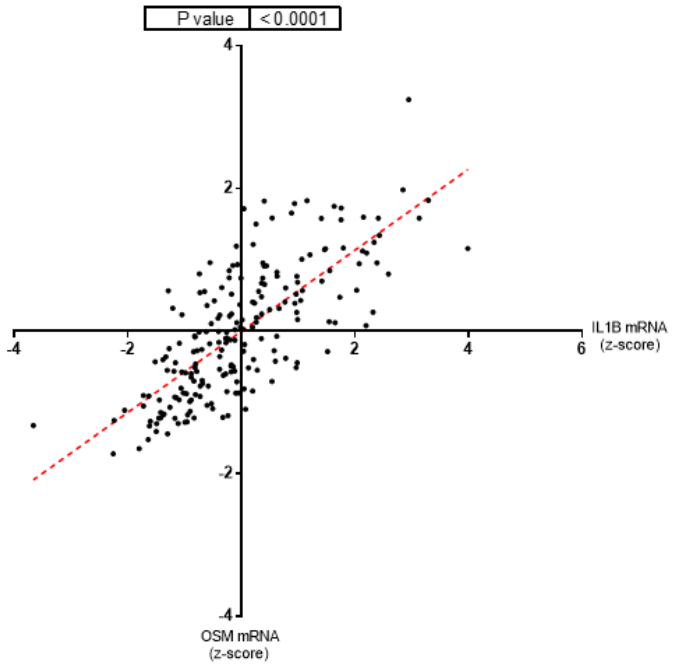

A
IL1B
OSM
TNFSF11
IL6
TNF
IL4
IL1A
IL18
TNFSF13B
TNFSF13
IL10
CSF1
IL15
CSF3
IL7
TNFSF12
CD70
IL20
TNFSF14
TGFB2
KITLG
LTA
TNFSF10
FASLG
LIF
TGFB3
IL2
IL13
IL17A
TGFB1
MST1
IL11
TNFSF8
TNFSF18
LTB
EPO
TPO
IFNG
IFNA1
IL5
CSF2
IFNB1
FLT3LG
0
10
20
30
40
50
60
70
B
IL1B
IL1R1
OSM
OSMR
*
High
Low
Percent survival
Months
0
50
100
150

3-2

Figure 3.3 Correlation of IL-1B and OSM expression in GBM patients.

A) Pearson correlation of IL-1B and OSM mRNA gene expression scores from TCGA GBM cohort. Normalized Z-score expression values were downloaded from TCGA using cBioPortal. Pearson correlation was conducted using cBioPortal, p-value and regression analysis was performed using the 'lm' function in the core R statistical package. B) Genome wide gene rank of Pearson correlation with IL-1B. Gene correlations were performed using the correlation functionality in cBioPortal.

A



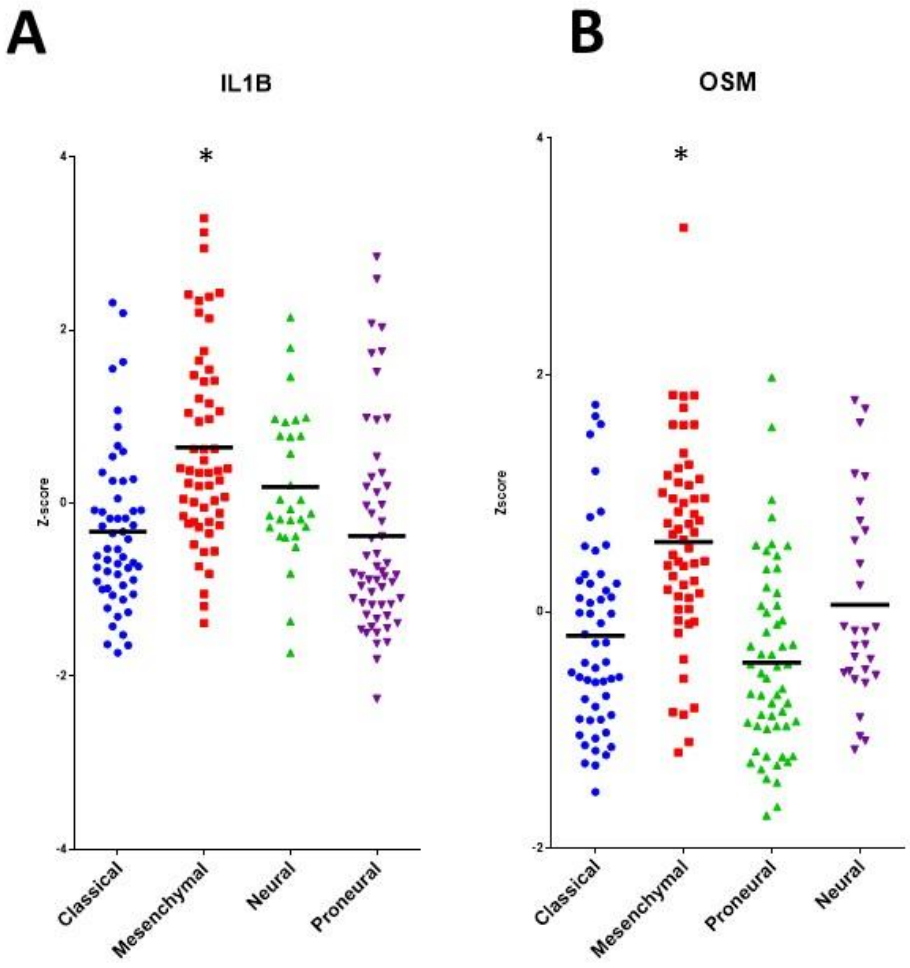
B

Rank	Gene	Pearson Correlation with IL-1β
1	GPR84	0.78
2	OSM	0.77
3	IL1A	0.71
4	IER3	0.69
5	THBD	0.68

3-3

Figure 3.4 Cytokine expression levels according to GBM subtype.

A-C) Patient subtype was downloaded using clinical expression information contained within TCGA. Statistical analysis of cytokine expression was conducted using a one way ANOVA analysis (* = $p<0.05$) using GraphPad Prism software.


A
IL1B
Z-score
4
2
0
-2
-4
*
Classical
Mesenchymal
Neural
Proneural
B
OSM
Zscore
4
2
0
-2
*
Classical
Mesenchymal
Proneural
Neural

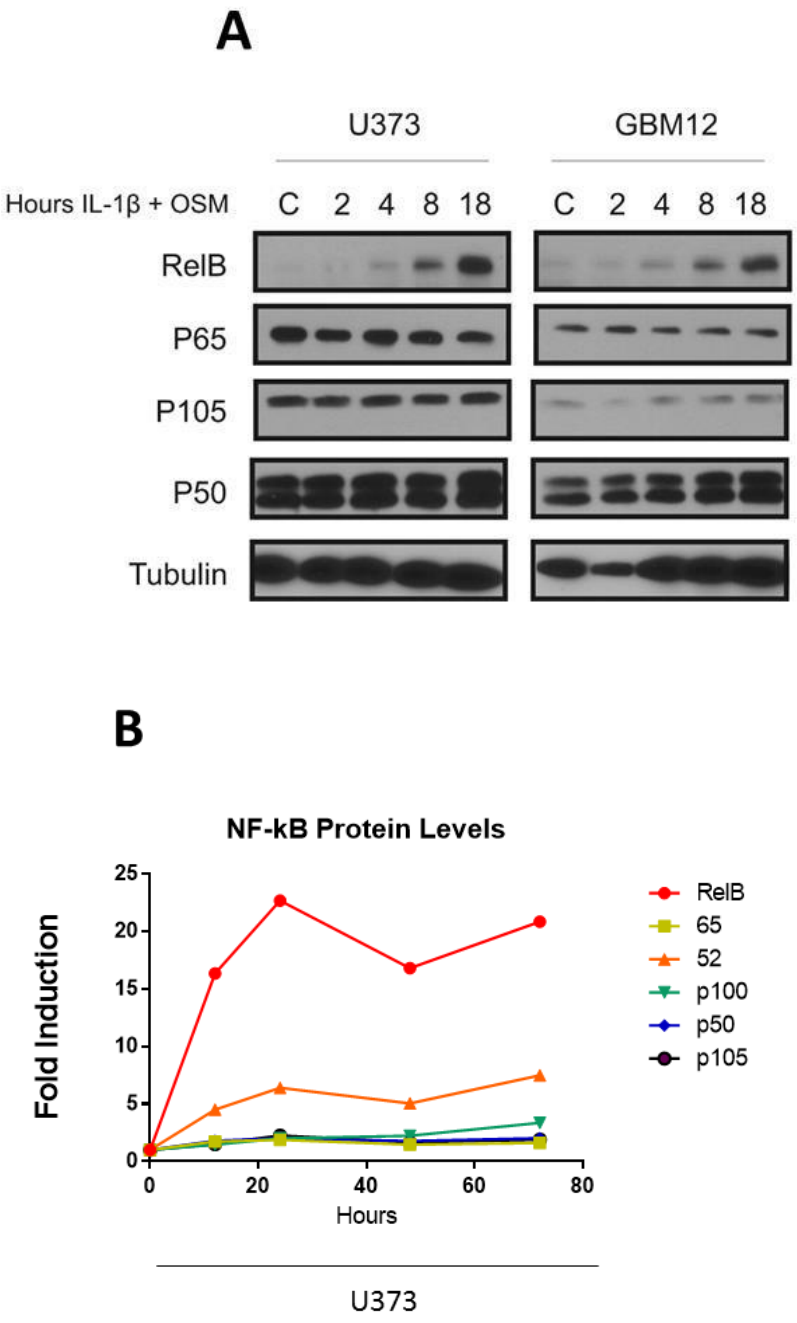
3-4

3.4.2 IL-1B and OSM activates RelB-p50 signaling

Interestingly, an excess of RelB forms RelB/p50/IκBa complexes, which are activated by canonical NF-κB stimuli, such as IL-1B [46,48]. Since RelB has higher affinity for p100/p52 than for p50 [97], canonical RelB activation is minimal in the absence of high RelB expression [56,96]. Since non-lymphoid cells, including astrocytes, express RelB at low levels, its dimerization with p50 does not occur basally. Nevertheless, p65/p50 induces RelB expression, which subsequently forms complexes with p50 [97]. These complexes have been shown to provide a negative feedback loop suppressing cytokines by several mechanisms including epigenetic silencing [51-52, 93-94]. While non-canonical RelB/p52 signaling has been suggested to play a role in GBM [61-64], our data show that IL-1B and OSM treatment promotes canonical RelB/p50 signaling in GBM cells and astrocytes. Similarly to what has previously been found in astrocytes [100], basal expression of RelB is relatively low in GBM cells. However, IL-1B induces expression of RelB and p50 in primary GBM and established GBM cell lines (Fig. 3.5). Interestingly, the fold increase in RelB far outpaces the fold increase in other NF-κB family members suggesting the production of an excess of RelB, to subsequently form RelB/p50/IκBa complexes. RelB translocates to the nucleus of GBM cells in response to IL-1B/OSM (Fig. 3.6), and IL-1B induces formation of RelB/p50 complexes (Fig 3.7). Intriguingly, OSM has been shown to increase RelB/p50 complex formation by a mechanism independent of increased RelB synthesis [105].

Figure 3.5 RelB induction in response to IL-1B and OSM treatment.

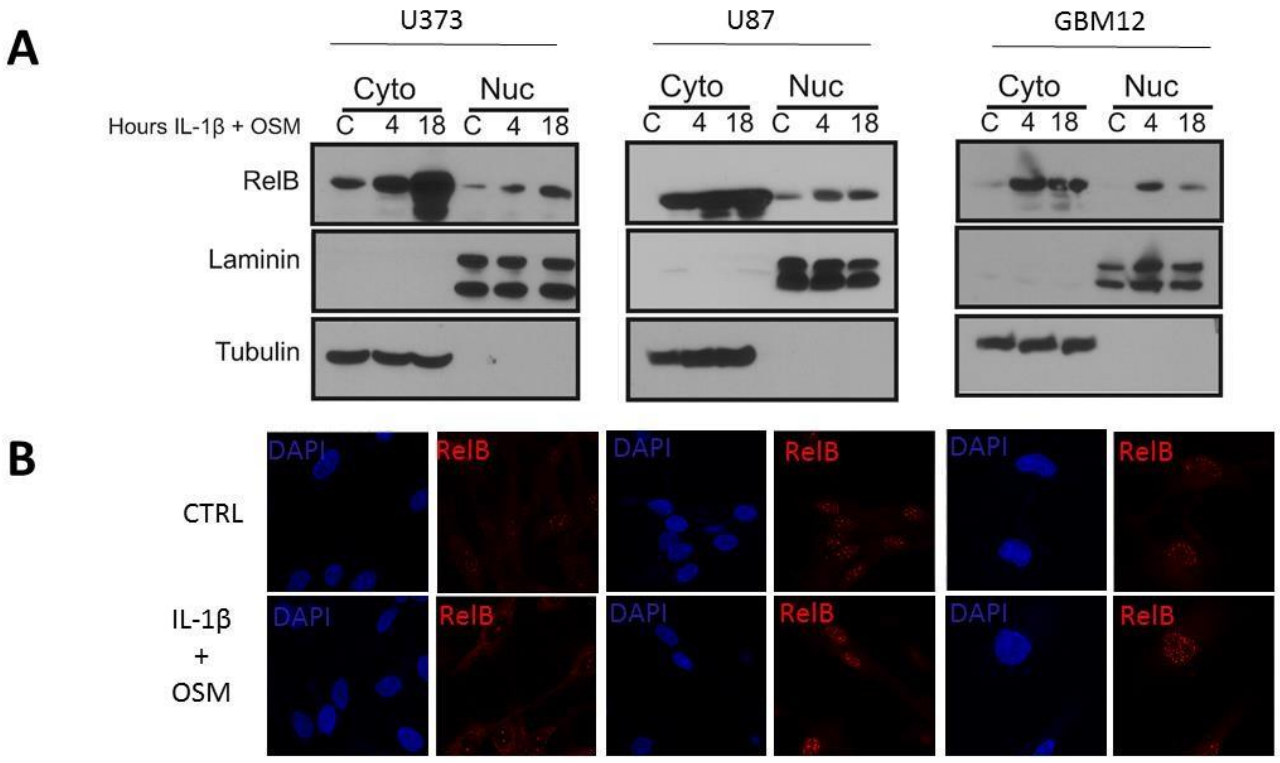
A) Protein levels of NF-κB family members in response to IL-1B and OSM treatment in U373 and primary GBM 12 cells. Cells were treated for the time periods indicated. B) 3 day induction study of NF-κB family members in response to IL-1B and OSM. Cells were treated with IL-1B and OSM, cell lysates were harvested at 12, 24, 48, and 72 hours, and protein levels of NF-κB family members assessed via western blot. Densitometery was calculated using ImageJ software.


A
U373
GBM12
Hours IL-1β + OSM
C 2 4 8 18
C 2 4 8 18
RelB
P65
P105
P50
Tubulin
B
NF-kB Protein Levels
Fold Induction
25
20
15
10
5
0
0 20 40 60 80
Hours
RelB
65
52
p100
p50
p105
U373

3-5

Figure 3.6 RelB Nuclear Translocation in GBM cells.

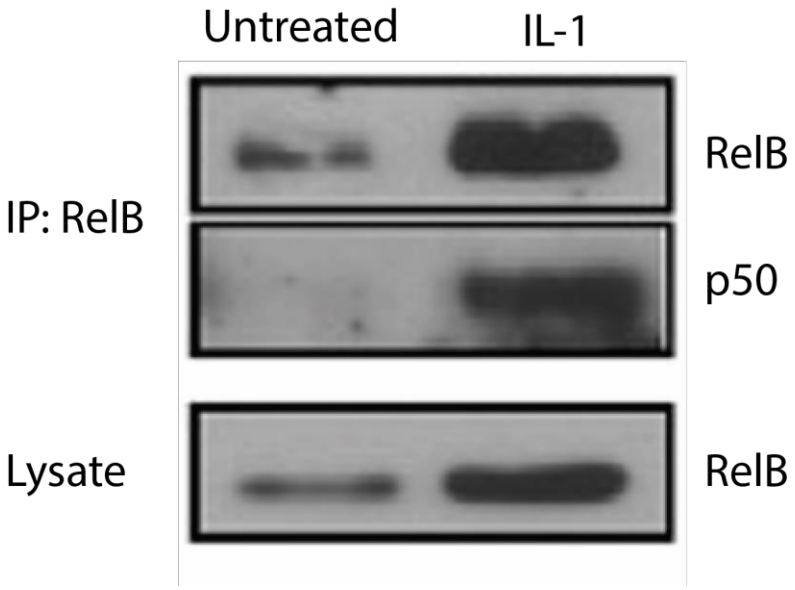
A) Nuclear and cytoplasmic fractionation for RelB nuclear translocation. Nuclear and cytosolic fractions of U373, U87 and primary GBM12 cells were isolated at indicated time points, after IL-1B and OSM stimulation, according to the protocol described in the materials and methods, and probed for RelB. B) 18 hours after IL-1B and OSM stimulation U373, U87, and primary GBM12 cells were fixed and stained and visualized via immunofluorescence for RelB to assess RelB nuclear accumulation after IL-1B and OSM treatment.


A
U373
U87
GBM12
Cyto
Nuc
Hours IL-1β + OSM
C 4 18 C 4 18
RelB
Laminin
Tubulin
B
CTRL
IL-1β + OSM
DAPI
RelB

3-6

Figure 3.7 IL-1B/OSM Induces RelB/p50 signaling

U373 were stimulated with IL-1 for 8 hours, RelB was immunoprecipitated, and co-immunoprecipitated p50 and RelB were detected by Western blotting, as indicated. Expression of RelB in the lysates is shown the lower panels (lysate).



3-7

3.4.3 RelB acts as a molecular switch of proinflammatory cytokine production in glioblastoma vs primary human astrocytes

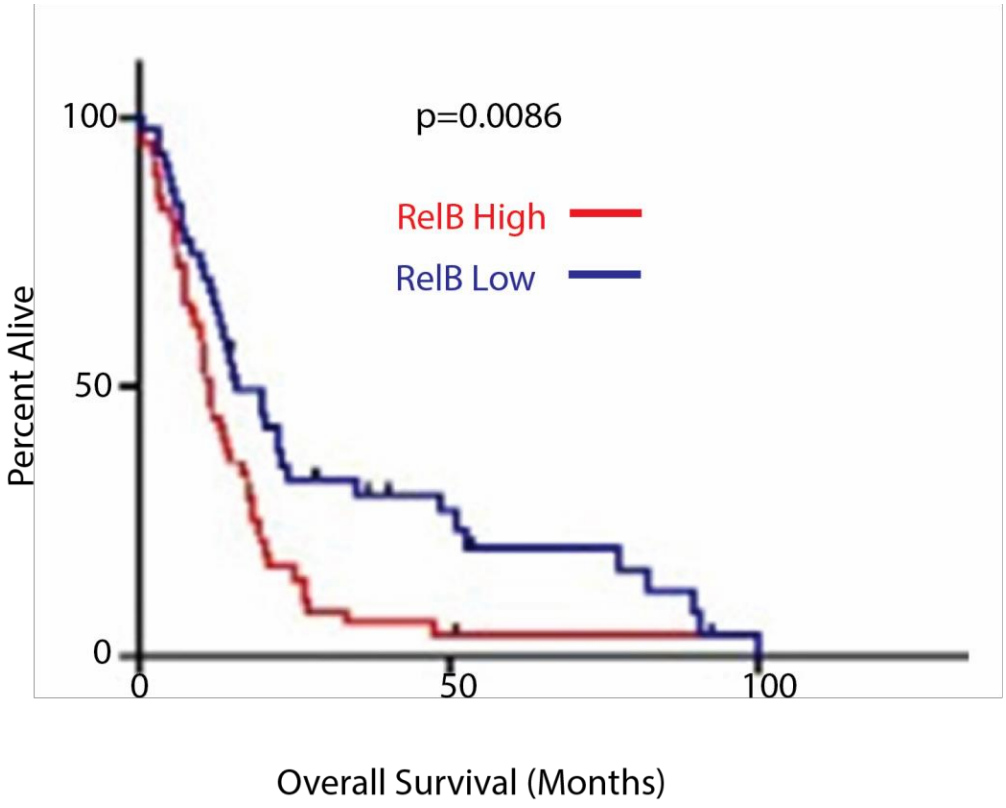
Our data suggested that RelB expression is similarly induced by IL-1B and OSM in astrocytes [100] and GBM cells. Additionally, we had shown that IL-1B and OSM cotreatement leads to RelB nuclear translocation, and activation of the RelB canonical NF-κB pathway. Previously, RelB has been shown to act as a critical negative feedback regulator of NF-κB signaling through histone modification [51,52].

Given that chronic inflammation drives GBM progression, we hypothesized that an intact RelB cytokine silencing mechanism would lead to less aggressive disease. Conversely however, Kaplan Meier analysis of RelB in GBM tumors suggest that increased RelB levels lead to more aggressive disease (Fig 3.8). Additionally, our unbiased cytokine analysis suggested that IL-1B and OSM are potential drivers of chronic inflammation. Given that IL-1B and OSM potently activate RelB canonical signaling, we investigated whether IL-1B/OSM induced RelB signaling indeed functions to silence cytokine production in GBM cells.

Strikingly, while RelB suppressed cytokine (IL-1, IL-6) expression in astrocytes (Fig 3.9) in a manner similar to what has been previously been shown in macrophages, RelB surprisingly enhanced cytokine expression in established and primary GBM cell lines (U373, U87, GBM12) (Fig 3.10). These data imply that the anti-inflammatory RelB-driven feedback loop present in astrocytes is converted into a feed-forward loop in GBM cells. Additionally, the aberrant RelB switch from silencing factor to activating factor controls not only proinflammatory cytokines, but also important proinflammatory enzymes previously implicated in GBM progression such as COX-2.

Figure 3.8 RelB Survival Analysis in GBM.

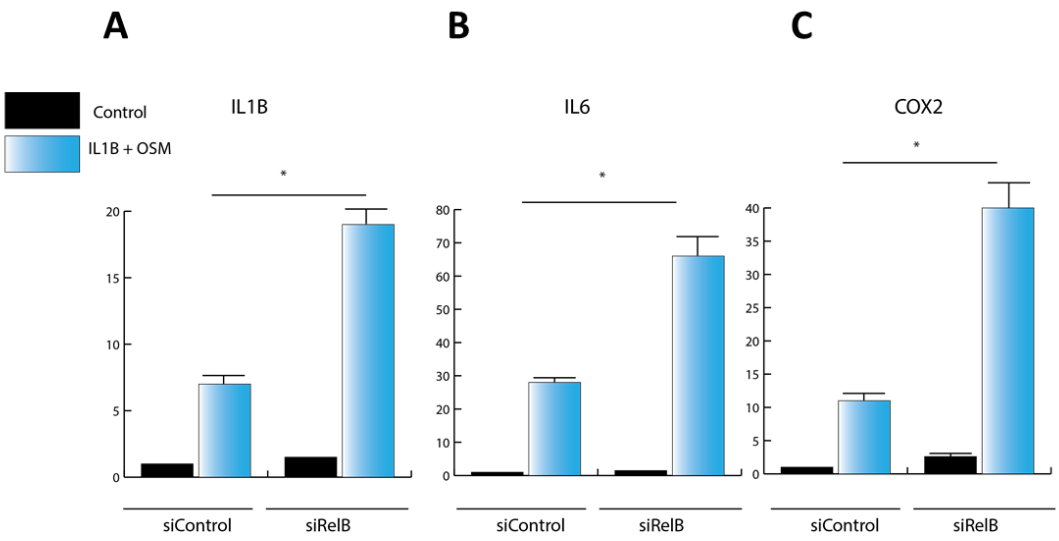
Kaplan Meier analysis of RelB high and low expressing patients in the TCGA GBM cohort (n=208). Z-score normalized RelB expression values from TCGA were obtained using the cBioportal web portal. Kaplan Meier analysis was conducted using GraphPad Prism software. Statistical significance was assessed using a Cox proportional hazard ratio test.


100
50
0
0
50
100
p=0.0086
RelB High
RelB Low
Percent Alive
Overall Survival (Months)

3-8

Figure 3.9 RelB effect on IL-1B and OSM induced inflammation in primary human astrocytes.

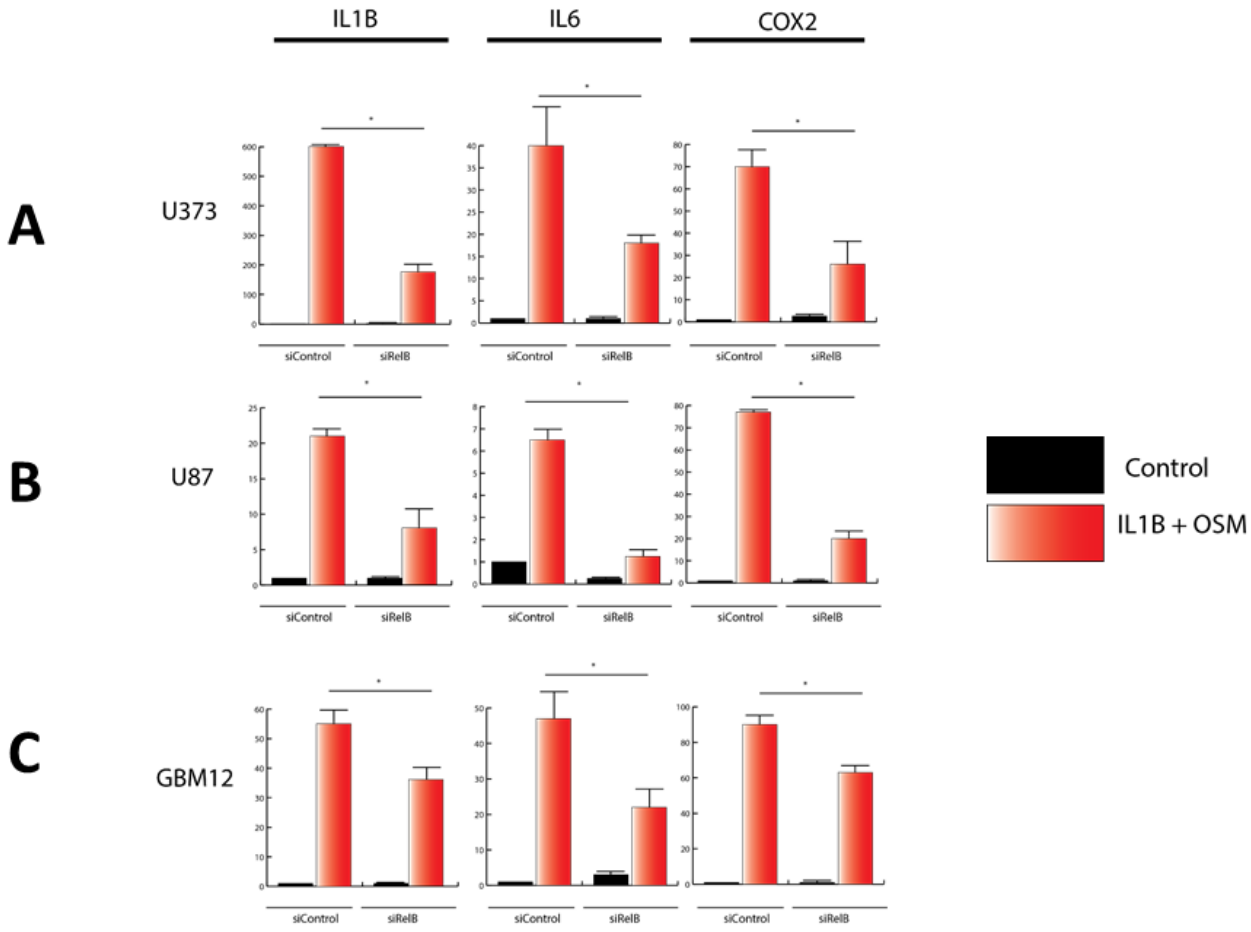
A-C) Primary human astrocyte were treated with siControl or SiRelB as indicated. Cells were treated with IL-1B and OSM and incubated for 18 hours. mRNA levels of IL1B, IL6 and COX 2 was assessed via qPCR.


A
B
C
Control
IL1B + OSM
IL1B
IL6
COX2
*
*
*
20
15
10
5
0
80
70
60
50
40
30
20
10
0
40
35
30
25
20
15
10
5
0
siControl
siRelB
siControl
siRelB
siControl
siRelB

3-9

Figure 3.10 RelB effect on IL-1B and OSM induced inflammation in GBM cells.

GBM cells (U373, U87, and primary GBM12 cells) were treated with siControl or SiRelB as indicated. Cells were treated with IL-1B and OSM and incubated for 18 hours. mRNA levels of IL1B, IL6 and COX 2 was assessed via qPCR.


IL1B
IL6
COX2
A
U373
B
U87
C
GBM12
siControl
siRelB
Control
IL1B + OSM

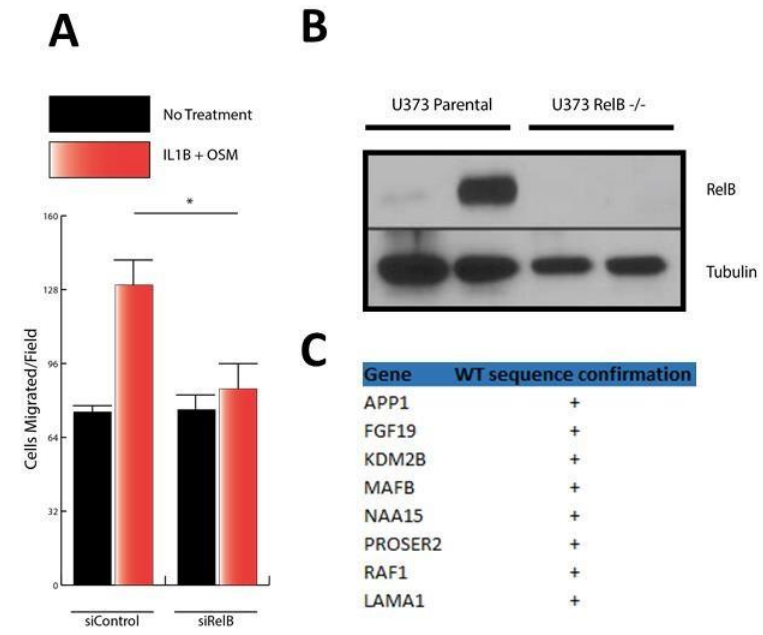
3-10

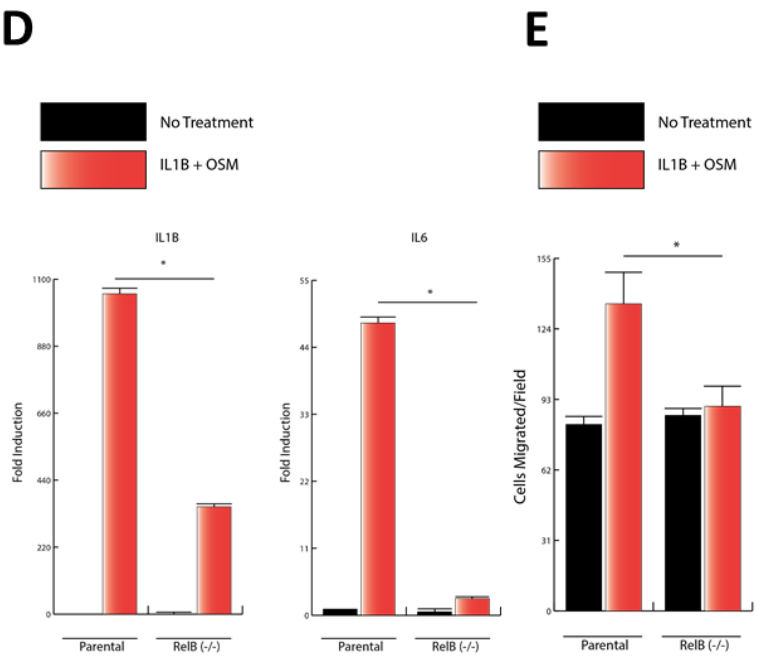
3.4.4 RelB Controls IL-1B/OSM induced migration in vitro

RelB has been previously implicated to induce GBM cell migration [61-64], however its actions have been attributed to its role in the noncanonical RelB-p52 signaling axis. Previously it has been shown that canonical NF-κB stimuli IL-1B induces GBM cell migration [40]. Additionally, other NF-κB dependent proinflammatory gene products such as IL-6 have been shown to increase GBM cell migration [98]. We hypothesized, given that GBM cells require RelB to upregulate proinflammatory gene products, that a genetic knockdown of RelB would attenuate an IL-1B/OSM induced migratory response. To test this hypothesis, we treated U373 cells with siRNA targeting RelB and measured IL-1B and OSM induced migration in siControl and siRelB treated cells. We found that RelB is vital for the IL-1B/OSM induced migratory response of GBM cells (Fig 3.11). Additionally, we confirmed these findings by generating RelB (-/-) U373 cell clones using CRIPSR/CAS9 technology. After confirming that the CRIPSR cloning process did not generate any off target effects, and that RelB (-/-) U373 cells are far less able to upregulate cytokine production in response to IL-1B/OSM cotreatment (Fig 2.11), we measured migration in response to IL-1B/OSM treatment in RelB(-/-), and parental U373 cells. Similar to our transient RelB knockdown, RelB(-/-) cells are unable to induce migration in response to IL-1B and OSM treatment (Fig 3.11).

Figure 3.11 The effect of RelB on IL-1B and OSM induced migration in GBM cells.

A) U373 cells were treated with siControl and siRelB as indicated and stimulated with IL-1B and OSM at time 0hrs. Cell migration was assessed via migration assay after 24 hour incubation. B) RelB levels in U373 parental and U373 RelB (-/-) cells assessed by western blot. C) WT sequence confirmation of possible off target effects of CRIPSR/Cas9 RelB targeting guide RNA assessed by DNA sequencing. D) Parental U373 cells and U373 RelB (-/-) cells were treated with IL-1B and OSM and incubated for 18 hours. mRNA levels of IL1B, IL6 and COX 2 was assessed via qPCR. E) Parental U373 cells and U373 RelB (-/-) cells were stimulated with IL-1B and OSM at time 0hrs. Cell migration was assessed via migration assay after 24 hour incubation.


A
No Treatment
IL1B + OSM
*
160
128
96
64
32
0
Cells Migrated/Field
siControl
siRelB
B
U373 Parental
U373 RelB -/-
RelB
Tubulin
C
Gene
WT sequence confirmation
APP1 +
FGF19 +
KDM2B +
MAFB +
NAA15 +
PROSER2 +
RAF1 +
LAMA1 +


D
No Treatment
IL1B + OSM
IL1B
IL6
Fold Induction
Parental
RelB (-/-)
E
No Treatment
IL1B + OSM
Cells Migrated/Field
Parental
RelB (-/-)

3.4.5 RelB is aberrantly active in GBM

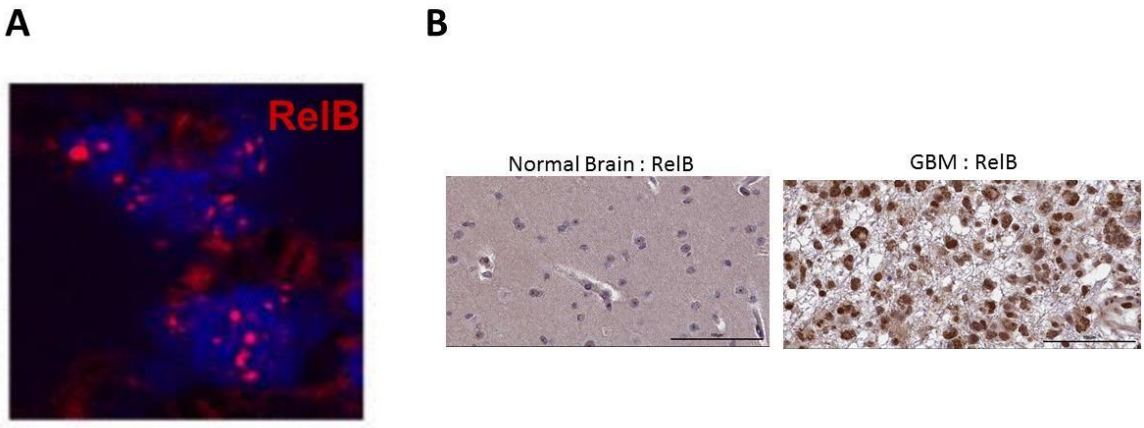
We had demonstrated that RelB drives a GBM-specific, proinflammatory gene expression loop in both GBM cell lines and primary GBM cells. Additionally, we showed that RelB is necessary for IL-1B/OSM induced GBM cell migration. To ascertain if targeting this pathway would be of therapeutic benefit, we evaluated RelB presence in the nucleus of human GBM tumors isolated *in vivo*. We obtained human GBM sections from the VCU Tissue and Data Acquisition and Analysis Core (TDAAC), and probed for RelB using immunofluorescence (IF). We observed a robust RelB signal in the nucleus of human GBM tumors (Fig 3.12). We confirmed this data using The Human Protein Atlas (HPA), a large-scale and unbiased IHC repository, and present the data according to HPA publication guidelines (http://www.proteinatlas.org/about/publications). Additionally, using HPA we observed greater staining of RelB in GBM tumor cells than in normal glial cells (Fig 3.12).

While our data suggest that RelB promotes GBM progression through a RelB/p50 signaling axis due to RelB activation through canonical NF-κB stimuli, previous reports suggest that noncanonical RelB-p52 signaling promotes GBM progression as well. We used genomic copy number analysis of TCGA patients to determine if either of RelB's dimerization partners are upregulated in GBM as opposed to normal glial cells and thus more available to form functional signaling dimers. Strikingly, over 80% of GBM patients have lost one allele of the NFKB2(p100/p52) (Fig 3.13). Additionally, over 30% of patients increased their genomic dose of the RelB gene. Given these data we sought to identify overexpressed NF-κB family members in GBM vs. normal brain tissue (Fig 3.13). We obtained RNA isolated from GBM tumors and normal brain via TDAAC and compared the expression of RelB, NFKB2, NFKB1 and RelA

between normal brain and GBM samples. We found that while RelA and NFKB1 levels are similar between the two groups, GBM tumors expressed more RelB and less NFKB2 (Fig 3.13). Previous studies have shown that increasing the NFKB1/NFKB2 ratio skews the signaling program of RelB toward RelB-cannonical signaling [96]. Thus, we concluded RelB is robustly upregulated in GBM, and that that RELB-canonical signaling is likely more prevalent in GBM compared to normal brain.

Figure 3.12 RelB nuclear presence in in vivo human samples.

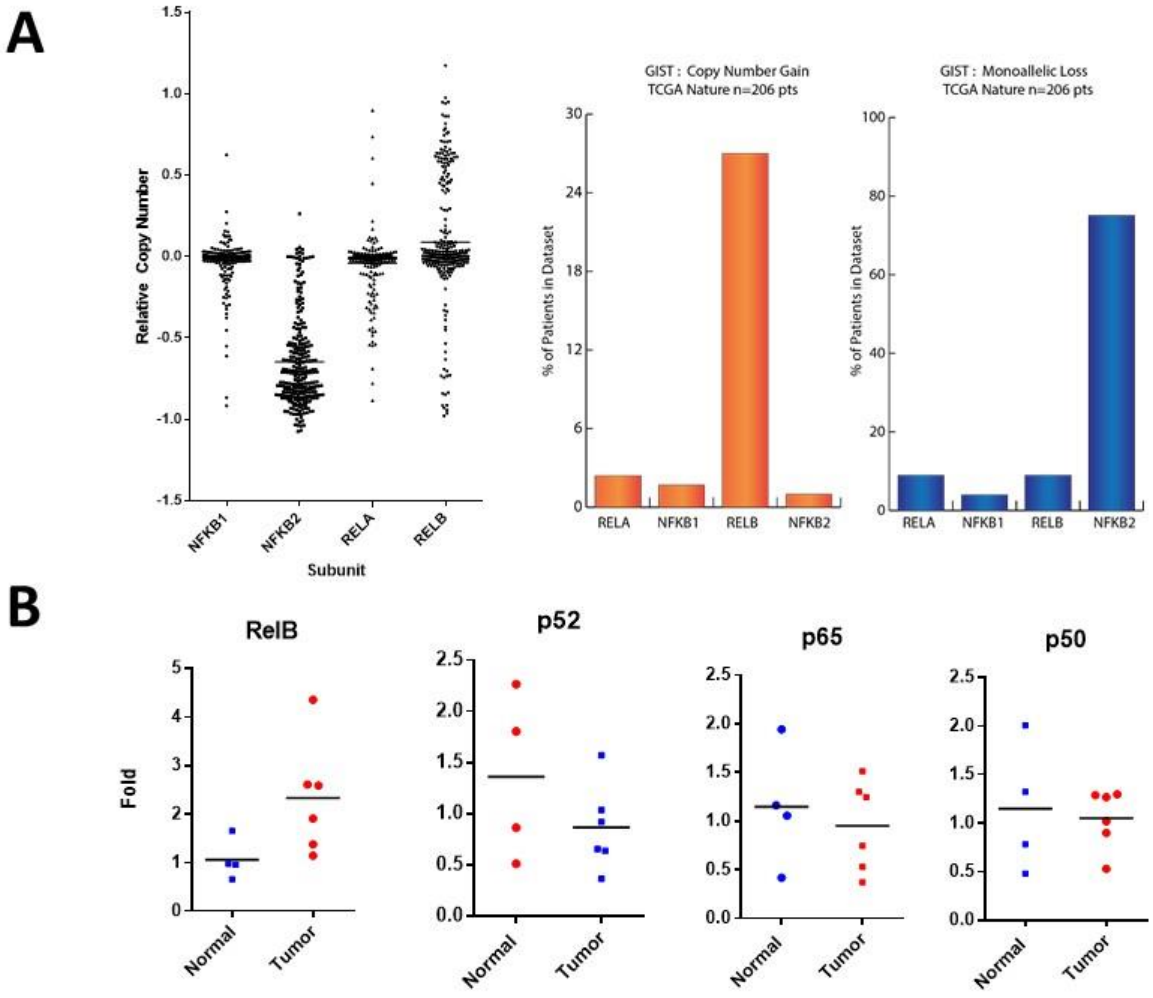
A) Human GBM samples were obtained from the VCU TDAAC and probed for RelB as described in the materials and methods. B) Images obtained from The Human Protein Atlas, displayed according to publications guidelines (http://www.proteinatlas.org/about/publications).



3-12

Figure 3.13 NF-κB genomic copy number analysis of GBM.

A-B) Genomic copy number analysis of NF-κB family members, GISTIC data was downloaded from TCGA via cBioPortal. C) mRNA expression of NF-κB family members from human GBM tumors. RNA was obtained from VCU TDAAC and NF-κB family member mRNA expression level was reverse transcribed and quantified by qPCR.


A
Relative Copy Number
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
NFKB1
NFKB2
RELA
RELB
Subunit
GIST : Copy Number Gain
TCGA Nature n=206 pts
% of Patients in Dataset
30
24
18
12
6
0
RELA
NFKB1
RELB
NFKB2
GIST : Monoallelic Loss
TCGA Nature n=206 pts
% of Patients in Dataset
100
80
60
40
20
0
RELA
NFKB1
RELB
NFKB2
B
RelB
p52
p65
p50
Fold
Normal
Tumor

3-13

3.4.6 RelB has a global diametrically opposed role in GBM vs Astrocytes

We observed that RelB stimulates the production of IL-1B, IL-6 and COX2 in GBM cells, whereas RelB inhibits the production of IL-1B, IL-6, and COX2 in astrocytes. Given these data we hypothesized that RelB signaling acts as a molecular switch to induce chronic inflammation in GBM. While we had demonstrated this differential control of IL-1B, IL-6 and COX2 expression in GBM cells and astrocytes, we sought to define the global role of RelB in response to inflammatory cytokines.

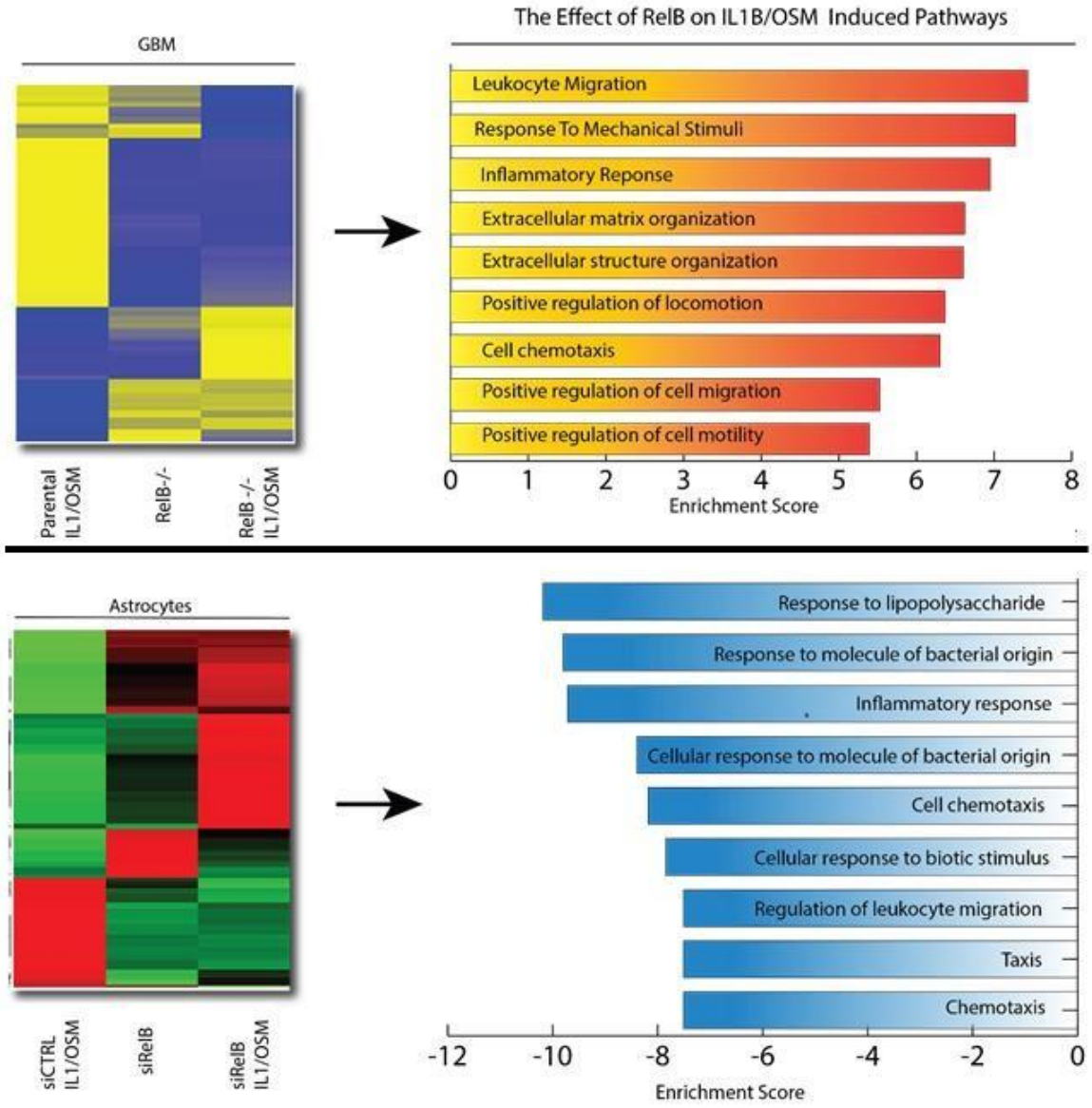
In order to define the global role of RelB in response to inflammation in GBM, we stimulated both parental and RelB (-/-) cells with IL-1B/OSM, performed RNAseq, and conducted differential expression testing and pathway enrichment between the two groups. In GBM cells, RelB assumes the role as a transcriptional activator; as the most significantly altered pathways were induced. Additionally, the genes activated by RelB in GBM are involved in the proinflammatory response ($p=1.37x10^{-7}$) and similar pathways involved in perpetuating inflammation and inflammatory cell chemotaxis (Fig 3.14). Thus, RelB serves as a transcriptional activator, and functions to perpetuate an initial inflammatory stimulus in GBM.

Next, we sought to define the global role of RelB in response to inflammation in astrocytes. To do this, we performed microarray analysis of IL-1B/OSM stimulated siControl and siRelB treated astrocytes, and conducted differential expression testing and pathway enrichment between the treatment groups. Conversely, in astrocytes, RelB functions as a repressor of transcription. Additionally, the genes inhibited by RelB in response to IL-1B/OSM are involved in the proinflammatory response ($p=1.92x10^{-10}$), and related pathways (Fig 2.14). Therefore, in astrocytes, RelB functions as a potent negative feedback mechanism to shut off

inflammation in response to an initial stimuli. Consequently, using genome-wide expression analysis, we concluded that RelB switches from a negative to a feedforward regulator of inflammatory stimuli in GBM.

Figure 3.14 Transcriptome wide analysis of RelB function in response to IL-1B and OSM induced inflammation.

(Top Panel) RNA was isolated from unstimulated and IL-1B/OSM stimulated Parental and U373 RelB (-/-) cells. Isolated RNA was used to perform global RNAseq analysis. After RNAseq data processing, differential expression testing and pathway enrichment analysis was performed. (Bottom Panel) RNA was isolated from IL-1B/OSM stimulated primary human astrocytes treated with either siControl or siRelB as indicated. Isolated RNA was reverse transcribed and the resulting cDNA was used for microarray analysis. After microarray data processing, differential expression testing and pathway enrichment analysis was performed.


GBM
Parental IL1/OSM
RelB-/-
RelB -/- IL1/OSM
The Effect of RelB on IL1B/OSM Induced Pathways
Leukocyte Migration
Response To Mechanical Stimuli
Inflammatory Reponse
Extracellular matrix organization
Extracellular structure organization
Positive regulation of locomotion
Cell chemotaxis
Positive regulation of cell migration
Positive regulation of cell motility
0 1 2 3 4 5 6 7 8
Enrichment Score
Astrocytes
siCTRL IL1/OSM
siRelB
siRelB IL1/OSM
Response to lipopolysaccharide
Response to molecule of bacterial origin
Inflammatory response
Cellular response to molecule of bacterial origin
Cell chemotaxis
Cellular response to biotic stimulus
Regulation of leukocyte migration
Taxis
Chemotaxis
-12 -10 -8 -6 -4 -2 0
Enrichment Score

3-14

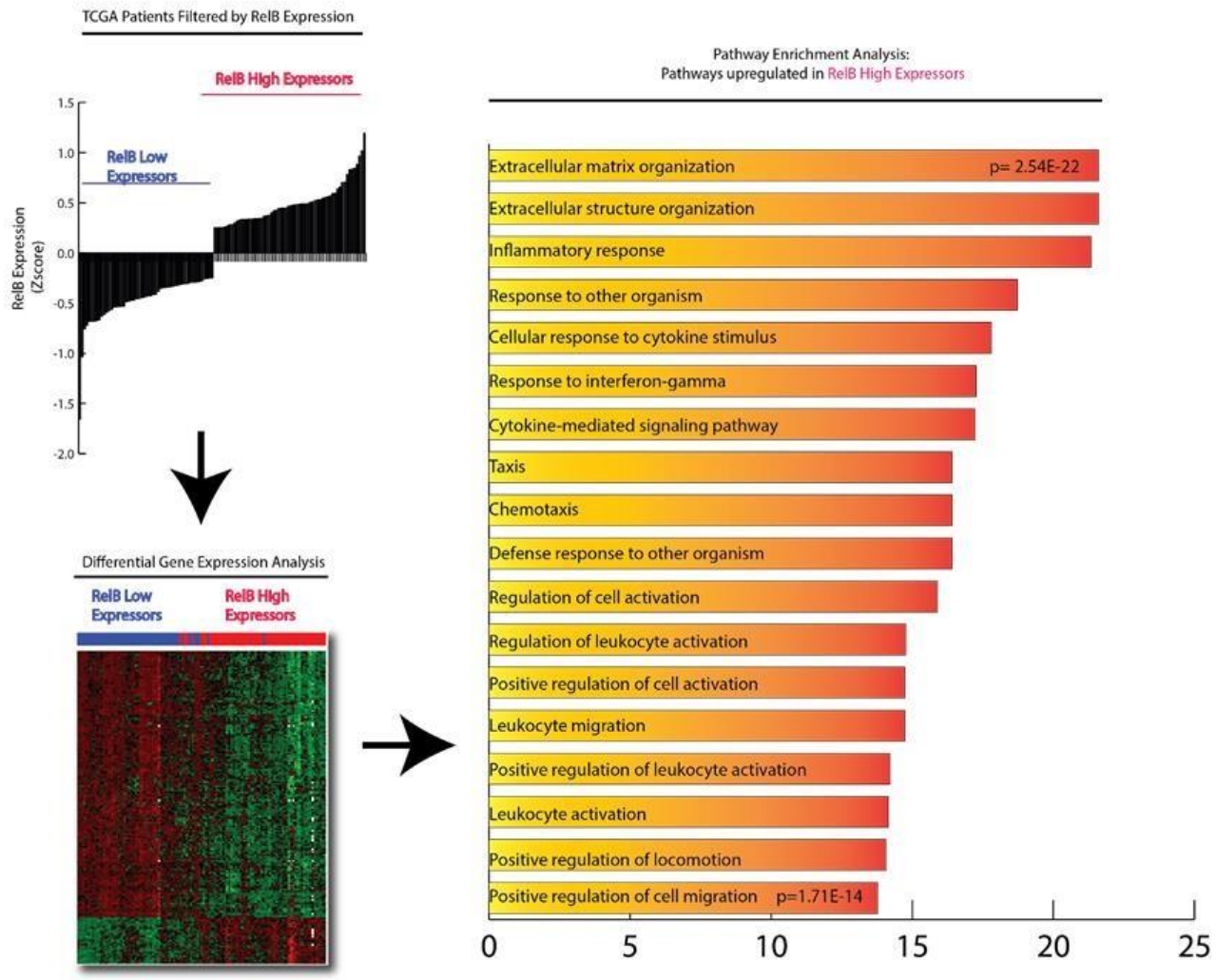
3.4.7 RelB controlled genes are mirrored in in vivo patient data.

Our data indicated that RelB acts as an aberrant, feedforward inducer of inflammation in GBM. Additionally, we had previously demonstrated that RelB is upregulated and constitutively active in human GBM samples *in vivo*. We hypothesized that if the aberrant action of RelB is biologically relevant in GBM patients, then GBM tumors, segregated by RelB expression levels, would demonstrate a similar upregulation of genes associated with a proinflammatory response. In contrast, if RelB maintained its putative function as a negative feedback regulator of inflammation, we would expect higher levels of RelB to be associated with decreased levels of proinflammatory mediators in human GBM patient samples.

To test our hypothesis that RelB acts to stimulate proinflammatory pathways in human GBM samples we filtered TCGA patients by their RelB expression (Fig 3.15). Next, we downloaded the full expression profiles of patients annotated to be either RelB high or low expressors and performed differential gene expression analysis. Finally, we obtained a list of genes upregulated in the high RelB group, and performed pathway enrichment analysis as described previously. We found that nearly identical proinflammatory pathways were enriched in the high RelB patient group as the pathways induced by RelB in GBM cells; examples include the upregulation of the inflammatory response, leukocyte migration and activation, and related pathways (Fig 3.15).

Figure 3.15 Pathway enrichment analysis between patients expressing high and low levels of RelB in GBM.

Z-score normalized RelB expression data was downloaded from TCGA via cBioPortal. Patients with a RelB expression Z-score of >0.25 or <-0.25 were classified as high and low RelB expressors, respectively. Differential gene expression testing between high and low RelB expressors was conducted using the 'limma' library of the R statistical package. Genes upregulated by the high RelB expressor group were used for pathway enrichment analysis.


TCGA Patients Filtered by RelB Expression
RelB High Expressors
RelB Low Expressors
RelB Expression (Zscore)
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
Differential Gene Expression Analysis
RelB Low Expressors
RelB High Expressors
Pathway Enrichment Analysis:
Pathways upregulated in RelB High Expressors
Extracellular matrix organization
p= 2.54E-22
Extracellular structure organization
Inflammatory response
Response to other organism
Cellular response to cytokine stimulus
Response to interferon-gamma
Cytokine-mediated signaling pathway
Taxis
Chemotaxis
Defense response to other organism
Regulation of cell activation
Regulation of leukocyte activation
Positive regulation of cell activation
Leukocyte migration
Positive regulation of leukocyte activation
Leukocyte activation
Positive regulation of locomotion
Positive regulation of cell migration
p=1.71E-14
0
5
10
15
20
25

3-15

3.4.8 RelB is a major regulator of macrophage chemotactic molecules and involved in the makeup of the inflammatory microenvironment

We observed that RelB acts as a global driver of proinflammatory signals in a cancer specific manner using RNA-seq, and confirmed these findings using the TCGA GBM patient cohort. While we had identified RelB as an aberrant inducer of inflammatory signals, we next asked what precisely the RelB-dependent program was doing to make tumors more aggressive. To answer this question, we combined our RNAseq data with gene expression and clinical outcome data from TCGA.

First, we wrote an R function to directly interface with TCGA, and perform iterative Kaplan Meier analysis of RelB-dependent genes identified by our RNAseq assay. Next we plotted all RelB-dependent genes which have a statistically significant impact on patient prognosis. Not surprisingly, we found that the vast majority of RelB-dependent genes, which are statistically important for patient prognosis, are markers of more severe disease (Fig 3.16). Lastly, we performed pathway enrichment analysis for the RelB-dependent genes which were independent markers of a poor prognosis. We found that the most overrepresented pathway were genes which are chemotactic for myeloid cells (Fig 3.16). Thus, we concluded that a major action of the aberrant RelB program is to recruit myeloid cells to the developing tumor.

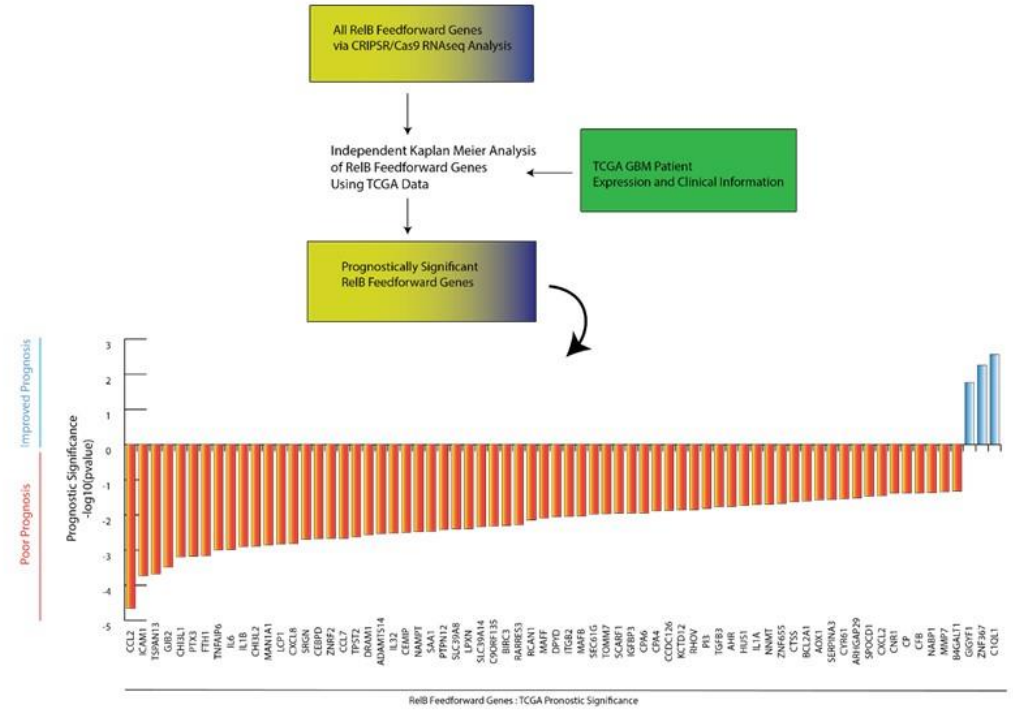
Myeloid cells have been shown to comprise from 5-30% of the tumor bulk, and increasing myeloid cell infiltration has been a marker of aggressive disease in GBM [18,19]. Classically, based largely on flow cytometric analysis of cell differentiation markers,

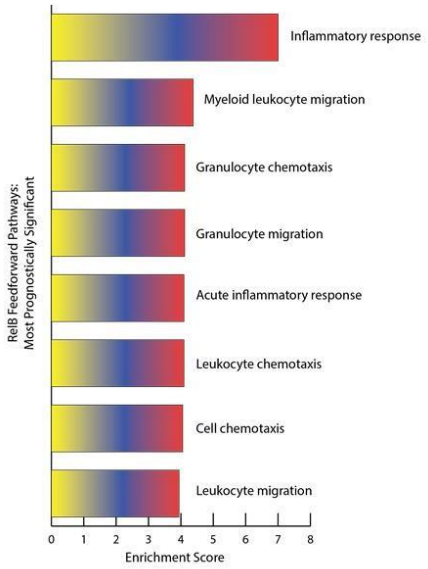
investigators have thought the GAM signature to be comprised mostly of M2 macrophages [19]. Recently however, via whole genome expression analysis, it has been shown that the majority of GAMs most closely resembled the M0, or undifferentiated macrophage phenotype [23]. While GAMs may most closely resemble an M0 macrophage, they are still activated, as determined by histologic analysis. Interestingly, GBM cells do not secrete classical drivers of the M1 phenotype (IFNy and TNFa) or the M2 phenotype (IL-4, IL-13) (Fig 2.17). GAMs rely on global stimulating factors such as the Macrophage Inflammatory Protein family (MIP), and the Colony Stimulating factor (CSF) family, and the Macrophage Chemotactic Protein (MCP) family for activation and chemotaxis [19].

Our combined analysis of prognostically important RelB driven genes closely aligns with these recent findings. Using our RNAseq assay we found that the RelB controlled genes which influence macrophage behavior, and demonstrate statistical prognostic importance belong to (i) the CSF family or proteins: CSF1 (M-CSF), CSF2 (GM-CSF), and CSF3 (G-CSF), (ii) the MIP family of proteins: CXCL2 (MIP2a), CXCL3(MIP2b), and (iii) the MCP family of proteins: MCP1, MCP3 (Fig 3.18). Importantly each of these genes is activated by RelB in a GBM specific manner (Fig 3.18). Thus, while it is likely that multiple inflammatory pathways important for patient prognosis are driven by the RelB feed forward loop, we suggest that a major role of the pathway is attraction and activation of GAMs.

3.16 Identification of most prognostically significant pathways controlled by the RelB-feedforward inflammatory loop.

A) All genes identified as RelB-controlled in GBM via RNAseq analysis were evaluated for their independent effect on patient prognosis using gene expression data and clinical outcome data downloaded from TCGA. B) Pathway enrichment analysis of RelB-controlled genes which had a statistically significant impact on GBM patient prognosis.

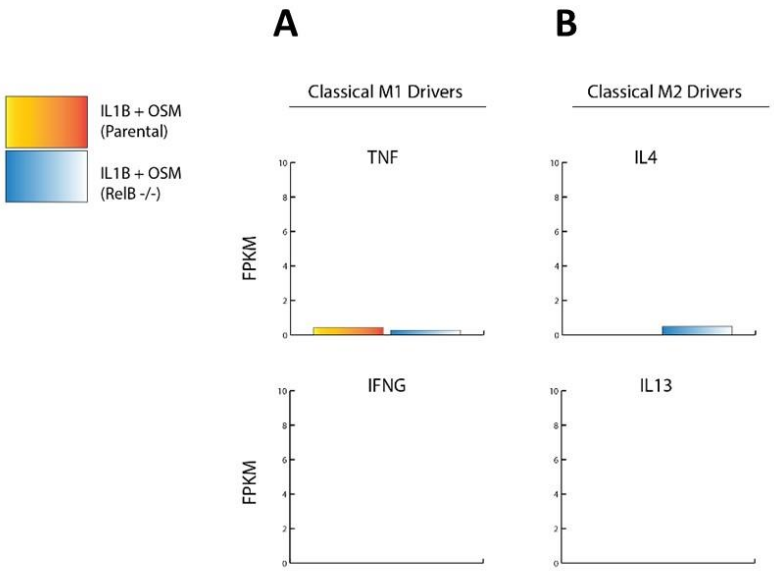

All RelB Feedforward Genes
via CRIPSR/Cas9 RNAseq Analysis
Independent Kaplan Meier Analysis
of RelB Feedforward Genes
Using TCGA Data
TCGA GBM Patient
Expression and Clinical Information
Prognostically Significant
RelB Feedforward Genes
Improved Prognosis
Poor Prognosis
Prognostic Significance
-log10(pvalue)
RelB Feedforward Genes : TCGA Pronostic Significance


RelB Feedforward Pathways:
Most Prognostically Significant
Inflammatory response
Myeloid leukocyte migration
Granulocyte chemotaxis
Granulocyte migration
Acute inflammatory response
Leukocyte chemotaxis
Cell chemotaxis
Leukocyte migration
Enrichment Score

3-16

3.17 GBM cell expression of M1/M2 drivers.

A-B) FPKM values of classical M1 (A) and M2 (B) drivers expressed by both cytokine induced parental cells and U373 RelB (-/-) cells.

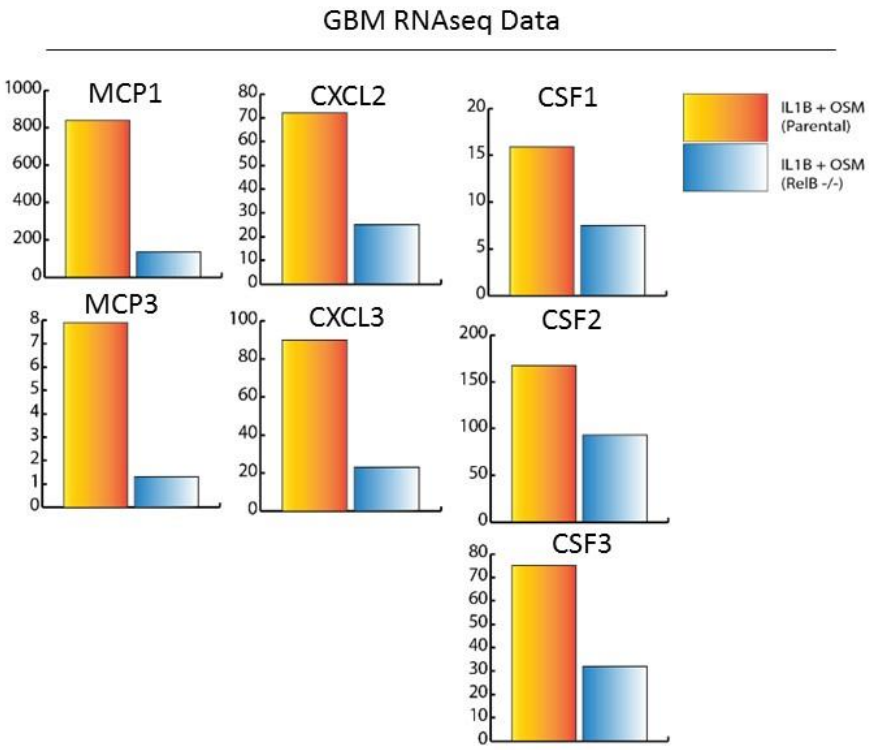

A
B
IL1B + OSM
(Parental)
IL1B + OSM
(RelB -/-)
Classical M1 Drivers
Classical M2 Drivers
TNF
IL4
IFNG
IL13
FPKM
FPKM
0
2
4
6
8
10

3-17

3.18 GBM cell expression of RelB-dependent prognostically significant macrophage activators and chemoattractacts.

A) FPKM values of prognostically significant RelB-controlled genes annotated to function as macrophage chemoattractants and activators. B) Comparison of previously identified macrophage activators and chemoattractants in GBM cells (U373) and primary human astrocytes.

A



B

Myeloid Leukocyte Migration	GBM	Astrocytes
CCL2 (MCP1)	Induced	-
CCL7 (MCP3)	Induced	Repressed
CXCL2 (MIP2a)	Induced	-
CXCL3 (MIP2b)	Induced	Repressed
CSF1 (MCSF)	Induced	-
CSF2 (GMCSF)	Induced	-
CSF3 (GCSF)	Induced	-

3-18

3.4.9 Genomic heterozygous deletion of the histone deacetylase SIRT1 leads to a loss of RelB mediated silencing in GBM

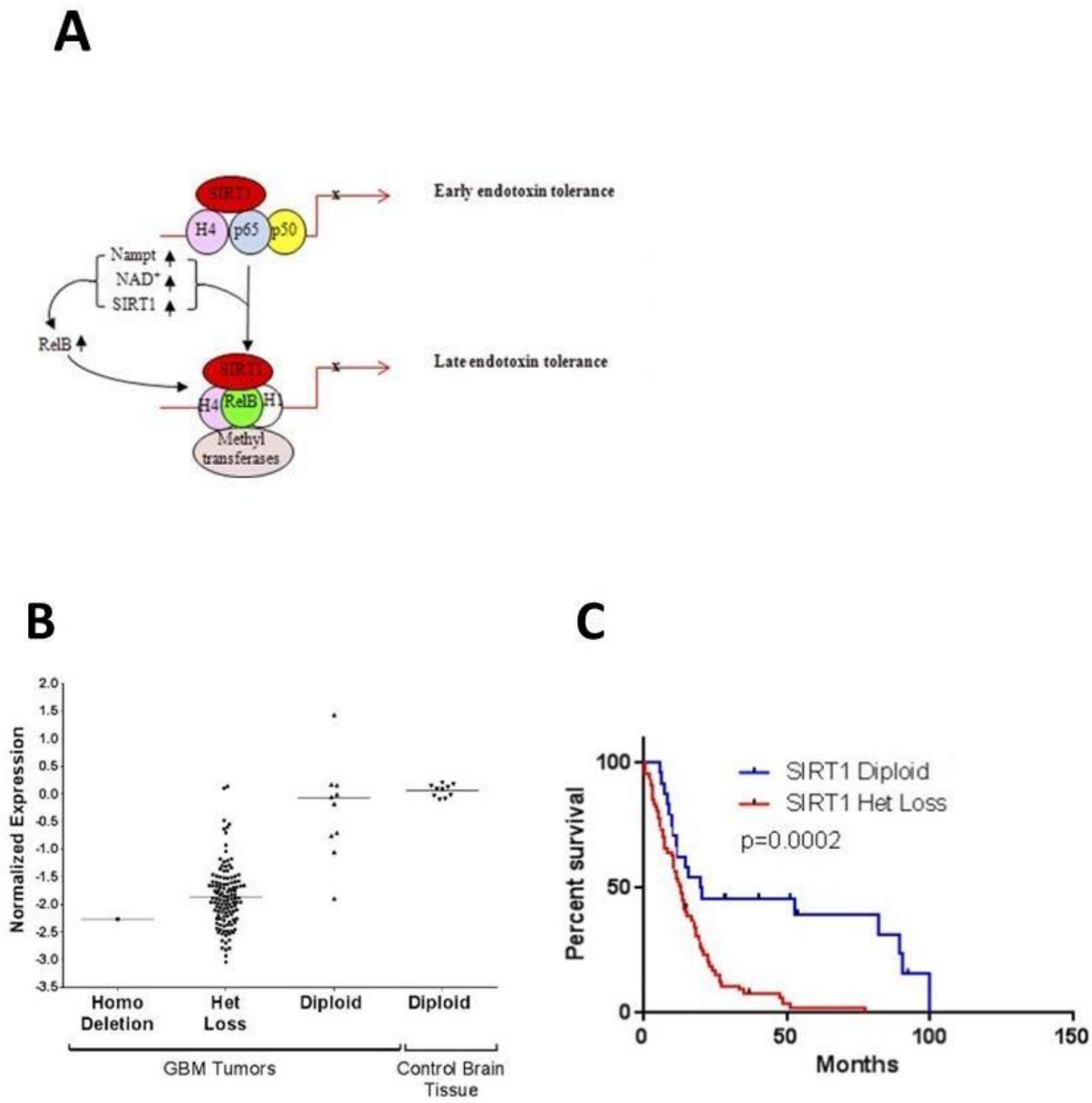
To understand the mechanism of "aberrant" cytokine activation by RelB in GBM cells, we investigated the mechanism by which RelB silences inflammatory gene production in astrocytes. The previously demonstrated model of epigenetic silencing of the il1b gene during endotoxin tolerance by RelB in macrophages [51,52], depends on the recruitment of histone deacetylase SIRT1 (Fig 3.19). We hypothesized that loss of SIRT1 expression could prevent epigenetic silencing.

Significantly, mining of TCGA database showed that one allele of the SIRT1 gene is deleted in 80% of GBM tumors (Fig 3.19) and leads to poor survival prognosis (Fig 3.19). Lower expression of SIRT1 mRNA was found in GBMs than normal (matching) brain (Fig 3.20), and confirmed on the protein level by IHC (Fig 3.20). These findings were further confirmed *in vitro* since SIRT1 mRNA was substantially decreased in GBM cell lines and primary GBM cells in comparison to astrocytes (Fig 3.20). SIRT1 activity was also significantly reduced in GBM cells in comparison to astrocytes (Fig 3.20). We addressed the importance of SIRT1 in RelB-mediated regulation by inhibiting SIRT1 activity in astrocytes and GBM cells. Remarkably, inhibition of SIRT1 activity enhanced expression of cytokines in astrocytes (IL- 1 and IL-6) but had no effect on cytokine production in GBM cells (Fig 3.21). Conversely, overexpression of SIRT1 in GBM cells diminished expression of cytokines, including IL-6, and IL-8 (Fig 3.22). Importantly, the effect of SIRT1 was RelB-dependent (Fig. 3.23). In another approach, we analyzed expression of cytokines in GBM cells treated with rosiglitazone, which activates SIRT1 (79). In agreement with data from SIRT1 overexpression, rosiglitazone diminished cytokine expression (Fig 3.24).

These data suggest that repression of cytokines in astrocytes depends on SIRT1; however, RelB/SIRT1-dependent repression does not function in GBM cells and thus allows activation of the aberrant RelB- dependent expression program promoting a feed-forward cytokine loop that promotes chronic inflammation.

3.19 Heterozygous loss of SIRT1 in GBM tumors.

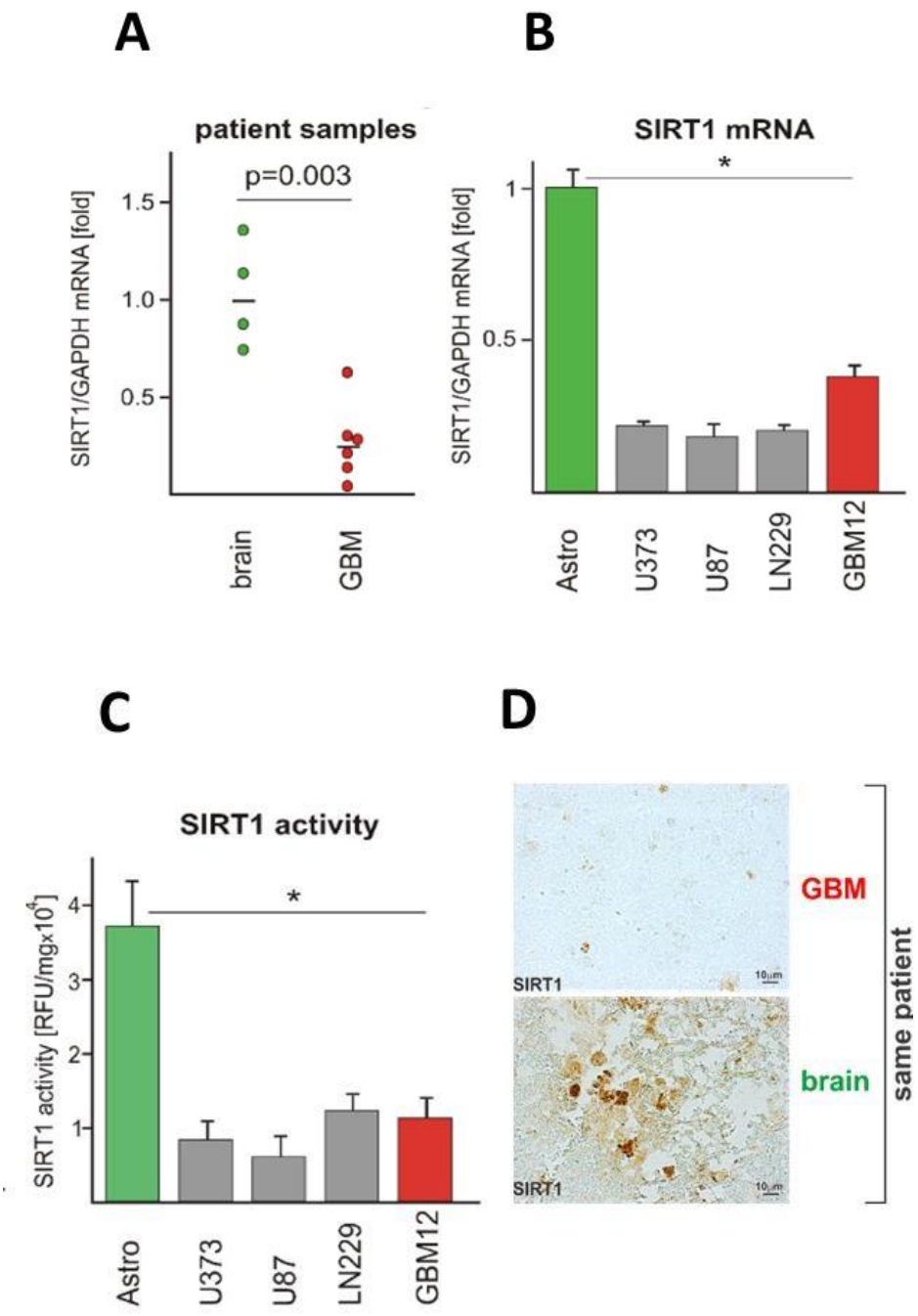
A) Model of the previously demonstrated SIRT1/RelB complex which functions to silence cytokine expression and establish immunologic “tolerance” in macrophages in response to sepsis (adapted from McCall et al. 2008). B) Plot of SIRT1 zygosity and SIRT1 expression in GBM tumors and control brain tissue. Z-score normalized SIRT1 expression values were correlated with patient SIRT1 zygosity established via GISTIC analysis. C) Kaplan Meier analysis of patients with diploid SIRT1 allele vs. patients with a heterozygous loss of the SIRT1 allele.


A
SIRT1
H4
p65
p50
Early endotoxin tolerance
Nampt
NAD+
SIRT1
RelB
SIRT1
H4
RelB
H1
Methyl transferases
Late endotoxin tolerance
B
Normalized Expression
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
-3.5
Homo Deletion
Het Loss
Diploid
Diploid
GBM Tumors
Control Brain Tissue
C
Percent survival
100
50
0
SIRT1 Diploid
SIRT1 Het Loss
p=0.0002
0
50
100
150
Months

3-19

3.20 Comparison of SIRT expression between GBM cells and primary human astrocytes and GBM section vs normal brain tissue isolated *in vivo*.

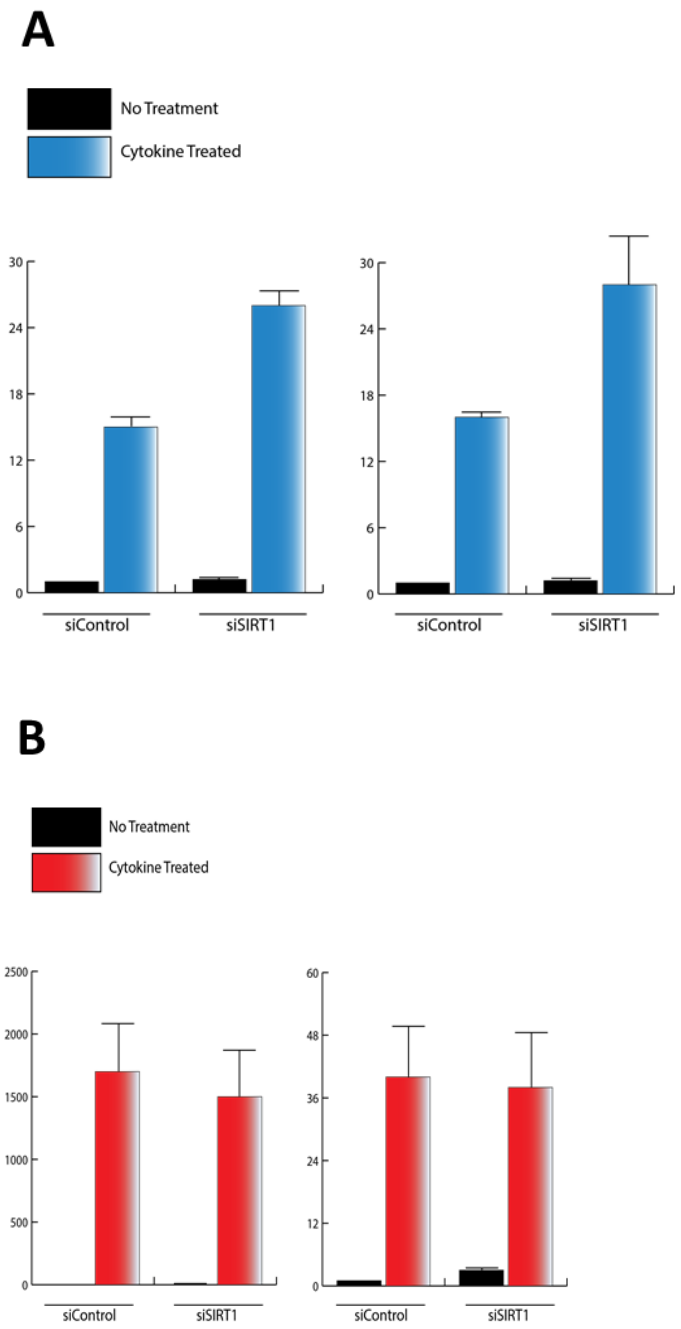
A) RNA from normal brain tissue and GBM tumor tissue was obtained from VCU TDAAC. RNA was reverse transcribed and SIRT1 levels were quantified via qPCR. B) *In vitro* Comparison of SIRT1 mRNA expression between primary human astrocytes and GBM cell lines (U373, U87, LN229, and primary GBM12). C) *In vitro* Comparison of SIRT1 activity between primary human astrocytes and GBM cell lines (U373, U87, LN229, and primary GBM12). D) Comparison of SIRT1 protein levels in GBM and normal brain tissue isolated *in vivo* via IHC.


A
patient samples
p=0.003
SIRT1/GAPDH mRNA [fold]
1.5
1.0
0.5
brain
GBM
B
SIRT1 mRNA
*
SIRT1/GAPDH mRNA [fold]
1
0.5
Astro
U373
U87
LN229
GBM12
C
SIRT1 activity
*
SIRT1 activity [RFU/mg x10^4]
4
3
2
1
Astro
U373
U87
LN229
GBM12
D
SIRT1
SIRT1
GBM
brain
same patient

3-20

3.21 SIRT1 effect on IL-1B and OSM induced inflammation in primary human astrocytes and GBM cells.

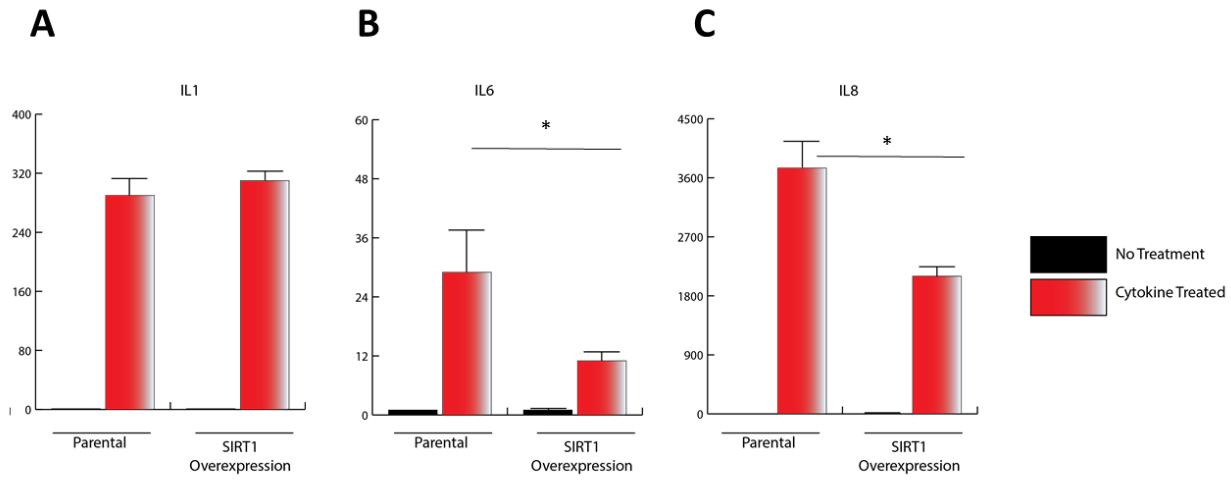
A) Primary human astrocytes and B) GBM cells (U373) were treated with siControl or siSIRT1 as indicated. Cells were treated with IL-1B and OSM and incubated for 18 hours. mRNA levels of IL-1B, and I-L6 levels were assessed via qPCR.


A
No Treatment
Cytokine Treated
30
24
18
12
6
0
siControl
siSIRT1
30
24
18
12
6
0
siControl
siSIRT1
B
No Treatment
Cytokine Treated
2500
2000
1500
1000
500
0
siControl
siSIRT1
60
48
36
24
12
0
siControl
siSIRT1

3-21

3.22 The effect of SIRT1 overexpression on cytokine production in GBM.

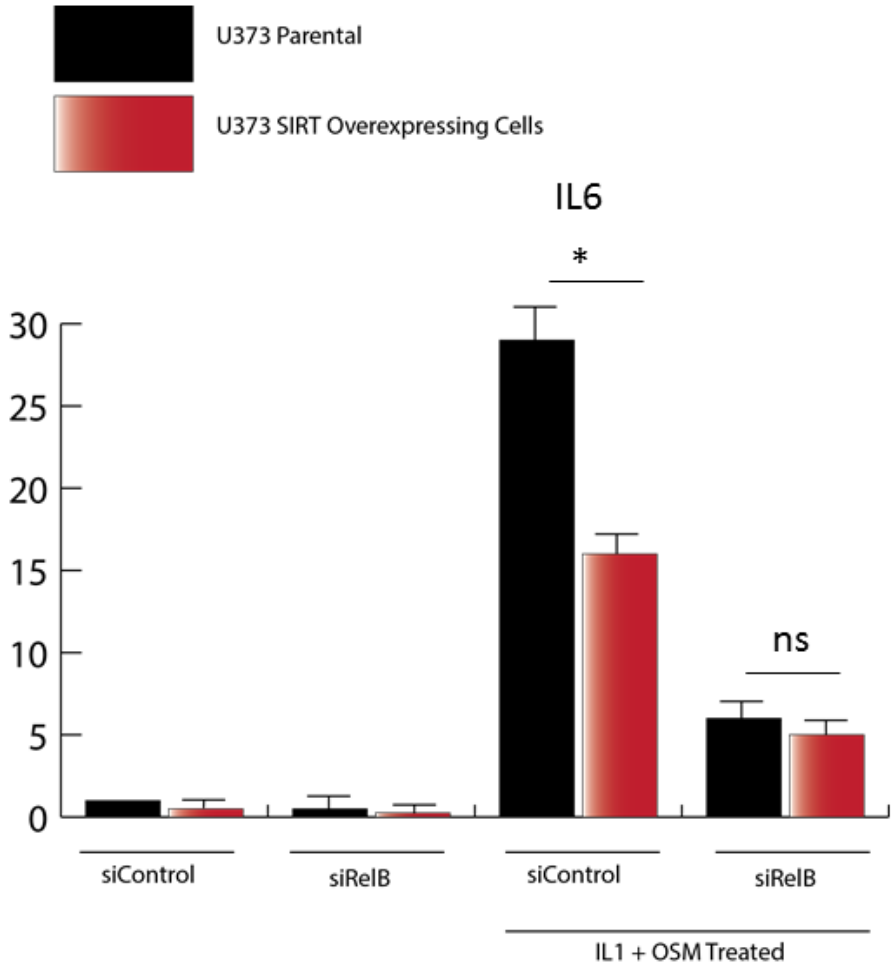
A-C) U373 parental and U373 overexpressing SIRT1 were treated with IL-1B and OSM and incubated for 18 hours. mRNA levels of IL-1B, IL-6, and IL-8 levels were assessed via qPCR.


A
B
C
IL1
IL6
IL8
400
320
240
160
80
0
60
48
36
24
12
0
4500
3600
2700
1800
900
0
*
*
Parental
SIRT1
Overexpression
No Treatment
Cytokine Treated

3-22

3.23 The effect of RelB on SIRT1 mediated cytokine silencing in GBM.

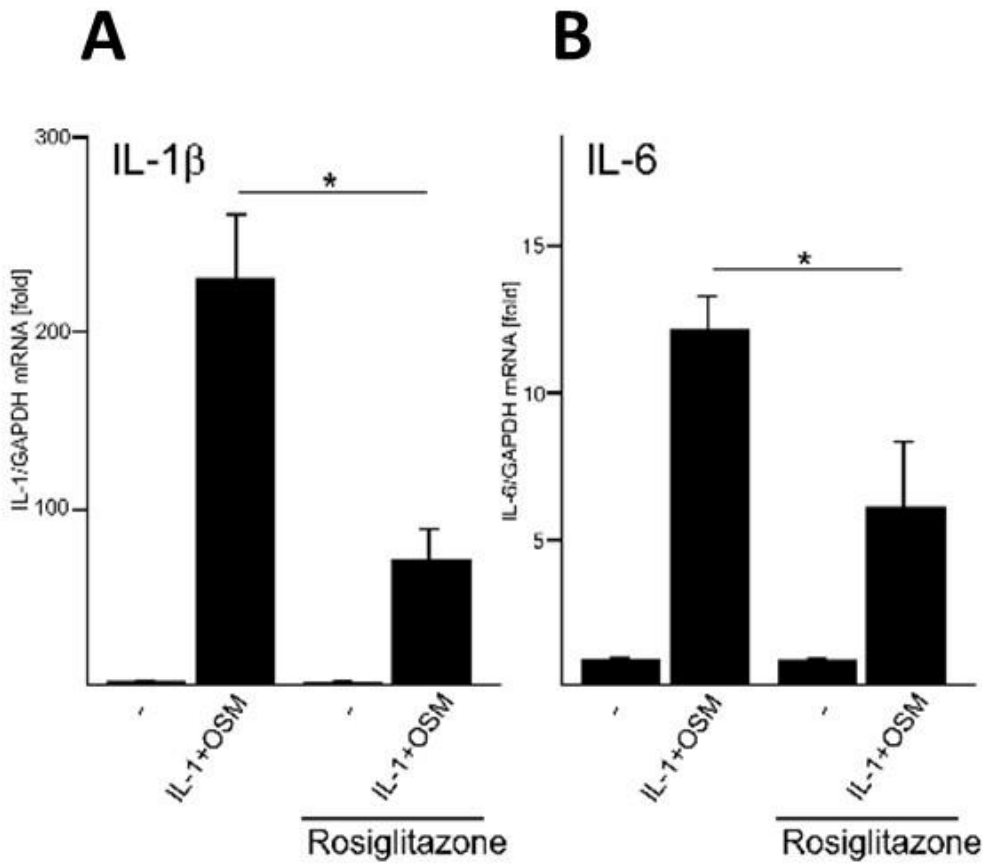
U373 parental and U373 SIRT1 overexpressing cells were treated with siControl and siRelB as indicated, induced cells were treated with IL-1B and OSM and incubated for 18 hours. mRNA levels of IL-6, was assessed via qPCR.


U373 Parental
U373 SIRT Overexpressing Cells
IL6
*
ns
30
25
20
15
10
5
0
siControl
siRelB
siControl
siRelB
IL1 + OSM Treated

3-23

3.24 The effect of the SIRT1 activator Rosiglitazone on IL-1B/OSM induced inflammation.

A-B) GBM cells (U373) were treated with Rosiglitazone for 2 hours as indicated, then treated with IL-1B and OSM as indicated and incubated for 18 hours. mRNA levels of IL-1B and IL-6 were assessed via qPCR.


A
B
IL-1β
IL-6
IL-1β/GAPDH mRNA [fold]
IL-6/GAPDH mRNA [fold]
300
200
100
15
10
5
*
*
-
IL-1+OSM
-
IL-1+OSM
Rosiglitazone
-
IL-1+OSM
-
IL-1+OSM
Rosiglitazone

3-24

3.5 Discussion

Although proinflammatory cytokines are secreted transiently during acute inflammation [38-39], chronic inflammation develops in GBM and promotes GBM cell proliferation, migration, invasion, resistance to apoptosis, and the maintenance of stem cell like properties [4,64,82-83,87-92]. To identify cytokines which specifically support GBM aggressiveness, we used an unbiased approach and correlated cytokine expression with clinical outcome data from TCGA. We identified IL-1B and OSM as two cytokine signaling programs, whose expression is negatively correlated with patient survival. Additionally, we demonstrated that IL-1B and OSM are frequently expressed together at high levels and that IL-1B and OSM are specifically expressed at high levels in mesenchymal GBM, which is the most common and deadly GBM [14]. Thus, we concluded that chronically elevated IL-1B and OSM levels initiate programs driving the progression of GBM.

NF-κB proteins include p65, p105/p50, RelB, and p100/p52. A wide variety of inflammatory stimuli, including IL-1B, activate the canonical NF-κB pathway triggering degradation of IκBa, translocation of p65/p50 heterodimers to the nucleus, and induction of p65/p50-dependent genes. RelB is a unique member of the NF-κB family mostly known as a downstream target of non-canonical NF-κB signaling triggered by CD40L, BAFF, and TWEAK. Interestingly, an excess of RelB forms RelB/p50/IκBa complexes, which can also be activated by canonical pathway [96].

Our data show that IL-1B and OSM treatment promotes canonical RelB/p50 signaling in GBM cells and astrocytes. Similarly to what we found in astrocytes [105], basal expression of

RelB is low in GBM cells. However, IL-1B induces expression of RelB in primary GBM and established GBM cell lines. Additionally, RelB translocates to the nucleus of the GBM cells in response to IL-1B/OSM, and IL-1B induces formation of RelB/p50 complexes. Interestingly, RelB was almost entirely localized in the nuclei in GBM patient samples. Thus, we demonstrated that in response to IL-1B and OSM, RelB/p50-canonical signaling is activated in GBM cells in vitro, and RelB is also activated in GBM *in vivo*.

RelB-canonical signaling has previously been shown to coordinate a negative feedback loop suppressing cytokines by several mechanisms including epigenetic silencing [51-52,93-94]. However, our patient prognostic data suggested that IL-1B/OSM induced RelB-p50 signaling correlated with a poor patient prognosis, and thus potentially drives chronic inflammation. We showed that IL-1B and OSM induced RelB signaling acts as a potent negative feedback mechanism of IL-1B, IL6 and COX2 expression in astrocytes. Strikingly, we found the opposite effect of RelB on IL-1B, IL6 and COX2 expression in GBM cells. Additionally, using high throughput expression analysis we confirmed that the global role of RelB in response to IL-1B and OSM induced inflammation is to act as a feedforward promoter of inflammatory genes. Conversely, this mechanism is cancer specific, as RelB globally acts as global suppressor of transcription, and genes annotated to be involved in the inflammatory response, in astrocytes. We therefore concluded that RelB acts as a molecular switch promoting chronic inflammation in GBM.

Additionally, our group sought to define precise programs stimulated by the cancer-specific RelB-dependent program which make tumors more aggressive. Utilizing an approach combining RNAseq data with gene expression and clinical outcome data from TCGA, we found that the RelB program stimulates a prognostically important program of genes which recruit and

activate myeloid cells. Additionally, this program corresponds with recently published papers indicating that GAMs are activated by global myeloid activators such as the CSF, MIP, and MCP family of proteins.

To understand the mechanism of "aberrant" cytokine activation by RelB in GBM cells, we investigated the mechanism by which RelB silences inflammatory gene production in astrocytes. The previously demonstrated model of epigenetic silencing of the il1b gene during endotoxin tolerance by RelB in macrophages [51,52], depends on the recruitment of histone deacetylase SIRT1. We hypothesized that loss of SIRT1 expression could prevent epigenetic silencing. Significantly, mining of TCGA database showed that one allele of the *SIRT1* gene is deleted in 80% of GBM tumors and leads to poor survival prognosis. Lower expression of SIRT1 mRNA was found in GBMs than normal brain and confirmed on the protein level by IHC. These findings were further confirmed *in vitro* since SIRT1 mRNA was substantially decreased in GBM cell lines and primary GBM cells in comparison to astrocytes. SIRT1 activity was also significantly reduced in GBM cells in comparison to astrocytes. Additionally, inhibition of SIRT1 activity enhanced expression of cytokines in astrocytes, but not GBM cells. Conversely, overexpression of SIRT1 in GBM cells diminished expression of cytokines in a RelB-dependent manner. These data suggest that repression of cytokines in astrocytes depends on SIRT1; however, RelB/SIRT1-dependent repression does not function in GBM cells and leads to activation of the "aberrant" RelB- dependent expression program promoting a feed-forward cytokine loop that promotes chronic inflammation.

In conclusion, we identified RelB as a molecular switch, promoting a cancer specific feedforward inflammatory loop, in response to the GBM driving cytokines IL-1B and OSM.

4 Chapter 3: YY1 switches RelB into a transcriptional activator to drive inflammation in GBM

4.1 Abstract

GBM tumors are characterized by necrosis and profound inflammation [3-4,15]; with cytokines secreted by both GBM cells and the tumor microenvironment. The mechanisms by which chronic inflammation develops and persists in GBM regardless of multiple anti-inflammatory feedback loops remain elusive. In preliminary studies, we found that expression of the NF-κB family member RelB is similarly induced in primary human astrocytes and GBM cells. Strikingly however, while RelB suppresses expression of cytokines in astrocytes, it unexpectedly enhances expression of cytokines in GBM cells. Thus, the anti-inflammatory RelB-driven feedback loop found in astrocytes is converted into a feed-forward loop fueling chronic inflammation in GBM. Additionally, we demonstrated SIRT1 loss as the molecular underpinning of a lack of RelB mediated silencing in GBM. However, still unknown is how RelB coordinates the transcriptional transactivation of a GBM-specific proinflammatory gene expression program. We analyzed regulatory elements of RelB-controlled genes using a genome-wide bioinformatics approach and identified a common regulatory motif that binds Yin Yang 1 (YY1), a transcription factor known to either repress or activate gene transcription by recruiting either corepressors or

coactivators [73]. Surprisingly, we found that while YY1 resides in the cytoplasm of astrocytes, its location is exclusively nuclear in established GBM cell lines and primary GBM cells, and it binds to the promoters of cytokine genes in GBM cells. In parallel with these findings, siRNA knock-down of YY1 profoundly suppressed expression of cytokines in GBM cells, but had no effect in astrocytes. We found that YY1 interacts with RelB and that RelB and YY1 stimulate cytokine transcription through the same molecular mechanism. Importantly, these complexes could provide long-lasting epigenetic effects that would be insensitive to classical NF-κB inhibitors. Therefore, our data demonstrates that YY1 plays an essential role in GBM-specific RelB-mediated inflammation, and support a mechanistic model which explains the development and persistence of this chronic inflammation in GBM.

4.2 Introduction

We had previously demonstrated that RelB expression is similarly induced by IL-1B/OSM in astrocytes and GBM cells. Additionally, we had previously ascertained whether RelB suppresses p65/p50-driven cytokine expression and in these cells according to previously published RelB actions. While RelB suppressed cytokine expression in astrocytes, it surprisingly enhanced cytokine expression in established and primary GBM cells. Thus, our data implied that the anti-inflammatory RelB-driven feedback loop present in astrocytes is converted into a feed-forward loop in GBM cells. Lastly, we showed that a lack of SIRT1 expression and activity prevented RelB mediated silencing of proinflammatory genes in GBM cells. However, these results failed to explain how RelB functions as a transcriptional activator of proinflammatory genes specifically in GBM. Given the current need to understand mechanisms by which chronic inflammation develops in GBM, we investigated RelB transcriptional activation of proinflammatory genes in an attempt to identify novel therapeutic targets which could potentially prevent the development of chronic inflammation.

We hypothesized that the aberrant-activation of cytokine genes by RelB in GBM cells may depend on an additional transcription factor that affects RelB activity specifically in GBM cells but not astrocytes. To identify this factor, we analyzed 2.5 kb-long regions surrounding the transcription start sites of RelB-controlled genes for the presence of regulatory elements using the 'PathView' and 'EnrichR' R packages. This bioinformatics approach identified several common regulatory elements, including a motif that can be bound by Yin-Yang 1 (YY1)

The Transcription factor Ying Yang 1 (YY1) is a ubiquitous transcription factor which controls many diverse functions including transcriptional and cell growth [73,76]. Transcriptionally, YY1 can function as either an activator or a repressor depending on its DNA binding site, associated transcription factors, and cell context. Studies using YY1 heterozygous mice and Xenopus YY1 homolog (-/-) demonstrate the importance of YY1 in the central nervous system. Namely, YY1 controls CNS development, neuronal differentiation and neurotransmitter transport [74,75]. Additionally, YY1 function has also been implicated in cancer biology [76]. Increased YY1 levels have been observed in many cancerous tissues, and YY1 levels have been shown to correlate with a poor prognosis in prostate, breast and bone cancers.

While the signaling mechanism by which YY1 exerts its effects is controversial, YY1 has been shown to be a member of the Polycomb Repressor 2 complex (PRC2), a histone modifier [77]. YY1 contains a Recruitment of Polycomb or REPO domain which allows YY1 to direct PRC2 to near genomic areas and modify histones. Interestingly, one study has shown that YY1 directly interacts with NF-κB family members to form a transcriptional activating complex made up of RelB, p50, and Oct2 in the promoters of the IgG heavy chain. Interestingly, YY1 was shown to interact only with RelB and p50 and not p65 or p52 [79].

Our unbiased approach suggested that YY1 may be a critical factor in influencing RelB behavior. Thus, we investigated the role of YY1 on proinflammatory signaling in GBM and its potential association with the RelB molecular switch.

4.3 Materials and Methods

JASPAR and TRANSFAC Positional Weight Matrix (PWM) Analysis Promoters of RelB controlled genes were downloaded from the hg37 genome using the UCSC genome browser. Promoter sequences were identified for enriched PWM of transcription factors using the JASPAR and TRANSFAC databases. These studies were conducted using the 'PathView' and 'EnrichR' R-packages.

Cell Culture Human cortical astrocyte cultures were established using dissociated human cerebral tissue. Cortical tissue was provided by Advanced Bioscience Resources (Alameda, CA), and the protocol for obtaining postmortem fetal neural tissue complied with the federal guidelines for fetal research and with the Uniformed Anatomical Gift Act. Human glioblastoma U373-MG cells were obtained from American Type Culture Collection (Rockville, MD), whereas human glioma U87 cells were obtained from Dr. Jaharul Haque (Cleveland Clinic Foundation, Cleveland, OH). Primary GBM 12 cells were obtained from Dr. Paul Dent (Virginia Commonwealth University, Richmond, VA). Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, antibiotics, and non-essential amino acids.

Down-regulation of target genes Expression of RelB and SIRT1 was down- regulated using SmartPool siRNAs (siRelB and siYY1) from Dharmacon (Dharmacon, Int., Lafayette, CO). siRNA was transfected into astrocytes and GBM cells using Dharmafect 1, according to the manufacturer's instructions.

RNA isolation and quantitative qPCR Total cellular RNA was prepared by Trizol (Invitrogen, Carlsbad, CA), following the manufacturer's protocol. Subsequently, 1 ug of total RNA was

reverse-transcribed using the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). IL1B, IL6, IL8, COX-2 and GAPDH mRNA levels were measured using pre-mixed primer-probe sets, and TaqMan Universal PCR Master Mix according to the supplier's instructions (Applied Biosystems, Foster City, CA). The cDNAs were diluted 10-fold (for the target genes) or 100-fold (for GAPDH), and amplified using the ABI 7900HT cycler. Gene expression levels were normalized to GAPDH mRNA levels, and presented as a fold induction with mean values +/- standard deviation.

Cytokines and Cell Stimulation Cells were stimulated with 25 ng/ml OSM (R&D, Systems, Inc., Minneapolis, MN), or 10 ng/ml IL-1B (a gift from Immunex Corp., Seattle, WA).

Clinical Samples Patient RNA samples and tumor slices were provided by the VCU Tissue Acquisition and Analysis core (TDAAC).

Nuclear and Cytosolic Fraction Assays Were prepared as previously described (2) described briefly Cells were washed with cold PBS and re-suspended in buffer containing 10 mM Hepes (pH 7.8), 10 mM KCl, 0.1 mM EDTA, 1 mM Na3VO4, 1 mM DTT, 1:500 proteaseinhibitors (Sigma) and 0.2 mM PMSF, and incubated on ice for 15 min. NP-40 was added (0.75%) and cells were vortexed for 10 sec. Nuclei and supernatant ("cytoplasm") were separated by centrifugation at 3000 rpm for 3 min at 4˚C. Nuclei were re- suspended in buffer containing 20 mM Hepes (pH 7.8), 0.4 M NaCl, 1 mM EDTA, 1 mM Na3VO4, 1 mM DTT and 1:500 protease inhibitors and incubated on ice for 15 min. Nuclear extracts were cleared by centrifugation at 14,000 x g for 5 min at 4˚C.

Immunohistochemistry Slides were fixed in ice cold acetone for 20 minutes, then incubated in 1% hydrogen peroxide. Next, sliders were washed in PBS and blocked in 5%BSA/1%Normal

Goat Serum for 1 hour. The slides were then incubated with primary antibody and blocking buffer overnight at +4 degrees C. The next day slides were washed in PBS and incubated with EnVision+ secondary reagent (Agilent Pathology Solutions) for 20 minutes, washed in PBS, and then exposed using DAB+ chromogen for 10 minutes.

Co-Immunoprecipitation Protein lysates (200–300 μg), prepared as described above, were precleared with 10 μl protein G–Sepharose beads (GE Healthcare, Pittsburgh, PA) for 1 h. The lysates were then incubated with 2 μg anti-RelB Abs overnight at 4°C, and 25 μl protein G–Sepharose beads were added and incubated for 1 h at 4°C. The beads were washed extensively with the lysis buffer, and immunoprecipitated proteins were eluted in sample buffer at 95°C for 5 min.

ChIP Cells were grown to 80-100% confluency in 10 cm dishes. Cells were crosslinked with 1% formaldehyde for 10 minutes. Cells were washed (125 mM glycine, 1mM PMSF in PBS). Cells were scraped and resuspended in SDS lysis buffer(1% SDS, 10 mM EDTA ph 8.0, 50 mM Tris-HCl). Cells were sonicated and centrifuged at 1300 rpm for 10 min and supernatant (chromatin) was collected. Chromatin was diluted 10x in ChIP IP buffer (0.01% SDS, 1.1% Triton-X, 1.2 mM EDTA, 16.7 mM Tris-HCl, 167 mM NaCl). Magnetic Beads (prewashed in ChIP IP buffer) were added to diluted chromatin and rocked for 2 hours. Beads were washed with ChIP IP buffer, High Salt Buffer (0.5 M HEPES, 5 M NaCl, 0.5 M EDTA, 10% SDS, 10% Triton-X, 10% Deoxycholate), LiCl Buffer (1M Tris-Hcl, 1 M LiCl, 10% NP-40, 10% Deoxycholate, 0.5 M EDTA), and TE buffer. DNA was eluted with Elution Buffer (1M Tris-HCl, 0.5 M EDTA, 10% SDS), and incubated with proteinase K at 55 degrees for 2 hours. Beads were incubated in 4.6 M NaCl overnight. The next day DNA was isolated using Qiagen Mini-Prep kit (Qiagen, NV).

ChIPseq Cooccupancy Analysis YY1 and RelB ChIPseq data files were downloaded from the NCBI Gene Expression Omnibus (GEO). BigWig files were realigned to hg37, and overlapping RelB and YY1 peaks were calculated and annotated to the nearest gene using the Genome Galaxy webserver (https://usegalaxy.org/).

Statistical Analysis All experiments were repeated at least three times with consistent results. Data are presented as mean ± SD. GraphPad Prism software was used for statistical analyses unless otherwise indicated. A Bonferroni posthoc test was used for one-way ANOVA comparisons, with $p < 0.05$ being considered statistically significant. Independent sample Student *t* test was used for unpaired observations.

4.4 Results

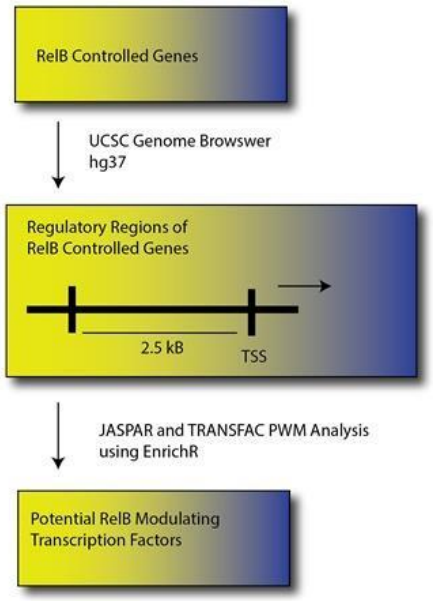
4.4.1 Unbiased approach reveals YY1 as likely coregulator of RelB.

Our group sought to understand the underlying mechanism of aberrant RelB transcriptional activation of proinflammatory genes in GBM. We hypothesized that the GBM-specific activation of genes by RelB may depend on an additional transcription factor that affects RelB activity specifically in GBM cells but not astrocytes. In an attempt to identify a GBM specific transcription factor, we used an unbiased approach using our genome-wide RelB expression analysis performed previously. In our approach, we analyzed 2.5 kb-long regions surrounding the transcription start sites of the RelB controlled genes for the presence of regulator elements using the JASPAR and TRANSFAC Positional Weight Matrix (PWM) databases. This approach assumes binomial distribution and independence, and tests for the probability for regulator element presence (RelB-regulated vs a random gene list) (Fig 4.1). This bioinformatics approach identified several common regulatory elements including a motif that can be bound by Ying-Yang 1 (YY1) ($p=0.000003$) (Fig 4.1). As YY1 has been known to differentially activate and repress genes depending on cell context we tested the hypothesis that YY1 modulates RelB activity at cytokine genes in GBM but not astrocytes.

4.1 Unbiased analysis of potential RelB modulating transcription factors.

A) 2.5 kb-long regions surrounding the transcription start sites of the RelB controlled genes were analyzed for the presence of regulator elements using the JASPAR and TRANSFAC Positional Weight Matrix (PWM) databases. B) Rank list of regulatory elements in RelB-Controlled genes.

A



B

Name	P-Value	Combined Score
YY1	0.000002773	12.25
POU2F2	0.000008425	11.78
SMAD4	0.00002774	11.03
TCF4	0.000008883	10.73
JUND	0.0002153	8.26
E2F1	0.001178	7.86
SP1	0.001866	7.7
NFYA	0.0002335	7.7
HNRNPK	0.001226	7.07
E2F6	0.0005407	6.74

4-1

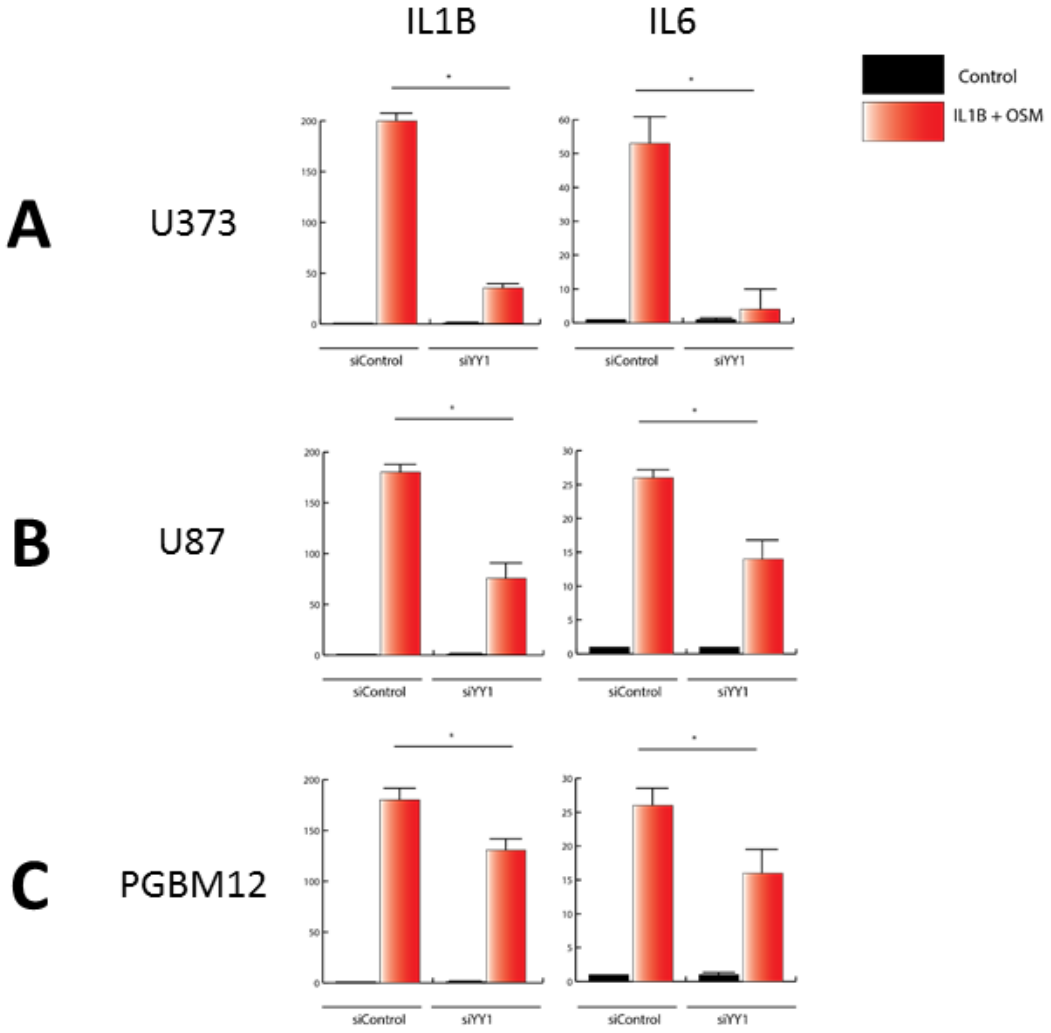
4.4.2 YY1 acts as a GBM specific activator of cytokines

We had identified the transcription factor YY1 as a potential modulator of RelB activity using an unbiased screen of RelB controlled genes. To test whether YY1 affects cytokine expression in GBM, we knocked down YY1 expression by treating U373 cells and primary GBM12 cells with either siControl or siYY1. Next, we stimulated each cell population with IL-1B/OSM, and measured IL-1B and IL-6 expression relative its unstimulated control. We found a robust attenuation of IL-1B/OSM induced IL-1B and IL-6 expression in siYY1 cell populations relative to siControl. These data clearly indicate that YY1 is necessary for cytokine induction in U373 and primary GBM cells (Fig 4.2). Thus, we concluded that YY1 modulates IL-1B/OSM induced cytokine production in GBM cells.

We next evaluated whether YY1 control of cytokine production was specific to GBM cells or persists in astrocytes as well. Consequently, we depleted YY1 expression from primary human astrocytes using transient siYY1 transfection, and measured IL-1B/OSM induced IL-1B and IL-6 expression relative to siControl treated astrocytes. Importantly, we found that YY1 depletion had no effect on IL-1B and IL-6 production (Fig 4.3). Therefore, we concluded that YY1 control of cytokine production is specific to GBM cells and not primary human astrocytes.

4.2 YY1 effect on IL-1B and OSM induced inflammation in GBM cells.

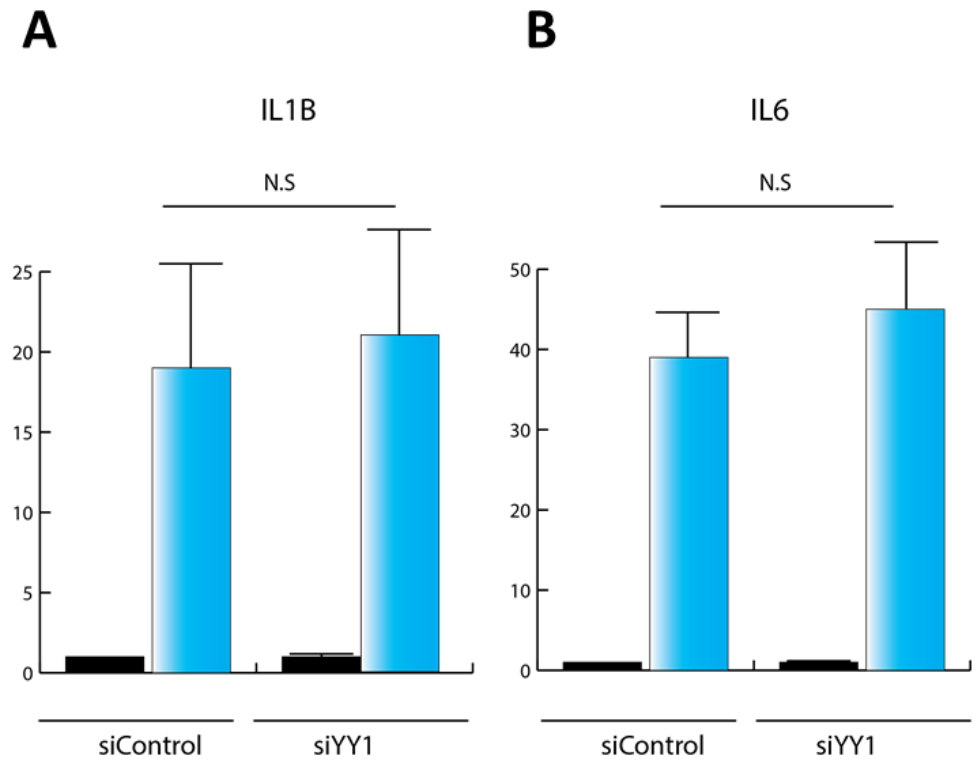
A-C) GBM cells (U373, U87 and primary GBM12 cells) were treated with siControl or SiYY1 as indicated. Cells were treated with IL-1B and OSM and incubated for 18 hours. mRNA levels of IL-1B, IL-6 was assessed via qPCR.


IL1B
IL6
Control
IL1B + OSM
A
U373
B
U87
C
PGBM12
siControl
siYY1

4-2

4.3 YY1 effect on IL-1B and OSM induced inflammation in primary human astrocytes.

A-B) Primary human astrocytes were treated with siControl or SiYY1 as indicated. Cells were treated with IL-1B and OSM and incubated for 18 hours. mRNA levels of IL-1B and IL-6 was assessed via qPCR.


A
IL1B
N.S
25
20
15
10
5
0
siControl
siYY1
B
IL6
N.S
50
40
30
20
10
0
siControl
siYY1

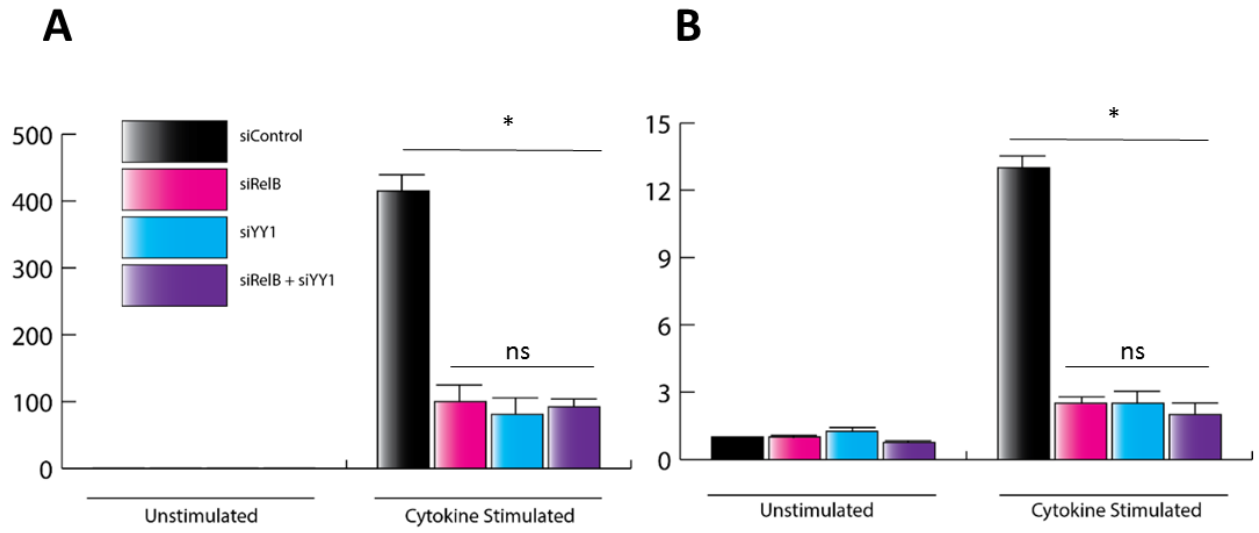
4-3

4.4.3 RelB and YY1 stimulate cytokines through the same molecular mechanism.

While our *in silico* analysis demonstrated a likely cooperation between YY1 and RelB we sought to determine whether RelB and YY1 induce cytokine expression through the same activatory pathway. To test this idea, we performed a dual knockout of YY1 and RelB in GBM cells using transient siRNA transfection (Fig 4.4). We hypothesized that if YY1 and RelB induced cytokine expression through separate pathways then mostly likely we would observe a difference in the degree of cytokine attenuation in siRelB alone or siYY1 alone treated cells; we additionally would expect an additive effect of cytokine attenuation in dual siRelB/siYY1 treated cells. However, we reasoned that if YY1 and RelB induced cytokine expression through the same mechanism, then a knockdown of YY1 alone, RelB alone or a dual knockdown would produce the same degree of cytokine attenuation. In agreement with our *in silico* analysis, no statistical difference was observed in attenuation of cytokine production in either the siRelB, siYY1, or siRelB/siYY1 treated groups (Fig 4.4). We therefore concluded that RelB and YY1 operate through the same molecular mechanism to stimulate cytokine production in GBM cells.

4.4 The effect of combined RelB and YY1 knockdown on IL-1B and OSM induced inflammation in GBM.

A-B) U373 cells were treated with siControl, SiRelB alone, siYY1 alone, and siRelB/siYY1 as indicated. Cells were treated with IL-1B and OSM and incubated for 18 hours. mRNA levels of A)IL-1B, B)IL-6 was assessed via qPCR


A
B
500
400
300
200
100
0
siControl
siRelB
siYY1
siRelB + siYY1
*
ns
Unstimulated
Cytokine Stimulated
15
12
9
6
3
0
*
ns
Unstimulated
Cytokine Stimulated

4-4

4.4.4 RelB and YY1 physically interact, bind the promoters of cytokine genes

Our dual knockdown experiment suggested that RelB and YY1 cooperate to stimulate cytokine production via the same mechanism in GBM cells, but not primary human astrocytes. We next aimed to distinguish whether this cooperation was through a direct physical interaction. Of note, YY1 has previously been shown to form a transcriptional activating complex with RelB and the homeobox containing transcription factor Oct-2. This complex was demonstrated to stimulate the production of the IgG heavy chain in malignant B-cells [79]. Interestingly, Oct-2 (POU2F2) binding elements were the second most enriched motif in the regulatory regions of RelB regulated genes via our previous in silico analysis (Fig 4.1). Thus, we tested whether a similar activating complex could be forming in GBM cells by immunoprecipitation (Fig 4.5). We found that YY1 and RelB directly interact in GBM cells, and that this interaction is enhanced with IL-1B/OSM treatment. Thus, we concluded that RelB and YY1 directly interact in GBM cells in a cytokine inducible manner.

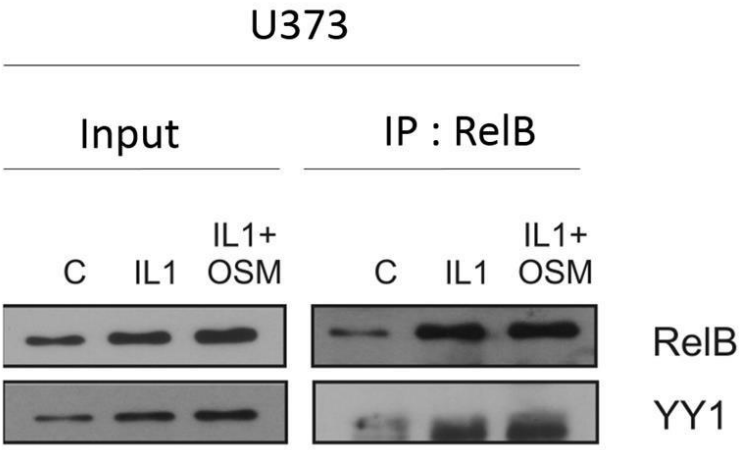
Given our *in silico* data and our observation that YY1 and RelB interact in GBM cells, we hypothesized that RelB and YY1 physically interact to form GBM specific transcriptional activating complex at the promoters of cytokine genes to induce transcription. We first tested this hypothesis *in silico* using publicly available YY1 and RelB ChIPseq datasets. At the time of our analysis 4 YY1 ChIPseq experiments and 1 RelB ChIPseq experiment were contained within the NCBI Gene Expression Omnibus. We utilized these files to align ChIPseq reads from all experiments to a common reference genome and computed overlap of the RelB and YY1 ChIPseq signals (Fig 4.6). We found significant genome-wide overlap between RelB and YY1 ChIP peak signals. Next, we annotated RelB/YY1 coincident peaks to their nearest gene and

performed gene-set enrichment analysis. Interestingly RelB and YY1 co-peaks were most statistically enriched in the promoters of genes involved in the immune response and organ growth and development (Fig 4.6).

Next, we sought to evaluate RelB and YY1 recruitment to cytokine genes directly in GBM cells. We isolated chromatin from U373 cells and performed ChIP to detect recruitment of RelB and YY1 to the promoters of cytokine genes. We found that YY1 and RelB are recruited to the IL-6 promoter upon cytokine stimulation (Fig 4.7). Thus, we concluded that RelB and YY1 are recruited to the promoters of cytokine genes upon cytokine stimulation, and this complex likely regulates a variety of genes involved in organ growth and development.

4.5 RelB directly interacts with YY1.

U373 cells were stimulated with IL-1B and IL-1B/OSM, RelB was immunoprecipitated, and co-immunoprecipitated RelB and YY1 was detected by Western blotting, as indicated. Expression of RelB and YY1 in the lysates is shown the left panels (lysate).

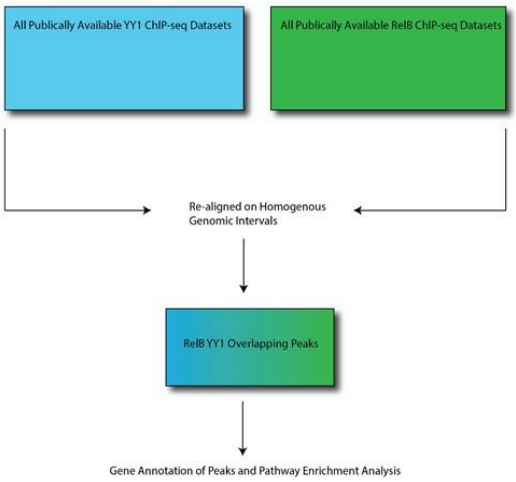


4-5

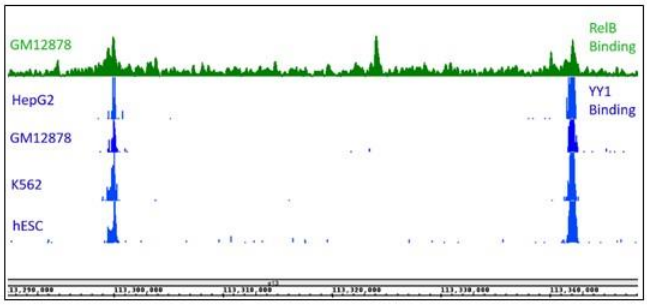
4.6 ChIPseq Cooccupancy Analysis

A) Workflow for data processing. YY1 and RelB ChIPseq data files were downloaded from the NCBI Gene Expression Omnibus (GEO). BigWig files were realigned to hg37, and overlapping RelB and YY1 peaks were calculated and annotated to the nearest gene using the Genome Galaxy webserver (https://usegalaxy.org/). B) Representative RelB/YY1 peaks were visualized using the Broad Institute Integrated Genome Browser. C) Enriched Pathways were discovered using the meme-suite webserver ChIP peak processing tools http://meme-suite.org/.

A



B

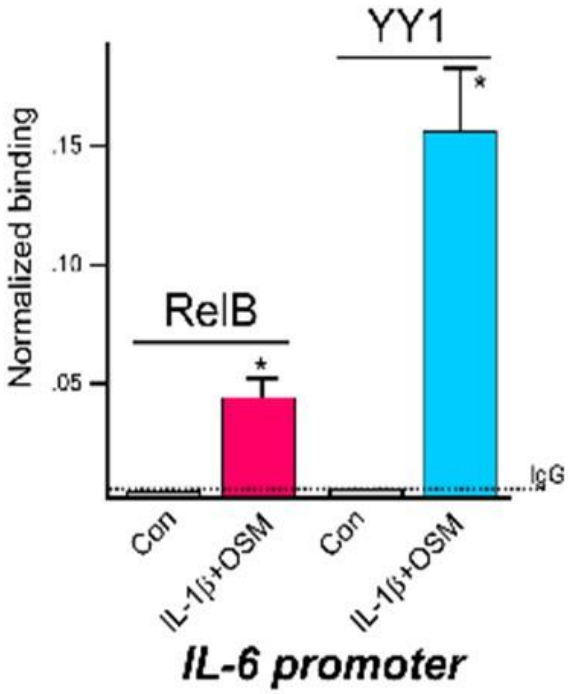


GO:0046620	BP	regulation of organ growth
GO:0050896	BP	response to stimulus
GO:0031975	CC	envelope
GO:0052564	BP	response to immune response of other organism during symbiotic interaction
GO:0052572	BP	response to host immune response
GO:0043498	MF	cell surface binding
GO:0050776	BP	regulation of immune response
GO:0042953	BP	lipoprotein transport
GO:0002282	BP	microglial cell activation during immune response
GO:0052031	BP	modulation by symbiont of host defense response
GO:0052033	BP	pathogen-associated molecular pattern dependent induction by symbiont of host innate immunity

4-6

4.7 RelB and YY1 promoter occupancy in cytokine genes

U373 cells were stimulated with IL-1B/OSM for 8h, and ChIP was performed using primers designed to the IL-6 promoter.


YY1
RelB
Normalized binding
.15
.10
.05
IgG
Con
IL-1β+OSM
Con
IL-1β+OSM
IL-6 promoter

4-7

4.4.5 YY1 is only in the nucleus of GBM cells, and constitutively in the nucleus of patient samples

We demonstrated that YY1 and RelB function to activate a GBM specific feedforward proinflammatory loop. However, we did not know the molecular mechanism explaining why RelB/YY1 cytokine activation was specific to GBM and not normal glial cells. Importantly, YY1 has been shown to be sequestered in the cytoplasm by the Rb protein [101]. Rb is only degraded during cell division; therefore, we hypothesized that in non-dividing normal glial cells YY1 would be sequestered in the cytoplasm, and thus unable to transactivate cytokine genes. However, in GBM, not only are cells dividing constitutively, but over 80% of patients possess a mutation in the Rb pathway, leading to Rb degradation. Thus, we reasoned that in GBM YY1 would constitutively be located in the nucleus, and able to transcriptionally regulate proinflammatory genes. To test this hypothesis, we performed cytosolic and nuclear fractions of both primary human astrocytes and GBM cells (U373 and primary GBM12), and blotted for YY1. In accordance with our hypothesis, we found that YY1 is localized almost exclusively to the cytoplasm of astrocytes whereas it is almost entirely nuclear in GBM cells (Fig 4.8). These results directly correlate with YY1 dependent regulation of cytokine expression in these cells.

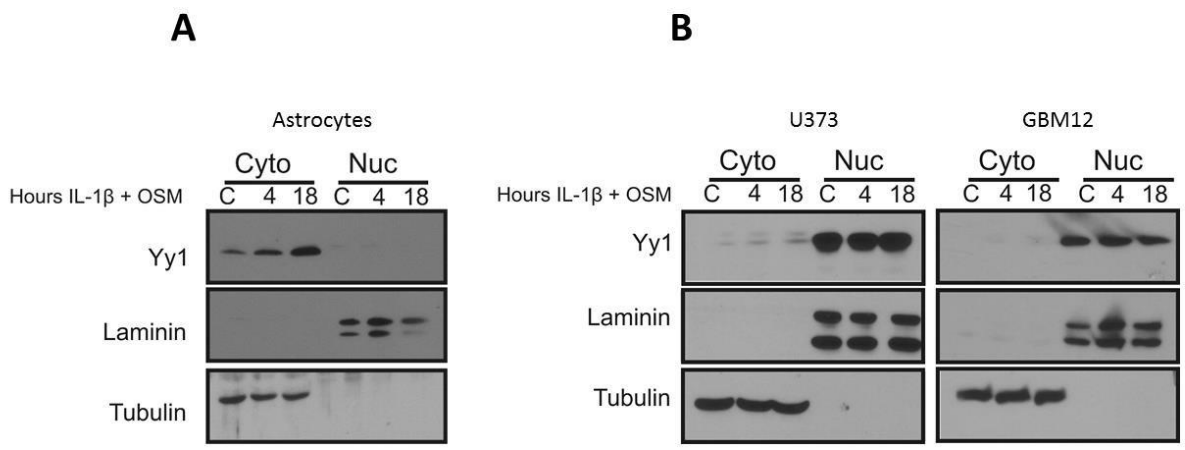
Next, we sought to confirm these findings in human tumors *in vivo*. We obtained tissue from VCU TDAAC and performed IHC for YY1. Our findings corroborated our *in vitro* data as YY1 was present in abundance in the nuclei of GBM cells, however its localization was primarily cytoplasmic in patient matched normal brain samples (Fig 4.9).

In conclusion, our results suggest a delicate role for RelB in controlling the inflammatory microenvironment in different cell contexts. In non-dividing cells RelB maintains its putative

function in the literature in response to inflammation, and acts as a negative feedback regulator to prevent chronic inflammation in collaboration with SIRT1. However, in the physiological context of a wound or a developing organ, when cells are proliferating, it would be of physiologic benefit to maintain the inflammatory microenvironment for growth and migratory ques. In this case, YY1 would translocate to the nucleus to drive a RelB dependent inflammatory signal. Importantly, this mechanism would be self-limiting physiologically because once a wound is healed, or an organ formed, cells stop dividing and YY1 would be sequestered in the cytoplasm. GBM cells however exploit this balance; not only are GBM cells are constantly dividing, but also genomic alterations ensure that Rb and SIRT 1 is inactive (Fig 4.10).

4.8 YY1 nuclear and cytoplasmic presence in primary human astrocytes and GBM cells.

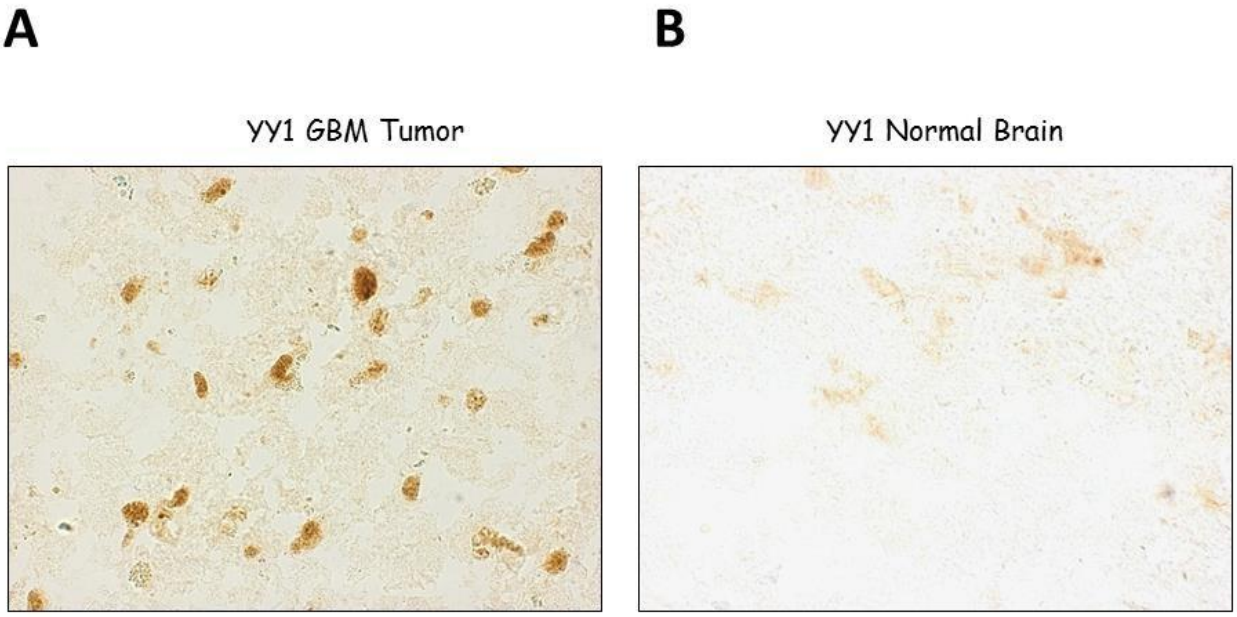
Nuclear and cytoplasmic fractionation probed for YY1 nuclear translocation. Nuclear and cytosolic fractions of A) primary human astrocytes and B) U373 cells and primary GBM12 cells were isolated at indicated timepoints, after IL-1B and OSM stimulation, according to the protocol described in the materials and methods, and probed for YY1, Laminin, and Tubulin.


A
Astrocytes
Cyto
Nuc
Hours IL-1β + OSM
C 4 18 C 4 18
Yy1
Laminin
Tubulin
B
U373
GBM12
Cyto
Nuc
Hours IL-1β + OSM
C 4 18 C 4 18
Yy1
Laminin
Tubulin

4-8

4.9 **YY1 nuclear and cytoplasmic presence in human GBM and normal brain isolated *in vivo***

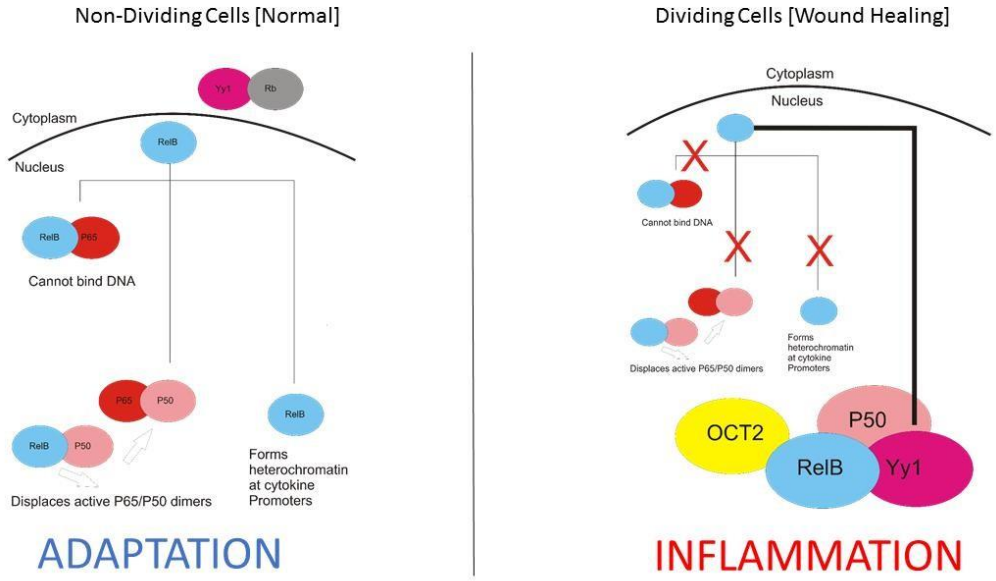
Human GBM and normal samples were obtained from the VCU TDAAC. YY1 protein levels were assessed by IHC as specified in the material and methods.



4-9

4.10 Diagram of RelB response to inflammation in dividing and non-dividing cells.

In non-dividing cells RelB maintains its putative function in the literature in response to inflammation, and acts as a negative feedback regulator to prevent chronic inflammation in collaboration with SIRT1. However, in the physiological context of a wound or a developing organ, when cells are proliferating, it would be of physiologic benefit to maintain the inflammatory microenvironment. In this case, YY1 translocates to the nucleus to drive RelB dependent inflammation. GBM cells however exploit this balance as GBM cells are constantly dividing, and both Rb and SIRT1 are inactive.


Non-Dividing Cells [Normal]
Yy1
Rb
Cytoplasm
RelB
Nucleus
RelB
P65
Cannot bind DNA
P65
P50
RelB
P50
RelB
Displaces active P65/P50 dimers
Forms heterochromatin at cytokine Promoters
ADAPTATION
Dividing Cells [Wound Healing]
Cytoplasm
Nucleus
Cannot bind DNA
Displaces active P65/P50 dimers
Forms heterochromatin at cytokine Promoters
OCT2
P50
RelB
Yy1
INFLAMMATION

4-10

4.5 Discussion

We had previously demonstrated that RelB expression is similarly induced by IL-1B/OSM in astrocytes and GBM cells. Additionally, we had previously ascertained whether RelB suppresses p65/p50-driven cytokine expression in these cells. While RelB suppressed proinflammatory gene expression in astrocytes, it surprisingly enhanced proinflammatory expression in established and primary GBM cells. Thus, our data implied that the anti-inflammatory RelB-driven feedback loop present in astrocytes is converted into a feed-forward loop in GBM cells. Lastly, we showed that a lack of SIRT1 expression and activity prevented RelB mediated silencing of proinflammatory genes in GBM cells.

We next sought to understand the mechanisms of GBM-specific RelB-dependent transcriptional activation using an unbiased approach. We hypothesized that the aberrant-activation of cytokine genes by RelB in GBM cells may depend on an additional transcription factor that affects RelB activity specifically in GBM cells but not astrocytes. To identify this factor, we analyzed the transcription start sites of RelB- controlled genes for the presence of regulatory elements. This bioinformatics approach identified YY1 as a common regulator motif.

To test whether YY1 differentially functions in GBM cells versus astrocytes, we analyzed cytokine expression in cells depleted of YY1. We found that although cytokine expression is YY1-independent in astrocytes, it is upregulated by YY1 in GBM cell lines and primary GBMs (IL-1 and IL-6, U373 and GBM12). To explain the difference in the regulation of cytokine expression between astrocytes and GBM cells, we analyzed the cellular localization of YY1. We found that YY1 is localized almost exclusively to cytoplasm of astrocytes whereas it is almost

entirely nuclear in GBM cells. In order to determine whether RelB and YY1 were functioning to stimulate cytokine expression through the same molecular mechanism, we performed a dual knock-down of YY1 together with RelB which demonstrated no additive effect on cytokine expression. Thus, our findings suggest that YY1 functions as a cancer-specific modifier of RelB function in response to inflammation.

Since YY1 can activate expression by forming complexes with RelB and Oct-2 [79], we tested whether YY1 directly interacts with RelB in GBM cells. We demonstrated a direct YY1/RelB interaction using immunoprecipitation. Furthermore, we also found both YY1 and RelB were bound to the IL-6 promoter in cytokine-treated cells suggesting that RelB/YY1 control of cytokine expression occurs through direct, proximal promoter binding of genes and subsequent transcriptional activation. Thus, we concluded that YY1 functions to modify RelB action through a direct physical interaction and accumulation on cytokine promoters, and that this mechanism is active in freshly isolated human GBM tumors.

Our data suggest that although RelB coordinates anti-inflammatory feedback in astrocytes (and likely other non-transformed cells in the tumor microenvironment), this mechanism does not function in GBM cells due to the presence of YY1 in the nuclei of these cells. As a result, GBM cells continuously secrete RelB/YY1/p50-induced proinflammatory cytokines which establishes and supports chronic inflammation promoting GBM progression.

5 Discussion

Although proinflammatory cytokines are secreted transiently during acute inflammation, chronic inflammation develops in GBM and promotes GBM cell proliferation, migration, invasion, resistance to apoptosis, and the maintenance of stem cell like properties [4,64,82-83,87-92]. To identify cytokines which specifically support GBM aggressiveness, we used an unbiased approach and correlated cytokine expression with clinical outcome data from The Cancer Genome Atlas (TCGA). We identified IL-1B and OSM as two cytokine signaling programs, whose expression is negatively correlated with patient survival. Additionally, we demonstrated that IL-1B and OSM are frequently expressed together at high levels and that IL-1B and OSM are specifically expressed at high levels in mesenchymal GBM, which is the most common and deadly GBM [14,15]. Thus, we concluded that chronically elevated IL-1B and OSM levels initiate intracellular signaling programs which drive the progression of GBM.

A wide variety of inflammatory stimuli, including IL-1, activate the canonical NF-κB pathway triggering degradation of IκBa, translocation of p65/p50 heterodimers to the nucleus, and induction of p65/p50-dependent genes [47]. RelB is a unique member of the NF-κB family mostly known as a downstream target of non-canonical NF-κB signaling. Interestingly, when large amounts of RelB is produced an excess of RelB forms RelB/p50/IκBa complexes, which can also be activated by the canonical pathway [96]. We show that IL-1B/OSM stimulation induces RelB expression, which subsequently forms complexes with p50 in GBM cells, similar to astrocytes; additionally, we show that IL-1B and OSM treatment induces RelB/p50 nuclear

translocation. Interestingly, it has previously been demonstrated that OSM induces RelB/p50 complex formation via a mechanism independent of increased RelB synthesis [100].

RelB/p50 signaling has previously been shown to drive a negative feedback loop suppressing cytokines by several mechanisms including epigenetic silencing [51,52]. However, previously published reports demonstrating RelB as a silhouette marker for mesenchymal GBM, and our analysis of patient outcome data which suggested that RelB and IL-1/OSM signaling were potential drivers of chronic inflammation, suggested that RelB cannot function to limit inflammation in GBM. Therefore, we compared RelB-canonical negative feedback in astrocytes and GBM cells. We showed that IL-1B/OSM induced RelB-canonical signaling acts as a potent negative feedback mechanism of IL-1b, IL-6 and COX2 expression in astrocytes. Strikingly, RelB canonical signaling drives IL-1b, IL-6 and COX2 expression in GBM cells. Additionally, using high throughput expression analysis we confirmed that the global role of RelB in response to IL-1B and OSM induced inflammation is to act as a feedforward promoter of inflammatory genes. Conversely, this mechanism is cancer specific, as RelB globally acts as a suppressor of transcription and genes annotated to be involved in the inflammatory response in astrocytes. Thus, we demonstrate that RelB-canonical signaling acts as a molecular switch promoting chronic inflammation in GBM.

Additionally, our group sought to define precise pathways/actions the GBM-specific RelB-dependent program induced to make tumors more aggressive. Utilizing an approach combining RNAseq data with gene expression and clinical outcome data from TCGA, we found that the RelB program stimulates a prognostically important program of genes which recruit and activate GAMs. Additionally, this program corresponds with recently published papers indicating that

GAMs are activated by global myeloid activators such as the CSF, MIP, and MCP family of proteins instead of classical M1 and M2 drivers of macrophage activation.

To understand the mechanism of "aberrant" cytokine activation by RelB in GBM cells, we investigated the mechanism by which RelB silences inflammatory gene production in astrocytes. The previously demonstrated model of epigenetic silencing of the il1b gene during endotoxin tolerance by RelB in macrophages [51,52], depends on the recruitment of histone deacetylase SIRT1. We hypothesized that loss of SIRT1 expression could prevent epigenetic silencing. Significantly, mining of TCGA database showed that one allele of the *SIRT1* gene is deleted in 80% of GBM tumors and leads to poor survival prognosis. Lower expression of SIRT1 mRNA was found in GBMs than normal brain and confirmed on the protein level by IHC. These findings were further confirmed *in vitro* since SIRT1 mRNA was substantially decreased in GBM cell lines and primary GBM cells in comparison to astrocytes. SIRT1 activity was also significantly reduced in GBM cells in comparison to astrocytes. We addressed the importance of SIRT1 in RelB-mediated regulation by either inhibiting SIRT1 activity in astrocytes or expressing SIRT1 in GBM cells. Remarkably, inhibition of SIRT1 activity enhanced expression of cytokines in astrocytes. Conversely, overexpression of SIRT1 in GBM cells diminished expression of cytokines. Importantly, the effect of SIRT1 was RelB-dependent. These data suggest that repression of cytokines in astrocytes depends on SIRT1; however, RelB/SIRT1-dependent repression does not function in GBM cells.

However, still unexplained was how RelB "switched" to a transcriptional transactivator in a GBM specific manner. We next sought to understand the mechanisms of GBM-specific RelB-transcriptional activation using an unbiased approach. We hypothesized that the aberrant-activation of cytokine genes by RelB in GBM cells may depend on an additional transcription

factor that affects RelB activity specifically in GBM cells but not astrocytes. To identify this factor, we analyzed 2.5 kb-long regions surrounding the transcription start sites of the RelB-controlled genes for the presence of regulatory elements. This bioinformatics approach identified several common regulatory elements, including a motif that can be bound by Yin-Yang 1 (YY1) ($p=0.000003$), a GLI-Krüppel-related zinc-finger transcription factor and chromatin modifier known to recruit both corepressors and coactivators and thus regulating chromatin structure. Although YY1 has been known for 25 years, it has neither been shown to regulate GBM progression nor chronic inflammation.

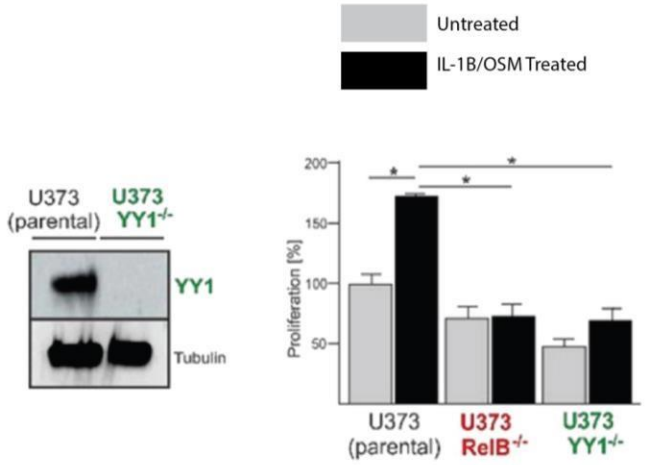
We tested whether YY1 differentially functions in GBM cells versus astrocytes, and we found that although cytokine expression is YY1-independent in astrocytes, it is upregulated by YY1 in GBM cell lines and primary GBMs. Additionally, we found that this GBM-specific control of cytokines is a result of the intracellular localization of YY1. We found that YY1 is localized almost exclusively to cytoplasm of astrocytes whereas it is almost entirely nuclear in GBM cells, which correlates directly with YY1- dependent regulation of cytokine expression in these cells. Additionally, we showed that knockdown of RelB does not attenuate cytokine expression in cells not expressing YY1 and visa versa indicating that YY1 is required for RelB dependent cytokine transactivation and that RelB is necessary for YY1 dependent cytokine transactivation, thus RelB and YY1 act to stimulate cytokine expression through the same molecular mechanism.

Furthermore, we demonstrated a direct YY1/RelB interaction using immunoprecipitation. We also found both YY1 and RelB were bound to the IL-6 promoter in cytokine-treated cells suggesting that RelB/YY1 control of cytokine expression occurs through direct, proximal promoter binding of genes and subsequent transcriptional activation. Furthermore, we showed

that YY1 was present in the nuclei of GBM cells in patient tumors while its localization was mostly cytoplasmic in nearby normal tissue. Thus, we concluded that YY1 functions to modify RelB action through a direct physical interaction and accumulation on cytokine promoters, and that this mechanism is active in *in vivo* human GBM tumors. Additionally, preliminary analysis of YY1 knockout CRIPSR pools suggests that the RelB/YY1 axis is necessary to mediate IL-1B/OSM induced proliferation (Appendix A).

Our data suggest that although RelB coordinates anti-inflammatory feedback in astrocytes, and that this mechanism does not function in GBM cells due to a loss of SIRT1, and the presence of YY1 in the nuclei of these cells. As a result, GBM cells continuously secrete RelB/YY1/p50-induced proinflammatory cytokines which establishes and supports chronic inflammation promoting GBM progression.

5.1 Appendix A: Preliminary Supplemental Figures



A YY1 knockout pool was generated using CRIPSR/Cas9 Technology. Both RelB and YY1 were necessary for IL-1B/OSM induced proliferation.

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5.3 Vita

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Education

Bachelors of Science in Biology with Honors, May 2010

Davidson College, Davidson, NC

Academic and Professional Honors

Scholarships and Honors

2016 Evans Award (VCU Biochemistry Department)
2015 Massey Cancer Center Excellence in Cancer Research Award (2nd place)
2013 M2 Course Award (highest grade in course): Pathology
2012 M1 Course Award (highest grade in course): Histology
2010 B.S. awarded with Honors
06-07 Lowell L Bryan Scholarship

Grants

F30 NCI Fellowship Training Grant:
Cytokine induced RelB/p50 complexes drive mesenchymal glioma progression.
[1F30CA200252-01A1]

2006 Davidson College Undergraduate Honors Research Grant:
Designing a modular single stranded DNA production device using msDNA.

Publications

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2) Sun D., Daniels T, Wolfe A, **Waters M**, Hamm R. Inhibition of Injury Induced Cell Proliferation in the Dentate Gyrus Impairs Cognitive Recovery Following Traumatic Brain Injury. **2015**. Journal of Neurotrauma.

3) Bhardwaj R, Yester JW, Singh SK, Biswas DD, Surace MJ, **Waters MR**, Hauser KF, Yao Z, Boyce BF, Kordula T. RelB/p50 complexes regulate cytokine induced YKL-40 expression. J Immunol. 2015 Mar 15; 194(6): 2862-70

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8) **Waters MR**, Raymond PW, Tusukamoto H, Puri P. Gene expression changes suffered during alcoholic liver injury persist to influence prognosis in hepatocellular carcinoma. [manuscript in preparation]

9) Asim A, Rabender C, **Waters M**, Arfeen A, Sperlazza J, Gobalakrisna S, Zweit J, Mikkelsen R. Nitric Oxide Synthase Activity and its Modulation in the Treatment of Colorectal Cancer. [manuscript in preparation]

10) Gupta AS, **Waters MR**, Biswas DD, Surace MJ, Siebenlist U, Kordula T. Phosphorylation of RelB on Serine 472 coordinates SIRT1-dependent epigenetic silencing of cytokine genes and tolerance in astrocytes. [manuscript in preparation]

------ **Pending Lab Publications (Projects)**

1) Biswas DD

2) Waters MR