REGULATION OF YKL-40 IN STERILE INFLAMMATION AND ITS ROLE IN GLIOBLASTOMA IN VIVO

Reetika Bhardwaj
Virginia Commonwealth University

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REGULATION OF YKL-40 IN STERILE INFLAMMATION AND ITS ROLE IN
GLIOBLASTOMA IN VIVO

By Reetika Bhardwaj

Biomedical Science B.S., Delhi University, 2007

A DISSERTATION submitted in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY at Virginia Commonwealth University.

Virginia Commonwealth University, 2014

Major Director: TOMASZ KORDULA
Full Professor, Department of Biochemistry and Molecular Biology
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I will forever cherish the time at Virginia Commonwealth University; the memories of graduate school will forever be with me. I have made lifelong friends, learned life’s most essential lessons of living honesty, being patient, and simply forgiving. I have found my true love and the passion for doing impactful and meaningful science is forever ignited within.
I dedicate this thesis to

my family, my husband, Rahul, and my beloved friends

for their constant support and unconditional love.

I love you all dearly.
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<td>Activating protein 1</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood Brain Barrier</td>
</tr>
<tr>
<td>BCL-3</td>
<td>B-cell lymphoma 3-encoded protein</td>
</tr>
<tr>
<td>BLI</td>
<td>Bioluminescence imaging</td>
</tr>
<tr>
<td>BRP-39</td>
<td>Breast regression protein-39</td>
</tr>
<tr>
<td>BSMC</td>
<td>Bronchial smooth muscle cells</td>
</tr>
<tr>
<td>CHI3L1</td>
<td>Chitinase-3-like protein 1</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CNTF</td>
<td>Ciliary Neurotrophic Factor</td>
</tr>
<tr>
<td>CNTFR</td>
<td>Ciliary Neurotrophic Factor Receptor</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>DAMPs</td>
<td>Damage associated molecular patterns</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DD</td>
<td>Death domain</td>
</tr>
<tr>
<td>DOX</td>
<td>Doxycycline</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGFR</td>
<td>EGF receptor</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>Extracellular signal-regulated kinases</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>GBM</td>
<td>Glioblastoma multiforme</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
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<td>GLAST</td>
<td>GLutamate ASpartate Transporter</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<tr>
<td>GRR</td>
<td>Glycine-rich region</td>
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<tr>
<td>GTP</td>
<td>Guanine triphosphate</td>
</tr>
<tr>
<td>HAD</td>
<td>HIV associated dementia</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin and eosin</td>
</tr>
<tr>
<td>HC gp-39</td>
<td>Human cartilage glycoprotein-39</td>
</tr>
<tr>
<td>HSP</td>
<td>Heat shock proteins</td>
</tr>
<tr>
<td>HUVECs</td>
<td>Human umbilical vein endothelial cell</td>
</tr>
<tr>
<td>IBD/DimD</td>
<td>IKK-binding domain/dimerization domain</td>
</tr>
<tr>
<td>IKK</td>
<td>IkB kinase</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin-1</td>
</tr>
<tr>
<td>IRAK</td>
<td>IL-1 Receptor Associated Kinase</td>
</tr>
<tr>
<td>IRF-1</td>
<td>Interferon Regulatory Factor-1</td>
</tr>
<tr>
<td>IκB</td>
<td>Inhibitor of nuclear factor B</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus Kinase</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinases</td>
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<tr>
<td>LIF</td>
<td>Leukemia Inhibitory factor</td>
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<tr>
<td>LIFR</td>
<td>LIF receptor</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MAPKK</td>
<td>MAPK Kinase</td>
</tr>
<tr>
<td>MMPs</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>NEMO</td>
<td>NF-κB essential modifier</td>
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<tr>
<td>NFI-X3</td>
<td>Nuclear factor 1 X3</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor kappa B</td>
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<tr>
<td>NIK</td>
<td>NF-κB inducing kinase</td>
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<tr>
<td>NLRs</td>
<td>NOD-like Receptors</td>
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<td>NRS</td>
<td>Normal Rabbit serum</td>
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</table>
OD  Oligodentroglioma
OSM  Oncostatin M
OSMR  OSM receptor
OVA  ovalbumin
PAC  Puromycin resistance
PAK  p21 activated kinase-1
PAMPs  pathogen associated molecular patterns
PBS  Phosphate buffer
Saline
PDGF  Platelet-derived growth factor
proline-rich,
glutamic acid-rich,
serine-rich, and
threonine-rich
Phosphatidylinositol-4,5-bisphosphate 3-kinase
PI3K  Pattern recognition receptors
dephosphorylate receptor and kinase phosphorylation sites
PRPs  retinoblastoma
RFP  Red Fluorescent Protein
RHD  REL homology domain
RIG-1  retinoic acid-inducible gene 1
RTK  Receptor Tyrosine Kinase
SCF  Stem cell factor
SDD  scaffolding and dimerization domain
SH2  Src Homology 2
SHP-1/2  domain containing protein tyrosine phosphatase
Simian
SIVE  Immunodeficiency Virus Infection
SOCS  Suppressors of
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TAD</td>
<td>Trans-Activator of Transcription</td>
</tr>
<tr>
<td>TAT</td>
<td>Tetracycline</td>
</tr>
<tr>
<td>Tet</td>
<td>Tissue inhibitor of metalloproteinase</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>Toll like receptors</td>
</tr>
<tr>
<td>TLRs</td>
<td>Tumor Necrosis Factor α</td>
</tr>
<tr>
<td>TNFα</td>
<td>U1242 cells with GFP Luciferase</td>
</tr>
<tr>
<td>U1242 GL</td>
<td>ubiquitin-like domain</td>
</tr>
<tr>
<td>ULD</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGF</td>
<td>cytokine signaling proteins</td>
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Abstract

REGULATION OF YKL-40 IN STERILE INFLAMMATION AND ITS ROLE IN GLIOBLASTOMA IN VIVO

By Reetika Bhardwaj, Ph.D.

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

Virginia Commonwealth University, 2014

Major Director: Tomasz Kordula, Ph.D.

YKL-40 is a secreted glycoprotein, which is a shared biomarker of chronic inflammation and oncogenic transformation. Indeed, YKL-40 expression is up-regulated in many diseases including multiple sclerosis, Alzheimer disease, viral encephalitis, HIV-associated dementia, brain infarction, and traumatic brain injury. YKL-40 is also expressed by several solid tumors, such as osteosarcoma, ovarian carcinoma and glioblastoma multiforme (GBM). It promotes the migration and invasion of astrocytes as well as GBM cells. Serum YKL-40 levels have been shown to directly correlate with tumor grade and potentially tumor burden in GBM. In contrast to the numerous reports documenting elevated
expression of YKL-40, relatively little is known about inflammatory mediators and specific molecular mechanisms that control its expression. For my PhD project, I decided to elucidate the mechanism regulating YKL-40 expression in inflammation and to understand how YKL-40 mediates the migration and invasion of GBM cells in vivo. As per my first project, I found that YKL-40 expression is up-regulated in many inflammatory models such as irritant induced inflammation (turpentine), EAE (experimental autoimmune encephalomyelitis), irradiation induced brain inflammation and in the HIV-TAT overexpression model. YKL-40 expression is also up regulated in oligodendroglioma patient samples. We show that YKL-40 expression is activated by major pro-inflammatory cytokines including IL-1, IL-6, and OSM in astrocytes, and that the activation of YKL-40 expression by cytokines depends on the STAT and NF-κB regulatory elements located within the YKL-40 promoter. Additionally, we discovered that STAT3 is a major regulator of YKL-40 expression in response to the IL-6 family cytokines, including OSM. Indeed, both depletion of STAT3 expression or overexpression of dominant-negative STAT3 abolishes activation of YKL-40 expression. In contrast, although IL-1 activates NF-κB (p65/p50) in astrocytes, IL-1-induced YKL-40 expression is p65/p50 independent, and instead regulated by the RelB/p50 complexes. Interestingly, OSM promotes formation of p50/RelB complexes. In conclusion, we found that YKL-40 is up regulated by major pro-inflammatory cytokines during inflammation associated with various disease models. Thus, we uncovered the regulatory mechanism that governs the expression of YKL-40 during sterile inflammation.
Next, I studied the role of YKL-40 in GBM in vivo. The major obstacle for treatment of GBM is the unique ability of GBM cells to diffusely infiltrate healthy brain tissue. GBM tumors express high levels of YKL-40, and we showed that administration of YKL-40 increases migration and invasion of U1242 cells in vitro. We used the U1242 cell line for our studies, due to its unique ability to form infiltrative tumors, which reflects the real brain pathology in GBM. Additionally, knock-down of YKL-40 suppresses the migration and invasion of GBM cells in vitro. To study this phenomenon in vivo, we utilized an inducible lentiviral vector containing shRNA to YKL-40. We generated stable GBM cells containing this shYKL-40 vector and used them in a mouse xenograft model for glioma to evaluate the effect of YKL-40 in invasion and migration of GBM cells.

RelB expression is increased in GBM patients, and it is associated with up-regulation of a distinct subset of gene. Here we show that RelB expression is increased during inflammation in response to IL-1, where it can enhance the expression of YKL-40, which can modulate the inflammatory response. Thus, we found that YKL-40 is up regulated by major inflammatory cytokines during inflammation associated with various diseases. In addition, YKL-40 secreted by brain cells other than GBM cells may have an effect on invasion of GBM cells in vivo.
Chapter 1

Introduction

1.1 Sterile Inflammation

Inflammation is putatively understood to be a response to pathogenic stimuli, which can result in redness, swelling, pain and increased temperature. However, sterile inflammation, “occurs in the absence of microbial or infectious stimuli, and instead occurs in response to physical, chemical or metabolic noxia”. Myocardial infarction, trauma, and ischemia, are some examples of diseases associated with sterile inflammation (1). Amplification of the immune response occurs once PAMPs (pathogen associated molecular patterns) are detected on bacterial surfaces and released by viruses. Pattern recognition receptors (PRPs), including toll like receptors (TLRs), NOD like receptors (NLRs) and RIG-1 are expressed by innate immune cells and bind to PAMPs to activate the innate immune cascade to overcome intruding pathogens (2). In case of sterile inflammation, a similar mechanism occurs to activate the immune response due to stimuli called DAMPs (damage associated molecular patterns). DAMPs include DNA, RNA, heat shock proteins (HSP), high mobility group box-1, cholesterol crystals (3, 4), which are normally sequestered but are released due to cell death and damage. Similar to infectious inflammation, activation of immune cells by DAMPs leads to increased expression of inflammatory cytokines and the recruitment of immune cells. Excessive inflammation can lead to various pathological conditions including gout, arthritis, type 1 diabetes,
Alzheimer’s disease and multiple sclerosis. Multiple sclerosis is an inflammatory disease, which results in demyelination, oligodendrocyte cell death, and axonal degeneration.
**Figure 1 Mechanism for Inducing Sterile Inflammation.** Sterile stimuli that include damage-associated molecular patterns (DAMPs), sterile particulates and intracellular cytokines released from necrotic cells can activate the host immune system to induce sterile inflammation through at least three pathways that are not mutually exclusive. (3)
1.2 Astrocytes

Astrocytes are the most abundant cell type in the CNS, which emphasizes their ability to modulate a range of homeostatic and pathologic condition. The most common markers of astrocytes are GFAP and vimentin, which are intermediate filament proteins (5). Astrocytes are evolutionarily conserved suggesting that their basic functions remain conserved as well. Astrocyte also exhibit morphological diversity as well as inherent functional specialization. They can be classified into five types based on their anatomical location. (1) White matter astrocytes, which have star like appearance due to numerous fine projections, some of which are GFAP positive; (2) Grey matter astrocytes, which also referred as ‘protoplasmic’, and have a less complex morphology, and are GFAP negative; (3) Ependymal astrocytes, which are closest to progenitor cells, are GFAP positive and are located in the stem cell niches; (4) Radial glia, which are located in the cerebral cortex, direct migration and provide a scaffold for migrating neurons during brain development; (5) Perivascular astrocytes, which form “vascular feet” which are specialized projections around the neurovasculature, and are GFAP positive as well.

In the past, these cells were considered to be space filling cells of the CNS (central nervous system), and only responsible for providing support to the neighboring excitable neuron. Astrocytes are located in close proximity of neurons and endothelial cells of the BBB (5), where they form a light association. Additionally, astrocytes provide energy and substrates to neurons to maintain homeostatic conditions. In regions of active neurotransmission at the synapse, astrocytes dilate the BBB to allow the exchange of oxygen and glucose in active regions of the
brain. Astrocytes, during early brain development, secrete factors that are critical for active synapse formation and synapse elimination (6). Radial glia, during early brain development, act as stem cells and differentiate to neurons and oligodendrocytes (7, 8). Many developmentally regulated tropic and trophic factors are secreted by astrocytes which help and direct exons during post natal brain development. Expression of these factors is increased during pathological conditions of the brain associated with astrogliosis. Hence, with the identification of more diverse functions of astrocytes in CNS homeostasis, it can be concluded that astrocytes control many functions in normal as well as pathological states of the brain.

1.3 Chitinase 3-like 1 (CHI3L1) or YKL-40

YKL-40 also known as human cartilage glycoprotein-39 (HC gp-39), Chitinase 3-like protein-1 (CHI3L1), mouse homologue breast regression protein-39 (BRP-39), is a secreted 40 kDa glycoprotein. In humans, the gene for YKL-40 is located on chromosome 1q31-q32. YKL-40 belongs to glycohydrolase family, which is comprised of many bacterial and eukaryotic proteins having a chitinase activity (9, 10). The YKL-40 protein is comprised 383 amino acids and is synthesized with an 8 amino acid N-terminal pro-peptide sequence which is cleaved before the protein is secreted. Even though it belongs to a family of chitinases, YKL-40 is an enzymatically inactive, due to a single amino acid substitution of glutamate to leucine in its core chitinase binding pocket; though it still binds to
chitin and many other carbohydrate molecules (10)

In normal individuals, YKL-40 is secreted by neutrophils, and chondrocytes, synoviocytes, and smooth muscle cells (10). It is also highly up-regulated during the differentiation of monocytes to macrophages, and during smooth muscle cell differentiation (11). However, recently it has been regarded as a marker of chronic inflammation and transformation. YKL-40 expression has been found to be highly up-regulated in many pathologies associated with ongoing inflammation, tissue remodeling and cancer such as rheumatoid arthritis, osteoarthritis, asthma, breast cancer, GBM, papillary thyroid carcinoma, extraskeletal myxoid chondrosarcoma, colon cancer, melanoma and metastatic prostate cancer. In addition, elevated levels of YKL-40 are also correlated with the disease severity and high mortality rates during various pathological states, including GBM. Recently, microarray data has indicated that, YKL-40 is one of the most up-regulated gene in GBM (12).

In vitro studies have shown that the expression of YKL-40 increases upon stimulation with pro-inflammatory cytokines in macrophages and other cell types including chondrocytes, and cancer cells (10) (13). In addition, YKL-40 knock-out mice showed impaired antigen induced Th2 responses, with YKL-40 being a potent inhibitor of death-receptor induced inflammatory cell apoptosis. In addition, the expression levels of IL-4 and IL-13 was decreased in YKL-40 null animals challenged with OVA (ovalbumin) antigen compared to the sham-treated animals (14). Even though YKL-40 is up-regulated in many inflammation associated pathologies, regulation
of its expression and the functions of YKL-40 are not well defined. Analysis of the -1.3 kb upstream region of YKL-40 promoter revealed presence of several transcription factor binding sites, including AP-1, CEBP, Sp1, Sp3, NF-κB and ETS (11). Previously it has been shown that during monocyte to macrophage differentiation expression of YKL-40 is mainly controlled by the SP-1 transcription factor (11). In response to pro-inflammatory cytokines IL-1 and TNFα, expression of YKL-40 is regulated by p65(NF-κB) in chondrocytes (13). In contrast, IL-1 and TNFα represses YKL-40 expression in glioma cells (15). Therefore, cell and tissue specific regulation in expression of YKL-40 cannot be ruled out. In addition, the level of YKL-40 expression increases upon stress stimuli including, hypoxia, cell confluence, serum starvation, and genotoxic stress (UV irradiation) in glioma cells (16). Interestingly, hypoxia induced expression of YKL-40 was independent of the HIF-1 transcription factor (16).

There have been studies indicating the functions of YKL-40. It has been shown to work as a mitogenic factor for several cell types including skin and lung fibroblasts, chondrocytes and synovial cells. YKL-40, also acts as a pro-survival factor when cells are exposed to stress conditions (10). Moreover, primary transformed astrocytes overexpressing YKL-40 express genes conferring resistance to irradiation treatment (17).

YKL-40 can also serve as chemotactic factor for HUVEC (Human umbilical vein endothelial cell), and as an adhesion and migration factor for vascular smooth muscle cells. Indeed, its overexpression significantly increases migration of transformed astrocytes (17). Thus, in
general, YKL-40 appears to function as a tissue remodeling, pro-survival, and pro-growth factor. In accordance with its prosurvival and pro-growth functions, YKL-40 has been shown to activate MAPK/ERK1/2 (Mitogen-activated protein kinases/Extracellular signal-regulated kinases) and PI3K/AKT (Phosphatidylinositol-4,5-bisphosphate 3-kinase) signaling when added exogenously (18, 19). Despite these findings, the precise mechanism of YKL-40 action is yet to be elucidated. Because YKL-40 contains a heparan binding motif it was postulated to bind carbohydrate moieties on the surface of cells. Interestingly, it has recently been shown that YKL-40 can bind to syndecan-1 (receptor for heparan sulfate binding protein) and that binding event further enhances syndecan-1 association with αvβ3 integrin. The syndecan-integrin complex formation leads to the phosphorylation and activation of FAK, and subsequent activation of ERK1/2 and PI3K (18). Recently, it was shown that YKL-40 binds to IL-13Ra2 and stimulates MAPK, Akt/PKB, and Wnt/β-catenin signaling and regulates oxidant injury, apoptosis, and pyroptosis via IL-13Ra2 (20). In addition, YKL-40 could promote BSMC (Bronchial smooth muscle cells) proliferation and migration directly via a protease-activated receptor-2–dependent pathway (21). YKL-40 has been identified during various studies, to recognize potential markers and targets for a subset of CNS pathologies. YKL-40 levels are elevated in the CSF of patients with schizophrenia, Alzheimer’s disease, Parkinson’s disease, multiple sclerosis, RETT syndrome, and GBM (12, 22, 23).

However, the regulation and role of YKL-40 in CNS cell types remains elusive. Recently, it has been shown that increased expression of YKL-40 is associated with the increase in CSF
(Cerebrospinal fluid) viral load in SIVE (Simian Immunodeficiency Virus Infection) and MS patients. This increased YKL-40 expression was found in association with the microglial nodules in SIVE (24). YKL-40 is expressed by astrocytes in vitro, and its expression is regulated by NFI-X3 and STAT3 (25). Even though regulation of its expression in normal brain (astrocytes) has been shown by our lab, regulation of its expression during various CNS pathologies remains open for further analysis.

1.4 Glioblastoma

Astrocytic tumors, which are widely represented by glioblastomas, are the most aggressive and fatal type of brain tumors (26). Four such astrocytic tumors are; (1) Pilocytic astrocytoma, grade I is benign and can be surgically removed, (2) whereas grade II diffuse astrocytoma, is of low malignancy and requires long-term clinical treatments; (3) Anaplastic astrocytoma, grade III, is malignant and leads to the patient’s death within a few years; (4) GBM, grade IV is highly malignant and causes death within months. GBM is the most common type of primary brain tumor, with a 5-year survival rate of less than 3%. A unique clinical problem in treating GBM is the extensive invasiveness of GBM cells. The mortality of patients with GBM remains high due to the ineffectiveness of current therapies, which include surgery, radio, and chemotherapy. The high rate of tumor relapse is due to the infiltrative nature of GBM. The high rate of tumor relapse points to the need for more targeted therapeutic approaches. There are several molecular markers that help to characterize these tumors (27), such as mutations in the p53 and
retinoblastoma (RB) tumor suppressor (25% of cases), EGF receptor (EGFR) amplification or overexpression (~40-60% of cases), mutations of the phosphatase and tensin homology gene (PTEN) (30% of cases), deletion or inactivation of cyclin-dependent kinase inhibitors p16INK4a/p19ARF (30-40% of cases), amplification or overexpression of transformed 3T3 cell double minute 2 (MDM2) gene (10-50%), and loss of heterozygosity (LOH) on chromosome 10 in 50-80% of cases. As a result of these genetic alterations, activation of signaling pathways such as PI3K Akt, Ras-MAPK and JAK/STAT (Janus Kinase/Signal Transducer and Activator of Transcription) leads to uncontrolled cellular proliferation and escape from apoptosis, which promotes tumor growth and progression.

Several proteases such as MMP-9, MMP-2 and MMP-7 contribute to the invasiveness of GBM cells by altering the ECM of brain parenchyma (28). Studies have documented the presence of tumor cells not only in the normal brain tissue immediately surrounding GBM tumors but also in distant brain tissue including the contralateral hemisphere (29).

The derogatory effects of tumor invasion are highlighted below (Figure 2). There is a presence of infiltrative cells even after surgical removal of bulk tumor (Figure 2C).
Figure 2 Pre and post-operative MRI images of glioblastoma multiforme (GBM) tumor. (A) Pre-operative T1-weighted MRI scan of GBM tumor. The area of contrast enhancement (grey arrow) corresponds to frank tumor with disruption of the blood-brain barrier. The surrounding brain tissue into which GBM cells are invading is clearly edematous (black arrow). (B) Pre-operative T2-weighted MRI scan of the same tumor. In this series, the frank tumor (grey arrow) and the surrounding brain tissue with edema and invading tumor cells (black arrow) are both bright. Note that the abnormal signal area extends across the corpus callosum into the contralateral hemisphere. (C) Post-operative T1-weighted MRI scan. The region of frank tumor has been resected resulting in the absence of contrast enhancement (grey arrow) but residual areas of edema and residual infiltrating tumor remain (black arrow) (30).
1.5 IL-1 Signaling

Interleukin-1 (IL-1) is an important pro-inflammatory cytokine associated with the host-defense response to injury and infection (31). It is a key regulator of both acute and chronic inflammation. It is an important mediator of inflammatory conditions in the CNS disorders including brain trauma, stroke, epilepsy, AD and PD (32, 33). The IL-1 family of cytokines consists of 11 related ligands, in which IL-1β, IL-1α, IL-1 receptor antagonist (IL-1Ra), and IL-18 are the most studied. IL-1α and IL-1β act as agonists and binds to the same type I receptor (IL-1R1). The IL-1R1 then associates with an accessory protein, IL-1R1AcP, and activates the downstream signaling cascades (34, 35). Subsequently, this leads to the production of secondary inflammatory mediators such as cytokines, chemokines and prostaglandins. IL-1R1 has a naturally occurring antagonist IL-1Ra, which is a soluble protein and competes with IL-1R and acts as a buffer for excess of IL-1 to inhibit downstream signaling (31, 35, 36).

The level of IL-1 expression during normal physiological conditions in a healthy individual is very low. However, IL-1 expression is dramatically increased in response to injury as well as infections (31). In the CNS, IL-1 is mostly produced by activated microglia. Chemokines and cytokines released by activated microglia can then stimulate astrocytes, oligodendrocytes and even neurons to further produce IL-1 (33, 37, 38). Except microglia, all other cells of the CNS express IL-1R1 and therefore respond to IL-1 (31).

IL-1, upon binding to its receptor, can activate numerous signaling cascades. In astrocytes, it is shown to activate the IKK-NF-κB pathway and the mitogen-activated protein kinases
(MAPKs) p38 MAPK, JNK and ERK (39). This activation leads to increased cell proliferation and hypertrophic changes in morphology also known as reactive astrogliosis. The IL-1 activated astrocytes, produce VEGF as well as many other factors regulating angiogenesis, and vessel plasticity. In addition, IL-1 also modulates astrocyte migration which is critical for recovery and repair of the injury site within the brain (40). In contrast, oligodendrocytes respond to IL-1 to promote their differentiation and maturation, indicating that IL-1 might have a role in the re-myelination and repair process after injury (31, 41).

Low level IL-1 expression in neurons is considered to regulate normal physiological functions including sleep, memory and long term potentiation. IL-1 concentration, and the duration of IL-1 activation of neurons determines its effects. In contrast to astrocytes, IL-1 does not signal through MAPK, JNK and ERK1/2 activation in neurons; instead, it activates Src kinase (42, 43). Neurons are more vulnerable to neurotoxic effects from IL-1 secreted by activated glial cells. High levels of IL-1 causes both glia and neurons to produce various neurotrophic and growth factors that promote neuronal survival (44). In the neurons, high concentration of IL-1 activates the c-Rel subunit of NF-κB instead of RelA, hence is responsible for its protective effects (45). Knock-out mice lacking IL-1α/β show protection to ischemic brain injury. Whereas, IL-1Ra (the antagonist) knock-out mice exhibit enhanced (3 fold) ischemic brain injury and caused increased mortality (36). IL-1 plays an important role in neuroinflammation as well as sterile inflammation and it can up-regulate various genes/factors that can lead to its toxic or protective effects during inflammation.
1.6 NF-κB pathway

There are five members of the mammalian NF-κB family, p65 (RelA), RelB, c-Rel, p50/p105 (NF-κB1), and p52/p100 (NF-κB2), that exist in unstimulated cells as homo or heterodimers bound to IκB family proteins. NF-κB proteins are characterized by the presence of a conserved 300 amino acid Rel homology domain (RHD) that is located toward the N terminus of the protein and is responsible for dimerization, interaction with IκBs, and binding to DNA (Fig.2). Upon stimulation, IκB kinase complex causes phosphorylation of IκBs, which is followed by ubiquitination, degradation of IκB proteins. This leads to the release of NF-κB dimers that translocate to the nucleus, bind specific DNA sequence, promoting transcription of target genes (46).
**Figure 3 Components of NF-κB pathway.** The mammalian Rel (NF-κB) protein family consists of five members: p65 (RelA), RelB, c-Rel (Rel), and the precursor proteins p100 (NF-κB 2) and p105 (NF-κB 1), the latter giving rise to p52 and p50, respectively. The IκB family consists of eight bona fide members, IκBα, IκBβ, IκBε, IκBζ, BCL-3, IκBNS, p100, and p105, which are typified by the presence of multiple ankyrin repeat domains. The IKK complex consists of IKKα (IKK1 or CHUK), IKKβ (IKK2), and NEMO (IKKγ). Relevant domains typifying each protein family are indicated. (ANK) Ankyrin repeat domain; (DD) death domain; (RHD) REL homology domain; (TAD) transactivation domain; (LZ) leucine zipper domain; (GRR) glycine-rich region; (SDD) scaffolding and dimerization domain; (ULD) ubiquitin-like domain; (Z) zinc finger domain; (CC) coiled-coil domain; (NBD) NEMO-binding domain; (a) α-helical domain; (IBD/DimD) IKK-binding domain/dimerization domain; (MOD/UBD) minimal oligomerization domain/ubiquitin-binding domain; (PEST) proline-rich, glutamic acid-rich, serine-rich, and threonine-rich (46).
The trademark of the NF-κB pathway is its regulation by IκB proteins such as IκBα, IκBβ, IκBε, IκBζ, and IκBNS and the precursor proteins, p100 and p105 which contain a multiple ankyrin repeat domain. NF-κB pathway is activated by phosphorylation of IκBs on conserved serine residues, termed as destruction box serine residues (DSGXXS), leading to recognition by bTrCP proteins. Once the phosphorylated destruction box is recognized it induces K48-linked polyubiquitination by the Skp1–Culin–Roc1/Rbx1/Hrt-1F-box (SCF or SCRF) family of E3 ligases together with the E2 enzyme UbcH5. The most studied member of the family is IκBα, which is rapidly degraded during activation of canonical NF-κB signaling pathways, leading to the release of multiple NF-κB dimers, although the p65:p50 heterodimer is considered the primary target of IκBα.

The NF-κB pathway has been classified into two types; canonical and non-canonical. Once the canonical pathway is triggered by various stimuli, it leads to the activation of IKKβ (also known as IKK2). IKKβ exists in a complex with the closely related kinase IKKα (also known as IKK1) and the regulatory protein NEMO (also known as IKKγ). Once activated IKKβ phosphorylates IκB proteins such as IκBα. In contrast to the classical NF-κB pathway, the non-canonical pathway depends on IKKα but is independent of NEMO. IKKα activation by these stimuli leads to phosphorylation of p100 and the generation of p52/RelB complexes (46).

Some studies have shown the presence of RelB/p50 dimers, as a result of RelB induction by the canonical pathway. In addition, a recent study showed the presence of RelB/p50 dimers in the cytoplasm bound to IκBα and IκBε. This complex is regulated by the canonical
signaling pathway during dendritic cell (DC) maturation (47). These recent studies using mathematical modelling, combined with the careful kinetic analyses of IκB degradation, RelB activation and gene expression and the use of knockouts of various members of the IκB family presents a definitive rejection of the idea that RelB is regulated by non-canonical signaling alone.(47)
Picture modified from- A less-canonical, canonical NF-κB pathway in DCs. Matthew S Hayden. Nature Immunology 2012
Figure 4 A RelB canonical pathway in dendritic cells. The canonical NF-κB signaling pathway (left) comprises of a NEMO-dependent IKK complex that phosphorylates (P) IκB–NF-κB complexes depicted by IκBα-p65-p50. IκB is then ubiquitinated (Ub) and degraded, allowing the NF-κB dimer to translocate to the nucleus and bind κB sites on the promoters of target genes. In the non-canonical or alternative pathway (right), different set of inputs induce the degradation of TRAF3, which leads to the stabilization of NIK and NIK-IKKα–dependent phosphorylation of p100 bound to RelB. Once phosphorylated the p100 protein is processed by the proteasome, releasing the RelB-p52 dimers, which translocate to the nucleus and bind κB sites. In DC, an additional third pathway in which overexpression of RelB and constitutive processing of p100 results in the formation of RelB-p50 complexes bound mainly by IκBα (and, to a lesser extent, by IκBε) in resting condition has been discovered. These RelB-p50 complexes are regulated by stimuli of the canonical pathway (47).
1.7 IL-6 signaling

IL-6 and IL-6 related cytokines including LIF (Leukemia Inhibitory factor), CNTF (Ciliary Neurotrophic Factor), OSM are a group of pleiotropic cytokines that regulate immunomodulatory activities, inflammation and oncogenesis. These cytokines bind and cause heterodimerisation of individual receptors that include IL-6R, LIFR, CNTFR, OSMR with gp130 receptor subunit (48). This binding of ligand to receptor causes receptor associated tyrosine kinases, Janus kinases (JAK1, JAK2, JAK3 and Tyk2), to phosphorylate tyrosine residue within the cytoplasmic domain of gp130 subunit. This creates a docking site for src-homology-2 containing proteins, including STATS. STAT proteins are then tyrosine phosphorylated by JAKs, which leads to their dimerization and subsequent translocation to the nucleus. Once in the nucleus they recognize and bind to specific DNA elements in the promoter region of target genes (49).

Many receptors with intrinsic tyrosine kinase activity (RTK’s) such as EFG, PDGF and non-RTK’s such as src can also activate STAT proteins (50-52). There are 7 types of STAT proteins: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6 (53). STATs form both homo and heterodimers dependent on ligand types and stimulus strength. All STATS can form homodimers except STAT2. Heterodimers are made of STAT1 and STAT2 as well as STAT1 and STAT3, whereas other STATs can form tetramer or higher ordered structure (53, 54).

STATS can undergo various post-translational modifications including acetylation, phosphorylation, sumoylation, and ubiquitination, which regulates its transcriptional activity as well as biological response. Specifically, serine-727 phosphorylation within the transactivation
domain of STAT1 and STAT3 enhances their transcriptional activity without affecting DNA binding. Once the phosphorylation mark is removed by nuclear phosphatases, such as TC45 in the case of STAT1 and STAT3, it favors the nuclear export of these proteins (55). Not only does JAK-STAT signaling regulate a plethora of physiological functions, but in turn itself it is under tight control of negative regulation at different levels. Several cytoplasmic tyrosine phosphatases such as SH2-containing phosphatases 1 (SHP-1), SHP-2 and protein tyrosine phosphatases 1B (PTP1B) dephosphorylate receptor and kinase phosphorylation sites, which disables the recruitment of SH2 domain containing proteins including STATs (56, 57). Suppressors of cytokine signaling (SOCS) proteins, which are a family of cytoplasmic inhibitors, that bind to the receptor sites and/or JAK catalytic sites to block further STAT activation. These molecules after being induced by cytokines can regulate the receptor protein turnover as well as that of associated molecules via ubiquitin-proteasome mediated degradation (58). In addition, there are some negative regulators of JAK-STAT signaling, such as protein inhibitors of activated stats 1 (PIAS1) and PIAS3. PIAS1 and PIAS3 interacts with tyrosine phosphorylated STAT1 and STAT3, respectively, and block their DNA binding in vitro (53).

1.8 Oncostatin M (OSM)

OSM belongs to IL-6 family of cytokines. It is a secreted glycoprotein, expressed mainly by monocytes, macrophages and microglia, neutrophils, T-cells, osteoblast, dendritic cells and testes.(49). OSM signals through type I (gp130/LIFR), and type II (gp130/OSMRβ) receptor
complexes. OSM activates STAT1, 3, 5 ERK1/2 and p38.
Figure 5 Proposed signaling pathways of OSM. (Top) OSM activation of the JAK/STAT pathway. Binding of OSM to its receptor subunits induces dimerization of receptor subunits, resulting in the phosphorylation and activation of JAKs. The activated JAKs phosphorylate (P) tyrosine residues (Y) on receptor subunits, providing docking sites for STATs. The STATs are recruited to the sites and are tyrosine phosphorylated by JAKs, followed by the homo or hetero dimerization of STATs. Dimerized STATs translocate into the nucleus, and bind to regulatory elements in DNA, and regulate gene expression. This process may be negatively regulated at multiple steps by PIAS and SOCS proteins.

(Bottom) OSM activates the MAPK pathway. OSM can also signal through the MAPK pathway. OSM binding induces tyrosine phosphorylation on gp130, which leads to the recruitment of SHP2 (Src homology-2 domain containing protein tyrosine phosphatase) SHP2 then provides a docking site for the SH2 domain of the adaptor protein Grb2, which is complexed with the GTP-exchange factor SOS. SOS interacts with Ras, and Ras binding recruits Raf. Activated Raf then transmits its signal via the MAPKKK–MAPKK–MAPK cascade, leading to gene expression (49).
OSM is a cytokine with both pro- and anti-inflammatory actions (49). It is associated with host defense, as is expressed by activated T-cells, macrophages and microglia. In the CNS, OSM is detected only in MS and HAD (HIV associated dementia) patients where it localized to activated microglia, hypertrophic astrocytes and infiltrating leukocytes but it is absent in normal brain. OSM stimulates the expression of MMP-1, 3 in astrocytes and IL-6, MCP-1 and TIMP-1 in astrocytes and brain endothelial cells.
Chapter 2

Materials and Methods

2.1 Mice.

The doxycycline (DOX)-inducible, brain-specific HIV-Tat1-86 transgenic mice have been described (59), C57BL/6 mice were obtained from Jackson Laboratory. All mice were housed at Virginia Commonwealth University according to guidelines of the Institutional Animal Care and Use Committee of Virginia Commonwealth University. The mouse protocols were approved by the Institutional Animal Care and Use Committee. Male and female mice 8–16 weeks of age were used. 4-6 weeks old Athymic Nu/Nu were purchased from NCI. The animals were housed for 10 days before utilizing them for experimentation.

2.2 Turpentine-induced inflammation.

Turpentine abscesses were initiated by subcutaneous injection of pure gum spirits of turpentine (50 µl) into anesthetized age-matched male and female mice (6–8 weeks of age). Mice were killed after 8h and the skin and underlying muscles at the site of injection was collected for mRNA analysis.

2.3 Experimental autoimmune encephalomyelitis (EAE).
Each mouse received intradermally 300 µg of MOG_{35-55} peptide (AnaSpec, Fremont, CA) emulsified in CFA containing 500 µg of Mycobacterium tuberculosis H37Ra (Difco, Detroit, MI) and intraperitoneally 200 ng of Pertussis toxin (Enzo Life Sciences, Farmingdale, NY). An additional dose of 200 ng of Pertussis toxin was administered two days post immunization. A booster dose MOG_{35-55}/CFA/Mycobacterium tuberculosis H37Ra was given seven days post immunization. Mice were examined daily (the first day following booster injection was assigned as day 1), weighted, and the severity of the disease was quantitated using a five point scale: 0 – no symptoms, 1 – limp tail, 2 – limp tail with loss of righting, 3 – paralysis of single hind limb, 4 – paralysis of both hind limps, and 5 – death.

2.4 Irradiation

Irradiations were carried out using a MDS Nordion Gamma-cell 40 research irradiator with a $^{137}$Cs source delivering a dose of 1.05 Gy/min to the head only. Mice received a dose of 20 Gy.

2.5 Clinical samples.

Biopsy samples of oligodendroglioma tumors were obtained from VCU’s brain tumor bank (Department of Neurosurgery, Richmond, VA). Normal cortical tissue samples were obtained from healthy brain regions of patients with oligodendroglioma or epileptic patients. The biopsy samples represent primary grade II and III, and recurrent tumors. All of the oligodendroglioma samples expressed significant amounts of myelin basic protein (data not shown), confirming the oligodendroglial origin of these tumors.
2.6 Cell culture.

Human glioblastoma U373-MG cells were obtained from American Type Culture Collection (Manassas, VA). Mouse astrocytes were prepared as described (59) Human cortical astrocytes were prepared from fetal tissue provided by Advanced Bioscience Resources and were cultured as previously described (60, 61). Cells were stimulated with 25 ng/ml IL-1, 25 ng/ml OSM, 25 ng/ml IL-6, or 25 ng/ml sIL-6R (all from R&D Systems, Minneapolis, MN). Cell Culture

The U1242 glioma cells were obtained from Dr. Kristoffer Valerie. The U1242 LG (Luciferase GFP-Green fluorescent protein) cells and variants were produced as described in 2.15. The U1242 and U1242 LG cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, antibiotics, sodium pyruvate, and nonessential amino acids. The lentiviral infected cells, received an additional Puromycin (1µg/ml) in their growth media.

2.7 Knock-down of target genes.

Expression of RelB, cRel, p50, STAT3, p65, YKL-40 and p52 mRNAs was down-regulated using SmartPool siRNAs transfected into astrocytes using Dharmafect 1 (all from Dharmacon, Lafayette, CO).

2.8 Quantitative PCR.

Total RNA was prepared utilizing the TRIzol (Life Technologies, Carlsbad, CA). 1 µg of RNA was reverse-transcribed using the high capacity cDNA Archive kit (Life Technologies).
Premixed primer-probe sets and TaqMan Universal PCR Master Mix (Life Technologies) were employed to examine mRNA levels. cDNAs were diluted 10-fold (for the target genes) or 100-fold (for GAPDH) and amplified using the ABI7900HT cycler. Gene expression levels were normalized to GAPDH and presented as a fold induction with the mean values ± standard deviation. A Power SYBRGreen PCR kit (Life Technologies) was used for chromatin immunoprecipitation qPCR assays as previously described (25).

2.9 Western Blotting.

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 & Schuell, Dassel, Germany). The anti-YKL-40, anti-RelB, anti-p65, anti-p50, anti-β-tubulin, and anti-GAPDH antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and anti-Stat3 antibody was obtained from Cell Signaling Technology (Danvers, MA). Antigen-antibody complexes were visualized by enhanced chemiluminescence using Immobilon Western blotting kit (Millipore, Temecula, CA).

2.10 Coimmunoprecipitation

200–300 μg of protein lysates, prepared as described above, were pre-cleared with 10 μl of the
protein G-Sepharose beads (GE Healthcare, Pittsburgh, PA) for 1h. The lysates were then incubated with 2 μg of anti-RelB or anti-p50 antibodies overnight at 4°C, 25μl of 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.

2.11 Synthetic Oligonucleotides.

The following oligonucleotides were synthesized to introduce mutations in the YKL-40 promoter: -669 NF-κB, 5’-CTGAATTTCGATAGCTGTCTTTCCCTCTAA-3’ and 5’-ACAGCTATCGAATTCAGAAGCAAAAT AG-3’; -717 NF-κB, 5’-ATCTCGAGAATAAACAGAAAGCAAAAT AG-3’ and 5’-TTATTCTCGAGATAAAGAGAGGATCTT-3’.

Following oligonucleotides were used in EMSA: -669 NF-κB probe, 5’-GATCTTTCTTGG GAATTTCCCTGTCA-3’ and 5’-GATCTGACAGGGAAATTTCCCAAGAAA-3’; -717 NF-κB probe, 5’-GATCTCTTTATGGGAATTTCAAAACAA-3’ and 5’-GATCTTTGTTTTGAAA TTCCCATAAAGA-3’.

2.12 Nuclear Extracts and EMSA.

Nuclear extracts were prepared as previously described (62). Double-stranded DNA fragments were labeled by filling in the 5’-protruding ends with Klenow enzyme using [α-32P] dCTP (3000
Ci/mmol). Briefly, 5 µg of nuclear extracts and ∼10 fmol (10,000 cpm) of probe were utilized. Anti-RelB, anti-p50, anti-p65 (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies were used for supershift studies.

**2.13 Chromatin Immunoprecipitation (ChIP) Assay.**

ChIP assays were performed as described previously (25) with the following exceptions. The cells were cross-linked with 1% formaldehyde for 10 min at 37 °C and then washed with ice-cold PBS containing 125 mM glycine. RelB and p50 antibodies or none-immune rabbit serum were used to precipitate cross-linked proteins. The following primers to the YKL-40 promoter were used in the qPCR: 5’-GTGCAGCCGCCGCTAG-3’ and 5’-GCCTGAAACTGAGCGCTCC-3’.

**2.14 Plasmids and transfections.**

The pYKL(-1300)Luc reporter was provided by Dr. Michael Rehli (University of Regensberg, Regensberg, Germany) (11). The site-directed mutagenesis was performed using QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA) and the oligonucleotides described above and mentioned before (25). Plasmids expressing dominant negative IκBα and constitutively active STAT3 were described before (25). Human astrocytes were transiently transfected in 12-well clusters using the FuGENE6 transfection reagent (Roche Applied Science). Twenty four hours post-transfection, the cells were stimulated with IL-1 or OSM for 20h and cell extracts were prepared. Luciferase assays were performed using a dual luciferase reporter assay kit.
Luciferase activities were normalized to Renilla luciferase activities. GIPZ plasmid containing shRNA to YKL-40 was purchased from Thermo-scientific Open Biosystems. The shRNA-YKL-40 was cloned into an inducible TRIPZ vector (inducible RFP expression). The pTRIPZ (Thermo-scientific) has a Tet (Tetracycline/Doxy-cycline) inducible shRNA expression and it permits reversible and regulatable gene knockdown.

2.15 Lentiviral Infection.

The U1242GL cells were obtained by infecting U1242 with infected with lentivirus expressing pTRIPZ which contained either scrambled shRNA or shRNA to YKL-40) packaged in a Lentiviral vector. The cells were then infected with LP-hLuc-lv201 (Genocopia), comprising of a eGFP and PAC (Puromycin resistance) gene. These cells were then selected in media with puromycin (1µg/ml) and were FACS sorted to obtain the two cell population expressing comparable GFP levels.

2.16 Wound Healing Assay.

The cells were plated in 6-well plates (3×10^5 cells/well for U1242 cells to obtain a single confluent monolayer. Twenty-four hours later, media from each well were removed and stored in labeled eppendorf tubes. Subsequently, scratches were made using 20µl sterile pipette tips, followed by washing of the wells either by PBS or fresh media, to remove the cell debris from the scratch. This was followed by adding back the original media from each well. The cells were
then stimulated at different stimulants as described in the figure legend. Pictures of the wounded area (six fields/well) were taken at time points mentioned in the respective figure legends. The number of cells that migrated in the wound area were counted in each field, and the data are presented as the cells migrated per field. The experiments were repeated twice. In the case of siRNA experiments, $2 \times 10^5$ cells/well were plated followed by siRNA treatment for 30 hrs, and subsequently scratches were made.

### 2.17 Transwell Migration Assay.

Transwell migration was measured using a modified Boyden chamber using polycarbonate filters (25×80 mm, 12-μm pore size, Neuro Probe Inc, MD) coated with fibronectin. First the cells were added to the upper chamber at $5 \times 10^4$ cells/well were allowed to migrate. After 8 hrs, the excess cells on the upper membrane surface were removed using cotton swabs, and the cells that migrated through the pores and spread on the lower surface of the filter were fixed with methanol and then stained with Diff-Quik (Fisher). The cells that migrated were counted using an inverted microscope with a 10X objective. Each data point is the average number of cells in six random fields and is the mean ± S.E. of three to six individual wells.

### 2.18 Invasion Assay.

The assays were performed exactly as described above in the Transwell migration assay section the only exception being that the filters were coated with Matrigel and not fibronectin.
2.19 Xenograft.

The stereotactic techniques used to implant glioma cells have been previously described (63). Briefly, cells were trypsinized, the pellet was then re-suspended in Matrigel at 1:1 ratio to obtain $5 \times 10^4$ cells/µl and then 1µl of cells (about $5 \times 10^4$ cells) was implanted in the right striatum of the mouse (seven animals/each group). All procedures have been approved by the Virginia Commonwealth Universities Animal Care and Use Committee protocols. The mice were checked regularly for post-operative morbidity, morbundity and neurological deficits. The tumor-bearing animals were imaged with Caliper IVIS 200Xenogen every week from week 1 to 4 post-implantation and sacrificed 30 days after post-implantation. Bioluminescence imaging (BLI) of the tumors was done using after subcutaneous injection of D-luciferin (Gold Biotechnology) at a concentration of 30 mg/mL in PBS. Post-mortem animals were perfused with 4%PFA. Brains were removed, allowed to sink in sucrrose for 24-48 hours, and embedded in OCT thereafter.

2.20 Immunohistochemistry.

The brain tumor samples were sectioned (6 µm), and stained with haematoxylin and eosin (H&E) at The Tissue and Data and Acquisition and Analysis Core (TDAAC) at VCU. 10 µm sections were used for Immunohistochemistry utilizing anti-RFP (Rockland, Pennsylvania) and Hoechst stain (Life technologies). The slides with brain sections, were fixed with acetone, followed by permeabilization with 1% Triton-X-100/PBS and blocked with 10% normal goat
serum in Triton-X-100/PBS before the exposure to primary antibodies followed by secondary antibodies. The sections were then incubated with anti-RFP overnight (primary). The section were then washed with wash buffer (0.01% Triton-X) (3x,5min). Secondary antibody conjugated to (Alexa-546) was added in wash buffer with 1% goat serum, and incubated at RT for 1 hour, followed by washing. The slides were then, stained with Hoechst nuclear stain for 5 min at RT, followed by 3 more washes, after which they were sealed using a coverslip and vectasheild (Vector Labs, CA). Whole brain (tiled images) were obtained using a Applied Imaging Ariol SL-50 system (Applied Imaging Corp.). All brain tumor sections were analyzed for invasion. The maximum distance between the center of tumor mass and the infiltrating cells (single or cluster cells) at the edge was measured by Image Pro 4.5 as described before (28). The mean of maximum distance was calculated from seven different infiltrating single cells/clusters per animal and seven different xenografts per group.

2.2.1 Statistical analysis.

All experiments were repeated at least three times with consistent results. Data are presented as mean ± SD or SEM (as noted in figure legends). For mouse studies, four to seven randomly chosen mice were used per experimental group. SPSS Statistics 21 software was used for statistical analyses. A Bonferroni post-hoc test was used for one-way ANOVA comparisons, with a P value of <0.05 being considered statistically significant. Independent sample Student's t-test was used for unpaired observations.
Chapter 3

Sterile inflammation-associated expression of YKL-40 is regulated by RelB/p50 complexes

3.1 Abstract

Secreted protein YKL-40 has been proposed as a biomarker of a variety of human diseases characterized by ongoing inflammation, including chronic neurological pathologies such as multiple sclerosis (MS) and Alzheimer’s disease. However, inflammatory mediators and the molecular mechanism responsible for enhanced expression of YKL-40 remained elusive. Using several mouse models of sterile inflammation, we now show that YKL-40 expression correlated with increased expression of both IL-1 and IL-6. Furthermore, IL-1 together with IL-6 or the IL-6 family cytokine oncostatin M (OSM), synergistically up-regulated YKL-40 expression in both primary human and mouse astrocytes \textit{in vitro}. The robust cytokine-driven expression of YKL-40 in astrocytes required both STAT3 and NF-κB binding elements of the YKL-40 promoter. Additionally, YKL-40 expression was enhanced by constitutively active STAT3 and inhibited by dominant-negative IκBα. Surprisingly, cytokine-driven expression of YKL-40 in astrocytes was independent of the p65 subunit of NF-κB and instead required subunits RelB and p50.

Mechanistically, we show that IL-1-induced RelB/p50 complex formation was further promoted by OSM and that these complexes directly bound to the YKL-40 promoter. Moreover, we found that expression of RelB was strongly up-regulated during sterile inflammation \textit{in vivo} and by IL-1.
in astrocytes *in vitro*. We propose that IL-1 and the IL-6 family of cytokines regulate YKL-40 expression during sterile inflammation via both STAT3 and RelB/p50 complexes. These results also indicate that IL-1 regulates the expression of a subset of specific anti-inflammatory genes in non-lymphoid tissues during sterile inflammation via the canonical activation of the RelB/p50 complexes.
3.2 Introduction

In the past several years YKL-40 (also known as chitinase 3-like protein 1, human cartilage glycoprotein 39, and breast regression protein 39) has attracted growing attention as a marker of ongoing inflammation and oncogenic transformation (64). This secreted glycoprotein, expressed in both invertebrates and vertebrates, belongs to the 18-glycosyl-hydrolase family of proteins but lacks glycolytic activity (65, 66). Although its biological functions are not fully understood, it is expressed by many cell types, including macrophages, neutrophils, synoviocytes, chondrocytes, smooth muscle cells, endothelial cells, microglia, and astrocytes, suggesting that its biological effects are not restricted to a particular cell type. Indeed, YKL-40 stimulates proliferation of synoviocytes and chondrocytes (67), promotes adhesion and migration of vascular smooth muscle and endothelial cells (68-70), and is a migration factor for astrocytes (25). Surprisingly, unchallenged YKL-40 knockout mice appear normal; however, when challenged with antigen they display impaired Th2-dependent immune responses, show diminished fibrosis and tissue inflammation, decreased accumulation of M2 macrophages, and increased apoptosis of CD4+ T cells and macrophages (14). In contrast, these mice display an exacerbated response to experimental autoimmune encephalomyelitis (EAE) (71) and enhanced inflammatory responses to hyperoxia (72), suggesting that YKL-40 differentially affects inflammatory responses depending on the type of immune activation and tissue involved. In agreement with the role of YKL-40 during inflammatory responses in mice, YKL-40 levels are elevated in patients with a wide array of human diseases characterized by ongoing inflammation, including rheumatoid arthritis, atherosclerosis, type 2 diabetes, pelvic inflammatory disease,
chronic pancreatitis and secondary diabetes, severe pediatric asthma, cirrhosis, Crohn’s disease, and others (73-80). Elevated levels of YKL-40 are also present in the cerebrospinal fluids of patients with a variety of acute and chronic neurological pathologies, such as MS, Alzheimer disease, viral encephalitis, HIV-associated dementia, brain infarction, and traumatic brain injury (22-24, 81), with activated microglia and reactive astrocytes both producing YKL-40 in the central nervous system. Thus, over the last decade it became evident that elevated YKL-40 expression correlates with both infection-induced inflammation and sterile inflammation, a paradigm triggered by physical, chemical or metabolic noxia. Accordingly, YKL-40 is also expressed by several solid tumors, such as osteosarcoma, ovarian carcinoma and glioblastoma multiforme (GBM) (9, 10), and promotes angiogenesis and radioresistance of GBM tumors and angiogenesis of breast and colon cancer cells (18, 82). Since elevated expression of YKL-40 correlates with ongoing inflammation that is a component of many diseases, YKL-40 has been proposed as a biomarker for many pathologies, including cardiovascular disease, asthma, arthritis, MS, Alzheimer disease, and many cancers, including GBM, melanoma, hepatocellular carcinoma, breast and pancreatic cancers (10, 81).

Despite the numerous reports documenting elevated expression of YKL-40, relatively little is known about the inflammatory mediators and specific molecular mechanisms that control its expression. Pro-inflammatory cytokines, including TNF and IL-1, induce expression of YKL-40 in chondrocytes (13) and astrocytes (25); however, to a much lower extent than the conditioned media of macrophages (83). Both IL-1 and TNF are known to trigger a classical IκB
kinase (IKK)γ-dependent activation of NF-κB, which involves IKKβ-mediated phosphorylation and subsequent degradation of IκBs, followed by the release and subsequent nuclear translocation of the p65/p50 NF-κB complexes (84). In contrast, these cytokines do not activate a non-canonical IKKγ-independent NF-κB pathway, which involves NF-κB-inducing kinase (NIK), IKKα-dependent processing of NF-κB2 p100, and generation of RelB/p52 complexes (85-87). Concordantly, the p65/p50 complex has been proposed to mediate TNF- and IL-1-induced YKL-40 expression in chondrocytes (13). In contrast however, p65/p50 has also been shown to recruit histone deacetylases-1 and -2 to the YKL-40 promoter and repress its expression in response to TNF in GBM cells (15). In addition, IL-6 and OSM moderately upregulate YKL-40 expression in human astrocytes, which requires STAT3 and formation of a complex between STAT3 and nuclear factor I-X3 on the YKL-40 promoter (25). However, profound activation of YKL-40 expression observed during ongoing inflammatory processes in vivo has not been adequately recapitulated in the in vitro experiments. Therefore in this study, we set up experiments to identify molecular mechanisms that govern YKL-40 expression during sterile inflammation.
3.3 Results

3.3.1 Sterile inflammation induces YKL-40, IL-1 and IL-6 expression.

To model the induction of YKL-40 expression during sterile inflammation, we first employed a turpentine mouse model of irritant-induced acute inflammation, which is IL-1R-dependent (88). Turpentine causes tissue destruction at the site of injection, local cytokine production, and infiltration of pro-inflammatory cells (61). The local reaction is subsequently followed by a systemic response, including the production of acute phase proteins in the liver (89). Indeed, YKL-40 mRNA expression was robustly activated in tissues surrounding the site of turpentine injection and its induction was accompanied by a very strong induction of both IL-1 and IL-6 mRNAs (Fig. 6). To corroborate these findings in the central nervous system model of sterile inflammation, expression of YKL-40, IL-1, and IL-6 was subsequently analyzed using a mouse EAE model of MS. Immunization of mice with MOG35-50 peptide resulted in the induction of EAE, and modest upregulation of YKL-40 expression in the spinal cords of animals (Fig. 7). The magnitude of YKL-40 induction was comparable to the results previously reported by others (71). In addition, expression of both IL-1 and IL-6 was also enhanced. We also found an increase in the expression of glial fibrillary acidic protein (GFAP), which indicated activation of astrocytes in EAE as reported previously (90). Subsequently, we analyzed expression of YKL-40, IL-1 and IL-6 in the brains of mice exposed to gamma irradiation, which is known to induce inflammation (91). In agreement with the turpentine and EAE data, levels of YKL-40, IL-1, and
IL-6 mRNAs were significantly higher in the irradiated brains (Fig. 8). We also used the HIV transactivator of transcription (TAT) transgenic mouse model in which induction of TAT protein expression in the brain induces local sterile inflammation and enhances expression of IL-1 and IL-6 (92). Using the TAT model, we found that YKL-40 expression was also enhanced upon activation of TAT expression (Fig. 9). Lastly, we analyzed samples of human oligodendroglioma tumors and found that the expression of YKL-40, IL-1, and IL-6 was strongly induced in comparison to normal brain (Fig. 10). We conclude that YKL-40 expression is enhanced during sterile inflammation, which coincides with the increased expression of IL-1 and IL-6.
Figure 6 Turpentine associated Sterile inflammation induces YKL-40, IL-1 and IL-6 expression. Mice were injected s.c. with 50 µl turpentine or PBS (n=6). Tissue at the site of injection was collected after 8h and the expression of YKL-40, IL-1 and IL-6 mRNA was analyzed by qPCR.
Figure 7 YKL-40, IL-1, IL-6 and GFAP expression is up-regulated during EAE. EAE was induced in mice (n=6) and scored as described in methods section (left panel). The expression of YKL-40, IL-1, IL-6 and GFAP mRNA was analyzed in spinal cords by qPCR (right panels).
Figure 8 YKL-40 expression is up-regulated in the brain in response to irradiation.

Expression of YKL-40, IL-1 and IL-6 was analyzed in the hippocampus of control (CON) or irradiated (IR) mice after 24 h.
Figure 9 YKL-40 expression is up-regulated in HIV-TAT transgenic mice. Tat expression was induced by DOX in Tat transgenic mice and the expression of YKL-40 mRNA was analyzed by qPCR.
Figure 10 YKL-40, IL-1, and IL-6 expression is up-regulated in Oligodendroglioma patients. Expression of YKL-40, IL-1 and IL-6 mRNA was analyzed by qPCR in biopsies of oligodendroglioma tumors (OD) and healthy brain tissue. Median values are indicated.
3.3.2. IL-1 and OSM synergistically induce YKL-40 expression in astrocytes. In the nervous system, both astrocytes and microglia express YKL-40 (24, 25) and these cell types also respond to a broad range of inflammatory stimuli (93). We used astrocytes as model cells to study activation of YKL-40 expression during sterile inflammation. Primary human astrocytes were stimulated with IL-1 and IL-6 together with soluble IL-6 receptor (sIL-6R) (due to the fact that human fetal astrocytes express limited amount of IL-6R (60)). We also included OSM, a member of IL-6 cytokine family that signals via OSM receptors abundantly expressed by astrocytes. Although IL-1, IL-6/sIL-6R, or OSM alone only moderately activated YKL-40 mRNA expression, stimulation of astrocytes with IL-1 together with OSM resulted in synergistic activation (Fig. 2A). Since IL-1-treated astrocytes express IL-6, their costimulation with IL-1 and sIL-6R also caused synergistic induction of YKL-40 expression; however, to less extent than OSM alone. Similar effects of IL-1 and OSM on YKL-40 expression were also observed at the protein level (Fig. 11A, insert) and in mouse primary astrocytes (Fig. 11B). These data together with our in vivo studies suggest that YKL-40 expression is coordinately regulated by IL-1 and cytokines of the IL-6 cytokine family during sterile inflammation.
Figure 11 Pro-inflammatory cytokines induce YKL-40 expression in astrocytes. Primary human (A) and mouse (B) astrocytes were stimulated with IL-1, IL-6, OSM and sIL-6R for 18h and expression of YKL-40 mRNA was analyzed by qPCR. Secreted YKL-40 was analyzed by western blotting in the culture medium (insert). ***P < 0.001 (one-way ANOVA).
3.3.3. STAT3 but not p65 (NF-κB) regulate cytokine-induced YKL-40 expression. NF-κB and STAT3 are the major transcription factors activated by IL-1 and OSM, respectively (84, 94). Since p65 subunit of NF-κB and STAT3 were previously implicated in cytokine-induced expression of YKL-40 (15, 25), their expression was knocked down in human astrocytes. Downregulation of STAT3 (Fig. 3C) dramatically diminished IL-1/OSM-induced YKL-40 expression (Fig. 12A). Surprisingly, knockdown of p65 had no effect on YKL-40 expression (Fig. 12A), but did drastically diminish the expression of IL-8, which is p65-dependent (Fig. 12B). To identify the mechanism of cytokine-induced YKL-40 expression, putative NF-κB, STAT3 and activatory protein 1 (AP-1) binding sites of the YKL-40 promoter were mutated and the generated reporters were analyzed in astrocytes (Fig. 12D). Although cytokine-driven expression of YKL-40 is p65-independent (Fig. 12A), mutation of the proximal NF-κB site (-669 to -660 bp) alone substantially diminished activation of the reporter, whereas mutation of the distal NF-κB site (-717 to -708 bp) alone had no effect (Fig. 12D). In addition, mutation of the STAT3 and AP-1 sites also drastically diminished reporter activity. In another approach, we overexpressed dominant-negative IκBα and constitutively active STAT3 and analyzed expression of the YKL-40 reporter (Fig. 12E). Constitutively active STAT3 enhanced, whereas dominant-negative IκBα diminished cytokine-responsiveness. Collectively, these results suggest that the proximal NF-κB and STAT3 elements of the YKL-40 promoter are required for full responsiveness to IL-1 and OSM; however and in contrast to previous reports (15), p65 is dispensable for this response.
Figure 12 Induction of YKL-40 expression by IL-1 and OSM is STAT3-dependent but p65-independent. Human astrocytes were transfected with the indicated siRNAs. Forty hours post transfection, cells were stimulated with IL-1 and OSM for 18 hours and expression of YKL-40 (A), IL-8 (B), and STAT3 (C) was analyzed by qPCR. (D) Astrocytes were transfected with the indicated reporters, stimulated with IL-1 and OSM for 18 hours, and luciferase and renilla activities were determined. Data are presented in comparison to the induced wild-type reporter (set as 100%). (E) Astrocytes were transfected with pYKL(-1300)Luc and plasmids expressing either vector, dominant-negative IκB (IκBSR), or constitutively-active (CA-STAT3). Cells were stimulated with IL-1 and OSM for 18 hours and processed as described in D. **P < 0.01 and ***P < 0.001 (one-way ANOVA).
3.3.4. **RelB and p50 regulate cytokine-induced YKL-40 expression.** To determine which component of the NF-κB complex regulates cytokine-driven YKL-40 expression, expression of p65, cRel, RelB, p50 and p52 was effectively knocked-down in astrocytes (Fig. 13B). Knockdown of either RelB or p50 significantly diminished cytokine-induced YKL-40 expression, whereas knockdown of p65, cRel and p52 had no effect (Fig. 13A). This finding implicates both RelB and p50 in YKL-40 regulation. Interestingly, although human astrocytes constitutively express low levels of RelB (Fig. 13B), IL-1 induced dramatic RelB protein accumulation in these cells (Fig. 14A). Similarly, RelB mRNA expression was also up-regulated by IL-1 in mouse astrocytes (Fig. 14B). RelB expression was also strongly activated in vivo by turpentine in an IL-1-dependent model of irritant-induced sterile inflammation (Fig. 14C). Cumulatively, these data suggest that increased expression of RelB may drive cytokine-mediated YKL-40 expression *in vitro* and *in vivo.*
Figure 13 Cytokine-induced RelB/p50 complexes regulate YKL-40 expression. Human astrocytes were transfected with the indicated siRNAs. Forty hours post transfection, cells were stimulated with IL-1 and OSM for 18 hours. Expression of YKL-40 (A) and p52 (B, bottom panel) was analyzed by qPCR. Expression of p65, RelB, p50 and β-tubulin was analyzed by western blotting (B, left panel).
Figure 14 Pro-inflammatory cytokines induce RelB expression. (A) Human astrocytes were stimulated with IL-1 and OSM for the indicated times and expression of RelB and GAPDH was analyzed by western blotting. (B) Mouse astrocytes were stimulated with IL-1 and OSM for 18 hours and expression of RelB was analyzed by qPCR. (C) Mice were injected i.c. with 50 µl turpentine or PBS (n=6). Tissue at the site of injection was collected after 24h and the expression of RelB mRNA was analyzed by qPCR. **P < 0.01 and ***P < 0.001 (one-way ANOVA).
3.3.5. RelB/p50 complexes bind to the YKL-40 promoter. To determine whether RelB regulates YKL-40 expression directly or indirectly, binding of RelB and p50 to the YKL-40 promoter was analyzed by ChIP (Fig. 16B). These experiments were performed in U373 glioma cells, which similarly to human and mouse astrocytes, up-regulate expression of both YKL-40 (Fig. 15A) and RelB (Fig 16B) in response to IL-1 and OSM, and this cytokine-induced expression is diminished by the knockdown of p50 and RelB. Although RelB and p50 did not bind to the YKL-40 promoter in unstimulated U373 cells, binding of RelB and p50 was apparent in cytokine-treated cells (Fig. 16B), suggesting that RelB/p50 complexes directly regulate YKL-40 expression. To determine if IL-1/OSM-treatment induces RelB/p50 binding to the previously identified NF-κB sites, EMSAs were performed using oligonucleotides containing distal and proximal NF-κB sites. In agreement with the mutational analysis that showed the importance of the proximal NF-κB site (Fig. 12D), strong protein binding to the proximal but not distal NF-κB site of the YKL-40 promoter was induced by IL-1 alone or together with OSM (Fig. 16C). Cytokine-induced proteins that bound to the proximal NF-κB element in vitro included complexes that were supershifted with anti-p65, anti-RelB and anti-p50 antibodies (Fig. 16D), indicating that the proximal NF-κB site can bind both p65/p50 and RelB/p50 complexes. We conclude that RelB/p50 complexes can directly bind to the proximal NF-κB site of the YKL-40 promoter.
Figure 15 Analysis of YKL-40 expression in U373 glioma cells. U373 were stimulated with IL-1 and OSM for 18h and expression of YKL-40 mRNA was analyzed by qPCR (A) and RelB protein expression by western blotting (C). (B) U373 cells were transfected with the indicated siRNAs. Forty hours post transfection, cells were stimulated with IL-1 and OSM for 18 hours. Expression of YKL-40 was analyzed by qPCR.
A

B

C

D

YKL-40 promoter

Normalized binding

NRS  p50  RelB

con  IL-1  OSM  IL-1 + OSM

***  *  

-669 NF-κB  -717 NF-κB

+  +  +  +  IL-1

+  +  +  +  OSM

-669 NF-κB

p65/p50  RelB/p50

IL-1 + OSM
Figure 16 RelB/p50 complexes bind at the distal NF-κB site of YKL-40 promoter. (A) YKL-40 promoter. NF-κB sites are indicated by grey boxes. Positions of ChIP primers (ChIP) and probes used in EMSA (-717 and -669 probe) are indicated. (B) ChIP was performed using chromatin prepared from U373 cells treated with IL-1 and OSM for 2h. Binding of p50 and RelB to the YKL-40 promoter was analyzed using the antibodies described in the experimental procedures. NRS indicates normal rabbit serum used for immunoprecipitation. Results are shown as normalized binding (binding of NRS-immunoprecipitated untreated samples were set as 1). Experiments were performed three times. *P < 0.05 and ***P < 0.001 (two-way ANOVA). (C-D) Nuclear extracts were prepared from U373 and glioma cells stimulated with IL-1 and OSM for 8 hours. The binding was then analyzed by EMSA using the 32P-labeled oligonucleotide probes derived from the 5’ flanking region of the YKL-40 (-717 NF-κB and -669 NF-κB, as indicated). (C) Binding to the -717 NF-κB and -669 NF-κB elements in U373 glioma cells. (D) Binding to the -669 NF-κB probe in U373 cells. Specific antibodies or NRS were added to the binding reaction.
3.3.6. OSM enhances IL-1-induced RelB/p50 heterodimer formation. OSM efficiently regulated YKL-40 expression via STAT3 (Fig. 12) and also promoted the recruitment of p50 to the YKL-40 promoter (Fig. 16). Since YKL-40 expression depends on RelB/p50 (Fig. 13), we subsequently asked whether OSM promotes formation of IL-1-induced RelB/p50 heterodimer. RelB and p50 were immunoprecipitated from cytokine-treated cells and analyzed for the presence of co-immunoprecipitated RelB and p50 (Fig. 17). Indeed, p50/RelB complexes were formed in response to IL-1. More importantly, OSM significantly enhanced IL-1-induced formation of p50/RelB complexes. These data suggest that in addition to STAT3 activation, OSM enhances YKL-40 expression by promoting formation of p50/RelB complexes, which bind to the YKL-40 promoter.
**Figure 17 OSM enhances IL-1-induced RelB/p50 heterodimer formation.** Human astrocytes were stimulated with IL-1 or OSM for 8 hours, p50 or RelB were immunoprecipitated, and co-immunoprecipitated p50 and RelB were detected by western blotting, as indicated. Expression of RelB and p50 in the lysates is shown the lower panels (lysate).
3.4 Discussion

Although YKL-40 is upregulated in a broad range of human diseases associated with ongoing sterile inflammation, its biological functions still remain elusive. Nevertheless, exacerbated EAE (71) and enhanced responses to hyperoxia (72) in YKL-40 knock-out mice suggest that cytokine-induced YKL-40 is needed for the proper resolution of inflammation. Once inflammation is resolved, YKL-40 expression ceases. In contrast, chronic inflammation leads to continuous aberrant upregulation of YKL-40 expression, which is continuously driven by pro-inflammatory cytokines. Our data from the turpentine, TAT-overexpression, and EAE mouse models together with human oligodendroglioma data, demonstrate that YKL-40 expression correlates with the expression of both IL-1\(\beta\) and IL-6. Importantly, IL-1\(\alpha\) and IL-1\(\beta\) are both critical initiators of sterile inflammation released in response to broad range of danger associated molecular patterns. IL-1\(\beta\) is processed from its inactive precursor in response to inflammasome activation and subsequently secreted from activated cells, while active intracellular IL-1\(\alpha\), which does not require processing, is liberated from damaged cells (95). Since IL-1 efficiently activates expression of IL-6 in many cell types (96, 97), it is not surprising that these two cytokines are found at the sites of local inflammation. The synergistic upregulation of YKL-40 expression by IL-1 and IL-6 (or OSM) in \textit{in vitro} experiments therefore likely recapitulates the regulation of YKL-40 \textit{in vivo}. Similarly to the regulation of YKL-40, IL-1 and IL-6 (or OSM) are known to control expression of acute phase proteins in the liver, which are released to the blood to limit
inflammation-associated damage and allow for the return to homeostasis. Thus, acute cytokine-driven expression of YKL-40 is likely highly beneficial and allows for proper resolution of inflammation. Nevertheless the mechanism by which YKL-40 limits inflammation remains elusive due to limited understanding of YKL-40 receptors and their signaling. In fact YKL-40 induces the interaction of α₃β₃ integrins with syndecan-1 in endothelial cells (82), it activates ERK, AKT, and Wnt/β-catenin signaling in macrophages via IL-13 receptor alpha 2-dependent mechanism (20), and activates ERK, AKT, and p38 via protease activated receptor 2 in bronchial smooth muscle cells (98). It remains to be established whether these proposed receptors/mechanisms are important for the biological functions of YKL-40 which are associated with inflammation. However, in agreement with the proposed role of YKL-40 in limiting inflammation, it has recently been shown that YKL-40 also inhibits NF-κB activation and expression of IL-6, IL-8 and MCP-1 by skeletal muscle cells by a protease activated receptor 2-dependent mechanism (99).

It is generally accepted that IL-1 triggers a classical NF-κB pathway in many cell types, which leads to the induction of p65/p50 target genes, including those encoding pro-inflammatory cytokines such as IL-8 and IL-6 (100). It is also accepted that RelB is activated in lymphoid cells, such as dendritic cells, by a non-canonical NF-κB pathway and that generation of RelB/p52 complexes that are important for proper dendritic cell functions (101). This non-canonical NF-κB activation pathway is activated by ligands such as CD40 and lymphotoxin-β (47) but cannot be activated by IL-1. However, it has recently been recognized that in dendritic cells, RelB can also
be activated by a canonical pathway in response to TNF, LPS and CpG, and this activation results in RelB/p50 complex formation (47). This canonical RelB activation depends on TRAF6 (102), which is further supported by findings that the RelB/p50 complexes are bound to IκBα and IκBe in the cytoplasm (47). It has been also suggested that canonical activation of RelB in dendritic cells is possible because of a higher level of RelB expression in these cells and the constitutive activation of non-canonical pathway (47). The physiological importance of the RelB/p50 complexes is strongly supported by a more deleterious phenotype of RelB-deficient than the NIK-deficient mice (103), suggesting that RelB has NIK-independent functions that likely can be attributed to RelB/p50 complexes. Nevertheless, the mechanism of specific RelB/p50-dependent gene regulation is not clear since RelB/p50 and p65/p50 complexes bind the same regulatory sequences.

Although existence of RelB/p50 complexes in non-lymphoid tissues has been shown more than a decade ago (87), the function of these complexes remained elusive. Our data clearly demonstrate that RelB expression is strongly induced during sterile inflammation in vivo, and this induction is likely mediated by IL-1. IL-1 also strongly activates expression of RelB in astrocytes in vitro. RelB subsequently forms complexes with p50 and activation of these complexes is controlled by IκBα. Once the RelB/p50 complexes are activated, they regulate expression of YKL-40 and likely other RelB/p50-responsive genes. Thus, it appears that RelB/p50 complexes may play critical role(s) during sterile inflammation that normally leads to
a return to homeostasis. However, chronic inflammation may also lead to pathological RelB/p50-dependent gene expression. Indeed, YKL-40 has recently been proposed as a marker of a particularly aggressive mesenchymal subtype of GBM (104). In agreement with our current findings indicating that RelB regulates YKL-40 expression, RelB is strongly expressed in mesenchymal subtype of GBM and promotes expression of mesenchymal genes, including YKL-40 (104). Furthermore, loss of RelB expression attenuates GBM cell survival, motility and invasion (105).

Our previous and current data suggest that the robust activation of YKL-40 expression requires both STAT3 and RelB/p50 binding to the YKL-40 promoter. Surprisingly, OSM also promoted formation of the RelB/p50 complexes by the mechanism which currently remains unclear but these complexes do not contain STAT3 (data not shown).

In summary, we propose that sterile inflammation leads to the expression of YKL-40, which is induced by IL-1 and cytokines of the IL-6 family. Mechanistically, both STAT3 and the RelB/p50 complexes drive the expression of YKL-40 via the elements within the YKL-40 promoter.
Figure 18 Proposed model of regulation of YKL-40 expression in astrocytes during sterile inflammation. During sterile inflammation, there is an up-regulation of inflammatory cytokines as well as an up-regulation of YKL-40 expression. We propose that IL-1 and OSM, a IL-6 family cytokine, can up-regulate YKL-40 expression in astrocytes. This up-regulation is mediated by the NF-κB and the JAK-STAT pathway. IL-1 leads to an up-regulation of expression of RelB, and p50. Concurrently, OSM activates the JAK-STAT pathway leading to the phosphorylation of STAT3. In addition, OSM enhances the binding of RelB to and p50 and this RelB/p50 heterodimer and STAT3 homodimer translocate to the nucleus and bind to the YKL-40 promoter, enhancing gene expression.
Chapter 4:

Role of YKL-40 in glioblastoma in vivo

4.1 Abstract

Glioblastoma multiforme (GBM) is the most common type of primary brain tumor, with a 5-year survival rate of less than 3%. A unique clinical problem in treating GBM is the extensive invasiveness of GBM cells. Due to the invasive phenotype and diffuse penetration of GBM into normal regions of the brain, standard treatments such as surgery and radiotherapy are ineffective, resulting in high rate of relapse and mortality. The high rate of tumor relapse points towards the need for more targeted therapeutic approaches. Recently, we reported that YKL-40 is secreted by primary human astrocytes and its levels are highly up-regulated in GBM cells (25). Although YKL-40 increases migration, invasion, growth, and survival of GBM cells in vitro (25, 106), the effect of YKL-40 in vivo on glioma tumor growth and invasion in the brain has not been established. We generated human glioma cells expressing inducible shRNA to YKL-40 to examine the role of GBM tumors in vivo. Tumor growth and invasion was analyzed after the down regulation of YKL-40 in the tumors grown intracranially. Our results show that although YKL-40 regulates invasion in vitro, does not alter tumor growth and invasion in vivo.
4.2 Introduction

Glioblastoma is the most common primary brain tumor in adults with a short survival period. The median survival time of glioblastoma patients is approximately 12 months, despite various treatments including surgery and radiotherapy. Even after surgical removal of the tumor, there is a high relapse and recurrence of the tumor. This is due to highly infiltrative nature of the tumor. In fact, invasion is the main cause for treatment failure. Some of the invasive cells that infiltrate the area surrounding the tumor can proliferate even after surgical removal of the bulk tumor causing relapse.

Rho family GTPases mediate key signaling elements that module GBM cell invasion. These GTPases regulate cell morphology as well as actin dynamics to stimulate cell squeezing through the narrow extracellular spaces that are typical of the brain parenchyma (107). The invasion phenomenon also depends on the activation or inhibition of proteinases and their inhibitors. These processes include two main proteolytic systems: the plasminogen activator system (PAS), which controls the activation of the proteinase plasmin from inactive plasminogen, and the matrix metalloproteinase (MMPs) and their inhibitors. The ECM of the brain parenchyma is modified by the GBM cells to facilitate migration and invasion. MMPs (especially MMP-2, MMP-9 and MT1-MMP) are overexpressed in GBM patients, which facilitate invasion by cleaving ECM components. Concordantly, down-regulation of MMP-9 decreases in vivo invasion and cell motility (28).

YKL-40 is a member of glycosyl hydrolase family 18, which can bind to chitin but lacks
catalytic activity. It has been shown to be a marker of chronic inflammation and oncogenic
transformation (64). It promotes adhesion and migration of vascular smooth muscle cells, and
acts as chemotactic agent for HUVECs. YKL-40 is highly up-regulated in various cancers
including breast, colorectal, ovarian, prostate, lung and glioblastoma (12, 16, 108-111). In GBM
patients, YKL-40 levels positively correlate with higher tumor grade, high relapse and poor
survival (12, 112-115). In addition, YKL-40 was identified as one of the most overexpressed
genes in glioblastoma (12, 17, 116-118). Overexpression of YKL-40 in transformed astrocytes
leads to expression of genes conferring radiation resistance and increased invasion. Furthermore,
GBM cells show enhanced migration and invasion in response to elevated YKL-40 \textit{in vitro} (25).
YKL-40 also induces VEGF expression in GBM cell lines, promoting angiogenesis and
radioresistance of GBM tumors (82). Administration of YKL-40 antibodies have been shown to
block growth, angiogenesis, and progression of xenografted tumors in flanks of mice (119).
Inflammation is associated with cancer incidence and actively promotes tumor progression (120).
The expression of pro-inflammatory cytokines including IL-1, IL-6, OSM, and TNFα as well as
prostaglandins are highly up-regulated in cancer cells as well as cancer associated inflammatory
cells (121-123). In addition, GBMs have higher expression levels of IL-6 and OSM leading to
constant activation of STAT3 signaling (124). GBM have also been shown to secrete
substantial amounts of IL-1, which results in the secretion of other cytokines, such as IL-6 and
IL-8, and promotes GBM proliferation. We have recently shown that YKL-40 expression is up-
regulated by IL-1 and OSM during sterile inflammation.
Collectively, these observations suggest that YKL-40 is a key mediator in GBM progression. However, the \textit{in vivo} function of YKL-40 in GBM is not well understood. In this study, we evaluate the role of YKL-40 in an invasive GBM model \textit{in vivo}. We found that YKL-40 expression is up-regulated in the presence of IL-1 and OSM in GBM cells. As reported before, YKL-40 effects the invasion and migration of GBM cells \textit{in vitro}; however it does not affect tumor growth or invasion \textit{in vivo}. 
4.3 Results

4.3.1. Gene expression profile of U1242 glioma cells: Invasiveness is a characteristic phenotype of human glioma, which is rarely seen in human xenografts established from tissue culture cell line, and is propagated as orthotropic tumor in mice. The U1242 glioma cell line is unique as it has invasive and aggressive phenotypes when grown in nude mice (28). We here characterized the expression profile of these cells as compared to U373, a non-invasive glioma cells commonly used and primary human astrocytes. We observed that U1242 cells express lower levels of GLAST (Glutamate Aspartate transporter), NESTIN, GFAP and CD44 but comparable levels of YKL-40 mRNA as compared to U373 glioma cells.
Figure 19 Gene expression profile of U1242 cells. RNA was isolated from U1242, U373 cells and astrocytes. Taqman qPCR was performed for NESTIN, CD44, YKL-40, GFAP and GLAST mRNA was normalized to expression of GAPDH.
4.3.2. YKL-40 controls migration and invasion of U1242 glioma cells *in vitro*. High expression of YKL-40 is associated with invasive GBM tumors (114). We have previously shown that YKL-40 promotes the migration of astrocytes and in vitro invasion of U373 glioma cells which are not invasive *in vivo*. We evaluated whether YKL-40 also regulates *in vitro* migration and invasion of U1242 glioma cells. The migration of U1242 cells was reduced by 50% when YKL-40 was downregulated (Fig 20A). Conversely, migration of U1242 cells was markedly increased in response to exogenous YKL-40 (Fig 20B). Similarly, invasion of U1242 cell was decreased by down-regulation of YKL-40 expression (Fig 21A) and increased in response to exogenous YKL-40 (Fig 21B).
Figure 20 YKL-40 controls glioma cell migration in vitro. (A) Transwell migration assay was done in U1242 cells transfected with si-Ctr, or si-YKL-40 30 hours post transfection. (B) U1242 cells were stimulated with YKL-40 (200 ng/ml) for 8hrs. Transwell migration assay was done on fibronectin coated membrane.
Figure 21 YKL-40 controls glioma cell invasion. Matrigel invasion assay was done with U1242 cells in Boyden chamber with cells transfected with siCtr or si-YKL-40 (A) or after stimulation of U1242 cells with YKL-40 (200 ng/ml). (B) Cells were allowed to invade through the matrigel for 8 hrs. Cells that invaded into the matrigel were counted from six random fields per experiments (represented as cells invaded per field).
4.3.3. **The expression of YKL-40 is induced by IL-1 and OSM.** GBM associated inflammation, like most cancer associated inflammation leads to an up-regulation of pro-inflammatory cytokine expressed both in infiltrating immune cells as well as resident tumor cells. YKL-40 expression has been shown to increase in response to IL-1 and OSM, a IL-6 family cytokine. To this end, we stimulated U1242 cells with IL-1 and OSM. Expression of YKL-40 mRNA was activated 14 fold in the presence of IL-1 and OSM (Fig 22A). Furthermore, we analyzed the migration and invasion of U1242 cells in the presence of IL-1 and OSM. We found that IL-1 and OSM enhance the migration and invasion of U1242 cells (Fig 22B,C).
Figure 22 IL-1 and OSM enhance YKL-40 expression, migration and invasion of U1242 cells. U1242 cells were stimulated with IL-1 and OSM for 20 hrs, followed by analysis of YKL-40 mRNA expression by qPCR, normalized to GAPDH (A). Transwell migration (B) and invasion (C) was analyzed as described before in the presence of IL-1 and OSM.
4.3.4. **Inducible down-regulation of YKL-40 in U1242 cells.** We constructed an inducible system, where YKL-40 expression could be knocked-down in response to doxycycline in U1242 glioma cells. We utilized the TRIPZ vector, which has a Tet-inducible shRNA expression (Fig 23). The Tet-inducible promoter is upstream of the shRNA, RFP and puromycin resistance genes. Therefore, the induction of this vector by doxycycline/tetracycline induces the expression of RFP in these cells, which is shown in Fig 24A and Fig 25A. The cells once infected, and then selected by puromycin, were assessed for the down-regulation of YKL-40 expression. Unstimulated U1242 produce low levels of YKL-40, making assessment of down-regulation rather difficult. Therefore, to better visualize the down-regulation of YKL-40, we utilized cytokine stimulated U1242 cells. Once stimulated U1242 cells showed an enhanced expression of YKL-40 (7.5 fold), which was downregulated in the presence of doxycycline (70% downregulation) (Fig 25). In addition we analyzed the migration (Fig 26) and invasion (Fig 27) of the U1242 shCtr (U1242 with scrambled shRNA) and U1242 shYKL-40 (U1242 with shRNA to YKL-40) cells after induction by IL-1 and OSM, followed by down-regulation of YKL-40 expression by doxycycline. We observed a decrease in migration and invasion of U1242shYKL-40 cells in response to YKL-40 down-regulation.
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Figure 23 Generation of U1242 cells with inducible knock down of YKL-40. (Top)

Experimental design to construct an inducible knockdown of YKL-40 is shown above. The U1242 cells were first infected with lentivirus to produce U1242 cells expressing inducible shCtr and shYKL-40. This was followed by infection of U1242 shCtr and U1242 shYKL-40 with lentivirus containing GFP and luciferase describe materials and methods. (Bottom) Schematic representation of TRIPZ lentivirus vector utilized to create stable cells with inducible knockdown of YKL-40.
A

TRE

\[ \text{tRFP} \quad \text{shYKL-40} \quad \text{Puro} \]

B

\[ \text{U1242-luc-GFP cells} \quad \text{+ Doxycycline} \quad \text{U1242-luc-GFP cells} \quad \text{+RFP shYKL activation} \]
Figure 24 Induction of RFP U1242 shCtr and U1242shYKL-40 cells. (A) A schematic diagram showing the position of RFP, shRNA and puromycin resistance gene down stream of tetracycline inducible promoter in the TRIPZ vector. (B) The cells were plated, treated with doxycycline (1µg/ml) for 48 hours, and RFP expression was visualized using microscope.
Figure 25 Knock-down of YKL-40 in U1242 stable cells. U1242GL shCtr and U1242 shYKL-40 cells were treated with doxycycline (1µg/ml) for 50 hours, with intermittent treatment with IL-1 and OSM after 30 hours for a time period of 20 hours. YKL-40 mRNA expression was analyzed by qPCR.
Figure 26 Down-regulation of YKL-40 decreases the migration of U1242 stable cells. U1242 shCtr and U1242shYKL-40 cells were treated with doxycycline for 50 hours, with intermittent treatment with IL-1 and OSM after 30 hours for a time period of 20 hours. Migration of these cells was analyzed using Boyden chamber as described in materials and methods.
Figure 27 Down-regulation of YKL-40 decreases the invasion of U1242 stable cells. U1242 shCtr and U1242 shYKL-40 cells were induced with doxycycline for 50 hours, with intermittent treatment with IL-1 and OSM after 30 hours for a time period of 20 hours. Invasion of these cells was analyzed using Boyden chamber as described in materials and methods.
4.3.5 GFP expression in U1242GL shCtr and shYKL-40. In order to perform in vivo experiments using U1242GL cells, we sorted U1242GL shCtr and U1242shYKL-40 to obtain cell populations with similar GFP and luciferase expression. First, cells were FACS sorted for the expression of GFP (Fig 28A) and populations expressing similar levels of GFP were used for further experiments. Subsequently, U1242GL shCtr (Fig 28A) and U1242GL shYKL-40 (Fig 28B) cells were analyzed for the expression of luciferase. Indeed, these cells expressed similar levels of luciferase (Fig 28C).
Figure 28 GFP and Luciferase expression in U1242GL shCtr and U1242GL shYKL-40 cells. The two cell types were analyzed using FACS. The population of cells expressing high and similar amount of GFP were isolated and utilized for further experimentation. U1242GL shCtr (A) and U1242GL shYKL-40 (B). Additionally, these isolated population of cells were then analyzed using the luciferase assay. Equal number of cells were plated overnight and were lysed using lysis buffer. Substrate with equal protein concentration was utilized for luciferase assay. Luciferase activity was then calculated (C).
Figure 29 In vivo experimental design. To assess the *in vivo* effects of YKL-40, we performed a tumor xenograft study. Athymic female mice were implanted with U1242GL shCtr and U1242GL shYKL-40 cells. The tumor was imaged using a Caliper IVIS-200 at 7, 14, 21, 28 days of tumor implantation. Half the mice from each group (n=7) were fed a doxycycline diet to induce the expression of shRNA control and YKL-40.
4.3.6. **YKL-40 does not affect tumor growth in vivo.** To assess whether tumor derived YKL-40 regulates glioma tumor growth *in vivo*, we implanted U1242GL shCtr and U1242GL shYKL-40 cells in brains of nude mice as described in materials and methods. Tumors were allowed to grow for 7 days and then mice were fed either normal or doxycycline diet to induce shRNA expression. Tumor growth was monitored by measuring luminescence. We found that there was no significant difference in tumor growth between the four groups, suggesting that YKL-40 does not influence tumor growth.
A

Tumor growth

B
**Figure 30 In vivo tumor growth after knock-down of YKL-40 expression.** The mice were imaged at day 7, 14, 21, 28 post tumor implantation using a Caliper-IVIS 200. Representative images are shown in (B). The calculated bioluminescence was used to calculate tumor growth for 4 groups of animals bearing tumors containing, U1242GL shCtr without doxycycline (shctr), U1242GL shCtr with doxycycline (shctr Doxc), U1242GL shYKL-40 without doxycycline (shYKL) and U1242GL with doxycycline (shYKL Doxc)
4.3.7. Down-regulation of YKL-40 in U1242GL cells does not affect their invasion in vivo.

We utilized the tumor xenograft model and immunohistochemistry to analyzed the effect of down-regulation of YKL-40 in GBM cells in vivo. 30 days post-tumor implantation, whole animal perfusion, followed by OCT embedding of brain was performed as described in materials and methods. Tumors from the four groups U1242GL shCtr without doxycycline (shctr), U1242GL shCtr with doxycycline (shctr Doxc), U1242GL shYKL-40 without doxycycline (shYKL) and U1242GL with doxycycline (shYKL Doxc) were sectioned and stained for Hoechst and anti-RFP. Representative images of 10µm sections, stained with Hoechst (nucleus) and anti-RFP (doxycycline induction), followed by tiled whole brain imaging using Ariol microscope are shown in Fig 31A. These images were analyzed for tumor invasion using Image pro4.5 software. The average of 7 points per tumor for 7 different tumors from each group were utilized to assess tumor invasion. The average distance of the center of the tumor to the farthest points within the four groups is depicted in Fig 31B. No significant difference in tumor invasion was observed within the four groups.
A

B

Maximal Distance of Invasion

<table>
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<td>shctr + Dox</td>
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**Figure 31 Invasion of U1242GL cells after YKL-40 knock-down.** (A) 10µm sections were stained for Hoechst (nucleus) and anti-RFP. (Top left) Hoechst stained whole brain slice. (Top right) GFP expression in the U1242GL tumor cells growing as an invasive tumor in the mouse brain. (Bottom) in the RFP expression in the tumor cells after induction by doxycycline. (B) Maximal Distance of invasion of four groups of U1242GL tumor cells: U1242GL shCtr without doxycycline (shctr), U1242GL shCtr with doxycycline (shctr Doxc), U1242GL shYKL-40 without doxycycline (shYKL) and U1242GL with doxycycline (shYKL Doxc), in the mouse brain 30 days post implantation was analyzed.

**4.4 Discussion:**
In the past, the standard of care for glioblastoma has been surgical resection, radiation therapy, and a frontline chemotherapeutic, such as temozolomide. Despite aggressive intervention, GBM patients have a poor survival rate with near universal relapse after surgical removal and chemotherapy. This high rate of relapse is attributed to the infiltrative nature of the GBM cells, which invade the brain, escape surgical removal, and resurface as a new tumor shortly thereafter.

YKL-40 is a secreted glycoprotein which is up-regulated in GBM (12, 106). Additionally YKL-40 plays a role in glioblastoma pathology as it has been shown to mediate angiogenesis, and radioresistance (82). Not surprisingly YKL-40 levels have been shown to be highly correlated with tumor grade, and alarmingly, YKL-40 is one of the most overexpressed genes in GBM when compared to low grade glioma and normal brain via competitive hybridization array (12, 125). Despite this, the exact role of YKL-40 at the site of tumor infiltration remains to be established. YKL-40 has been shown to promote invasion in transformed glial cells in vitro.

However, most cell lines used in in vitro settings to study invasion form encapsulated tumors in vivo when used in xenograft models, leading to poor recapitulation of the human disease. To address this, we utilized U1242 cells which spread invasively in the brain with a high mitotic index in xenograft models, in an effort to mimic human disease. We generated U1242 cells stably expressing shRNA to YKL-40 upon doxycycline induction after tumor implantation. We observed that there was no change in tumor growth in these mice as compared to control mice. In all three experimental conditions, we also observed no difference in the invasive capacity of the glioma cells. YKL-40 is a protein that has been shown to be elevated during inflammatory
conditions or pathologies associated with inflammation. We did not observe a decrease in invasive capacity in glioma cells down-regulating YKL-40, however the model that we used does present some shortcomings. One such drawback is the lack of an immune component of the athymic nude mice, which may prevent adequate recapitulation of the effects of YKL-40 seen in real pathologic inflammatory microenvironments due to cross talk between YKL-40 and the immune system. Furthermore our doxycycline induction occurred seven days after tumor implantation, and thus recapitulates the effects of YKL-40 on invasive capacity of tumor that has already substantially grown. Additionally, knocked down YKL-40 expression in glioma cells does not account for the effects of YKL-40 secreted by other cells in the brain. All of these factors may influence the data that we obtained and could have prevented us from observing YKL-40 function with respect to invasive capacity. Therefore, although our data suggest that YKL-40 does not affect invasion of tumors implanted in nude mice, it is possible that it promote the invasion of GBM cells in humans.

5. General Discussion:

RelB a member of the NF-κB family, has been shown to regulate dendritic cell maturation, and differentiation as well as development of endotoxin tolerance. Recently, it has been shown to be an oncogenic driver of mesenchymal glioma growth (105). In this study, we demonstrate that RelB expression is up-regulated in sterile inflammation in vivo. RelB expression is increased in
response to IL-1 both in primary human and mouse astrocytes as well as glioma cells. IL-1 induces RelB expression, which binds to p50, and RelB/p50 heterodimer then translocates to the nucleus and binds the YKL-40 promoter to enhance YKL-40 expression. YKL-40 has been shown to be up-regulated in variety of inflammatory diseases.

Our data raise several questions that still remain unanswered:

1. Why is IL-1 induction of YKL-40 expression controlled by RelB and not p65?
2. What up-regulates RelB expression in response to IL-1?
3. What could be the effect of YKL-40 on recruitment of other cells such as neural progenitors at the site of GBM as well as survival of glioma stem cells.

Pro-inflammatory vs Anti-inflammatory response: Why is IL-1 induction of YKL-40 expression controlled by RelB and not p65?

Studies in RelB knockout mice have shown that RelB is important in the development of medullary epithelium, mature dendritic cell function, secondary lymphoid tissue organization, and thymic negative selection (126, 127). These mice exhibit pre-mature mortality due to, multi-organ inflammation. Although the immune system lesions observed in RelB-deficient mice are consistent with the established role of NF-κB regulation of in hematopoietic tissues, the role of RelB in the inflammatory process remains obscure. We hypothesize that YKL-40
expression in inflammation is controlled by RelB and not p65 due to the role of RelB in resolution of inflammation.

RelB has been reported to possess potent anti-inflammatory properties (128-130). Targeted overexpression of RelB ablates cigarette smoke induced inflammation in mice in vivo (128). Elevated RelB expression has been correlated with the repression of pro-inflammatory gene expression (131). Furthermore, RelB has been shown to bind and sequester p65, which inhibits the binding of p65 to its promoter, hence inhibiting the expression of pro-inflammatory genes in LPS (Lipopolysaccharide) tolerant cells. LPS can induce RelB expression, which activates a negative feedback loop to balance the immune response.

In our study, we show that YKL-40 expression is modulated by RelB in response to IL-1 and OSM. YKL-40 is marker of chronic inflammation and is up-regulated in various inflammatory conditions but its role in inflammatory conditions is not well understood. YKL-40 knockout mice show an exacerbated response to EAE as well as an enhanced inflammatory response to hyperoxia (71, 72) Interestingly, YKL-40 knockout mice showed impaired allergen (OVA) sensitization as well as similar inflammation in response to cigarettes as compared to wild types (14, 132). Taken together, these studies insinuate that YKL-40 dependent regulation of inflammation may vary depending on the specific type of immune activation, and cell signaling, as well as the tissue involved. In the case of astrocytes, which are an important modulators of neuroinflammation, we show that up-regulation in YKL-40 expression is dependent on RelB
regarded as an anti-inflammatory member of the NF-κB family rather than p65, which is known to mediate pro-inflammatory gene expression. We hypothesize, that this alternate pathway of RelB instead of p65 to modulate YKL-40 gene expression highlights the potential role of YKL-40 to curb excessive inflammation to restore a balance during neuroinflammation. We speculate that YKL-40 expression in astrocytes is up-regulated in response to IL-1 via RelB to curb the overwhelming inflammatory response, via prevention of immune cell infiltration and activation of gliosis to prevent damage to the brain in case of excessive inflammation.

**What up-regulates RelB expression in response to IL-1?**

RelB promoter has two κB binding sites and RelB expression is modulated by RelA in monocytes (133). Once RelB is initially transcribed in a RelA dependent manner, it is then constitutively autoregulated in a transcriptionally dependent manner (133). In BMDC (bone marrow derived dendritic cells) its expression can be modulated by cRel (47). In our study, down regulation of p65, and cRel has no effect on cytokine mediated expression of YKL-40. We observed a 25 fold up-regulation in RelB gene expression when astrocytes were stimulated with IL-1. This up-regulation is not completely diminished by down-regulation of p65 or cRel in these cells (data not shown). Taken together these studies indicate that RelB expression in astrocytes in response to IL-1 might initially be p65 dependent followed by auto-regulation by RelB.
What could be the effect of YKL-40 on recruitment of other cells such as neural progenitors?

YKL-40 is a glycoprotein that is secreted and can have both auto as well as paracrine functions (19, 67). YKL-40 enhances the migration of astrocytes, GBM cells, and glioma stem cell (data not shown) (25, 106). In our study, we evaluated the effect of down regulation of YKL-40 secreted by GBM cells. In the future, the role of YKL-40 secreted by surrounding cells in the brain still needs to be evaluated. Neural stem cells are cells in the brain that show enhanced migratory capacity and have long been studied, to understand their role in GBM and potentially utilize them as targets to develop better therapeutics. Neural stem cell tropic studies in GBM, indicates that NSC migration can be extensive, even in the adult brain and along nonstereotypical routes, in GBM (134). GBM like other cancers, is accompanied by inflammation and up-regulation of cytokines like IL-1, IL-6 and OSM. We observed, an up-regulation of YKL-40 expression in NSC in response to IL-1 and OSM. The role of these NSC on glioma progression needs to be evaluated. In addition, the potential role of YKL-40 in attracting NSCs to glioma site, still needs to be explored.

References


