The Fli-1 Transcription Factor Regulates the Expression of IFN-gamma Inducible Protein 10 (IP-10)

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The Fli-1 Transcription Factor Regulates the Expression of IFN-gamma Inducible Protein 10 (IP-10)

BY

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A thesis submitted to the faculty of the Medical University of South Carolina in partial fulfillment for the degree of the Master of Science in the College of Graduate Studies

Department of Microbiology and Immunology

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Abstract

The innate immune system is the primary line of defense to protect the host from pathogen infection. Mammalian cells produce inflammatory cytokines and chemokines in response to innate immune signals and their expression is tightly regulated. IFN-gamma Inducible Protein (IP-10), also known as C-X-C motif chemokine 10 (CXCL10), is an inflammatory chemokine belonging to the CXC chemokine family. CXCL10 is chemotactic for many inflammatory cells, including macrophages, and altered expression of CXCL10 is associated with inflammatory diseases, including lupus nephritis and other autoimmune diseases. The Fli-1 transcription factor is a member of the Ets gene family and regulates the immune response, along with other cellular processes including its role in the pathogenesis of renal injury and systemic lupus erythematosus (SLE). Previous data has shown that Fli-1 heterozygous NZM2410 mice, a murine model of lupus with decreased Fli-1, had significantly decreased infiltration of inflammatory cells including macrophages in kidney. Similarly, in MRL/lpr lupus mice with decreased Fli-1, Decreased T cell infiltrates and CXCL10 levels in the kidney was founded. We hypothesize that Fli-1 is a critical regulator in directly modulating the expression of CXCL10. In this study, Fli-1 protein expression in endothelial cells transfected with Fli-1 specific siRNA was significantly decreased compared to the expression of Fli-1 in cells transfected with control siRNA. Additionally, endothelial cells transfected with Fli-1 specific siRNA produced significantly lower amounts of CXCL10 compared to cells transfected with control siRNA after stimulation by Toll-like receptor (TLR) 4 ligand, lipopolysaccharide.
(LPS). Chromatin immunoprecipitation (ChIP) assay was performed to show that Fli-1 binds Ets binding sites (GGAA/T) within the mouse CXCL10 promoter. The human CXCL10 gene promoter was used to perform a transient transfection to determine that Fli-1 actively promotes transcription from the CXCL10 promoter. Mutation of the DNA binding domain of Fli-1 demonstrated that Fli-1 activates transcription of CXCL10 in both indirectly and directly ways, likely with the assistance of co-factors or post-transcriptional modifications. Together, the results indicate that Fli-1 is a novel, critical transcription factor in regulating the expression of the pro-inflammatory chemokine CXCL10 and provides a possible mechanism for the protective effect of decreased Fli-1 expression in lupus and other inflammatory autoimmune diseases.
Chapter 1 - Introduction

Systemic Lupus Erythematosus

Systemic Lupus Erythematosus (SLE) is a chronic autoimmune disease that is involved in inflammation in many different organs of the body, including the kidney, joints, skin, and brain causing symptoms that range from rashes, arthritis, heart disease vasculitis, serositis, and nephritis [1-3]. Approximately 90% of SLE patients are women aged 20-40 years and the occurrence of lupus is high in young African American women [1, 3]. The recent peak in mortality of SLE patients over the past 30 years has brought major concerns about the development of the disease and implementing new therapies to treat it. Among SLE patients, 50-70% of the mortality is due to lupus nephritis [1]. Lupus nephritis is a type of glomerulonephritis that is commonly seen in SLE patients. It is frequently associated with tubulointerstitial and/or vascular lesions in combination with impaired renal function, edema, proteinuria and hypertension [1]. The pathogenesis of lupus nephritis is poorly understood and much of what we know of the disease comes from animal models that exhibit lupus-like symptoms, including glomerulonephritis. Glomerulonephritis is initiated through renal deposition of immune complexes that consequently activates complement and Fcγ receptors [1]. In the late 1960’s, researchers discovered that patients with lupus nephritis contain autoantibodies against double stranded DNA [1]. With these antibodies acting against self, the kidney initiates the release of inflammatory cytokines and chemokines attracting inflammatory cells including
macrophages, dendritic cells, T cells and B cells into the glomerular and tubulointerstitial areas of the kidneys [4]. The infiltration of inflammatory cells, such as T helper type 1 (Th1) cells, initiates a cascade of events that contributes to the progressive deterioration of kidney structure and function.

Taking a closer look at the inflammatory cell types that infiltrate the kidney in lupus nephritis patients, T lymphocytes and B lymphocytes initiate the accumulation and infiltration of these cell types within the kidney [5]. T lymphocytes are composed of two classes, CD4 and CD8 cells, identified by their distinct surface markers. CD8 cells are cytotoxic T cells [5]; therefore they kill foreign pathogens on site, however CD4 T cells differentiate into effector subsets, the dominant ones being Th1 and Th2 cells [5]. Interleukin-12 (IL-12) drives the differentiation of naïve CD4 T cells to their Th1 cell subset [5]. Th1 cells release chemokines, such as IFN-γ, to activate macrophages and recruit inflammatory mediators. Activation of monocytes causes them to mature into macrophages. Macrophages are antigen-presenting cells that induce phagocytosis of bacterial pathogens [5]. In addition to expressing IFN-γ, Th1 cells can express TNF-α and TNF-β, which facilitate expression of adhesion molecules in blood vessels [6]. The release of these chemokines may cause local tissue damage. Th1 cells also release granulocyte macrophage colony-stimulating factor (GM-CSF) that stimulates bone marrow stem cells to produce monocytes [5]. The release of IL-4 by CD4 lymphocytes leads to Th2 cell differentiation and, in turn, Th2 cells release a number of effector molecules including IL-4, IL-5, IL-6, IL-10, and IL-13 [7]. These chemokines target
extracellular parasites and play a role in asthma and allergies. Overall, Th1 cells initiate cell-mediated immunity and inflammation, while Th2 CD4 T lymphocytes regulate antibody-mediated immunity [5].

Dendritic cells (DCs) are equally involved in inflammation as macrophages, B-lymphocytes, and CD4 and CD8 cells and their subsets. DCs are derived from committed DC progenitors within the bone marrow and migrate to lymphoid and non-lymphoid tissue [8]. Between the two classes of DCs, conventional (cDC) and plasmacytoid (pDC), cDCs are antigen presenting and activate naïve T cells, and pDCs respond to viral and bacterial infections by producing large amounts of IFN-α and IFN-β [5].

The pathogenesis of lupus nephritis is not fully understood. As mentioned previously, due to autoantibody production, complement and Fcγ receptors (FcγR) are activated and initiates the formation of immune complexes [1]. The activation of complement leads to glomerular injury which essentially leads to glomerulonephritis, similarly with FcγRs [1]. There are a number of nuclear antigens and autoantibodies implicated in the pathogenesis of lupus nephritis including: dsDNA, ssDNA, chromatin, histone, SSA, SSB, ribonucleoprotein and anti-C1q antibodies [1]. All of which have been found in the kidneys of LN patients. How these anti-dsDNA and autoantibodies are deposited in the kidneys are not fully elucidated. There are a number of theories; however none have been proven or have any evidentiary support [1]. However, current studies are looking into possible ways to regulate inflammation within this progressive disease.
Like many inflammatory diseases, cytokines and chemokines are closely involved in lupus nephritis development [1]. Inflammatory cells are all recruited by inflammatory cytokines, and all of these cell types contain a common chemokine receptor, CXCR3. CXCR3 is a receptor for C-X-C motif chemokine 10 (CXCL10) the inflammatory chemokine this study is focused on. Detailed discussion regarding CXCL10 will be presented in another section.

**The Friend Leukemia Insertion Site 1 Transcription Factor**

The Ets gene family is composed of a number of transcription factors with a winged helix-turn-helix structure essential for protein-protein interaction and DNA binding [9]. Ets transcription factors are involved in various cellular processes, including the immune response, cellular proliferation, development, differentiation, transformation, and apoptosis [10]. All members of the Ets gene family contain an approximately 85 amino acid domain that binds to the conserved DNA binding motif (GGAA) [10]. One family member, the proto-oncogene Friend Leukemia Insertion site 1 (Fli-1) transcription factor, regulates the immune response along with other cellular processes such as cell growth, proliferation, maturation, and survival [9]. Fli-1 recognizes the consensus DNA binding motif GGAA/T and, upon binding, can be either a positive or negative regulator of transcription [9].

Several studies have implicated the Fli-1 transcription factor in SLE and lupus nephritis disease pathogenesis. The overexpression of the Fli-1 gene in transgenic mice has been shown to increase infiltration of B and T lymphocytes
within the kidneys, eventually leading to death due to tubulointerstitial nephritis and glomerulonephritis [11]. Because complete targeted disruption of the Fli-1 gene has resulted in embryonic death within the animal models, researchers decided to investigate the reduced expression of Fli-1 on disease development in lupus disease [12]. MRL/\textit{pr} mice and NZM2410 mice are widely used murine models of lupus [13]. Fli-1 heterozygous (Fli-1\textsuperscript{+/−}) congenic MRL/\textit{pr} mice with reduced expression of Fli-1 as well as Fli-1\textsuperscript{+/−} congenic NZM2410 mice were generated by backcrossing with B6 Fli-1\textsuperscript{+/−} mice. [11, 14]. As disease progressed, Fli-1\textsuperscript{+/−}MRL/\textit{pr} mice and Fli-1\textsuperscript{+/−} NZM2410 mice had significantly decreased serum levels of total IgG and anti-dsDNA antibodies compared to the Fli-1\textsuperscript{+/+} littermates [11]. Both \textit{in vivo} and \textit{in vitro} production of Monocyte Chemoattractant Protein-1 (MCP-1) chemokine was significantly decreased in Fli-1\textsuperscript{+/−} MRL/\textit{pr} mice and MS1 cells where Fli-1 is knocked-down. Also, the Fli-1\textsuperscript{+/−} MRL/\textit{pr} mice and Fli-1\textsuperscript{+/−} NZM2410 mice had markedly decreased proteinuria and pathologic renal scores. At 48 weeks of age, 100% of Fli-1\textsuperscript{+/−} MRL/\textit{pr} mice were alive, in contrast to only 27% of homozygous (Fli-1\textsuperscript{+/+} mice) littermates [11]. Notably, Fli-1\textsuperscript{+/−} NZM2410 mice with reduced expression of Fli-1 transcription factor had a marked reduction in nephritis, necrosis, and proteinuria compared to wild-type NZM2410 mice [14]. This is the same with the Fli-1\textsuperscript{+/−} MRL/\textit{pr} mice. Since infiltration of inflammatory cells into kidneys plays an important role in glomerulonephritis development, the inflammatory cells were quantitated and compared from Fli-1\textsuperscript{+/−}NZM2410 mice and wild-type littermates. In previous studies conducted in our lab, Fli-1\textsuperscript{+/−} NZM2410 mice and wild-type littermate controls were sacrificed, the kidneys
were removed, and inflammatory cell infiltration analyzed. In previous studies we’ve seen a statistically significant decrease in all inflammatory cells including CD3+ T cells, CD19+ B cells, Ly-6B.2+ monocytes/macrophages and CD11b+ dendritic cells in the kidneys from Fli-1+/NZM2410 mice compared to wild-type controls. Ly-6B.2+ cells decreased 44%, and all other inflammatory cells decreased more than 50% in the kidneys from Fli-1+/NZM2410 mice, as compared to wild-type controls. Notably, there was a 70% decrease in CD19+ cells in Fli-1+/NZM2410 mice versus wild-type controls [11].

Because chemokines are known to play an important role in inflammatory cell infiltration, the expression of inflammatory cytokines and chemokines in kidneys from wild type and Fli-1+/NZM2410 mice was also studied. The investigators observed significantly lower expressions of CCL5, MCP-1 and IL-6 in kidneys from Fli-1+/NZM2410 mice versus the kidneys of wild-type controls [14-16]. Both in vivo and in vitro production of MCP-1 was significantly decreased in Fli-1+/MRL/lpr mice and Fli-1 knockdown MS1 cells and in the kidney. Furthermore, our lab has demonstrated that Fli-1 directly regulates expression of inflammatory cytokines including MCP-1, Regulated upon Activation, Normal T Expressed and Secreted (RANTES), and IL-6, all of which are associated with lupus nephritis [11, 14, 16-19].

**Inflammatory signaling in innate immunity and Inflammation:**

Innate immunity is an important part of the immune system in an organism, which defends itself from invading pathogens in a non-specific manner
The Toll-like receptor (TLR) was initially discovered in *Drosophila*, and later it was discovered that *Drosophila* [20]. The first human TLR was reported in 1997, and now there are 10 functional Toll-like receptors in humans and 12 in mice [21]. TLRs are pattern recognition receptors (PRRs) that recognize pathogen associated molecular patterns (PAMPs) found on proteins, lipids, nucleic acids and lipoproteins, which are expressed on virus, bacteria, fungi and parasites [22]. Toll-like receptors have been identified in immune cells such as monocytes, dendritic cells, B cells and non-immune cells such as epithelial cells [22]. TLRs consist of an extracellular like receptor region, a transmembrane domain and a cytoplasmic Toll/IL-1 receptor domain [21]. Upon binding and activation by ligands, TLRs recruit a set of adaptor proteins such as Myeloid differentiation primary response protein 88 (MyD88) and TRIF-related adaptor molecule (TRAM), and then activate downstream kinases and transcription factors including Nuclear Factor-Kappa B (NF-kB), activator protein 1 (AP-1), Interferon regulatory factor 3 (IRF3) or IRF7 that regulate the expression of inflammatory cytokines and chemokines [23]. For example, the TLR-4 cascade can be MyD88 dependent, which in turn may lead to the stimulation of inflammatory cytokines, or MyD88 independent, which results in the expression of interferon inducible genes [24]. The Ets family is known to regulate inflammatory cytokine expression through TLR-4 [16, 25].
Chemokine (C-X-C motif) ligand 10 and CXCR3 receptor

Chemokines are a subfamily of cytokine-like molecules that induce chemotaxis and regulate functional properties of various leukocytes during inflammation [26]. The four groups of chemokines are distinguished by number and by the spacing of two conserved cysteine residues on the N-terminus (as shown in Figure 1). Two N-terminal cysteine residues, separated by a single variable amino acid, determine the CXC subgroup [27]. The CXC subgroup is further divided into two subdivisions. The ERL positive motif, containing glutamine, arginine, and leucine near the N-terminal, and the ERL negative motif, which do not contain glutamine, arginine, and leucine near the N-terminal CXCL9, CXCL10, and CXCL11, are part of the ERL negative motif [26, 27]. All three CXC chemokines have a similar structure and share a ligand receptor, CXCR3 [28]. A G-protein coupled receptor, CXCR3 is expressed on many immunological cell types, such as activated T-cells, specifically Th1 CD4+ cells, as well as natural killer cells (NK) and CD8+ T cells. Increased recruitment of each of those cells to the site of infection can lead to chronic inflammation [4, 29-31]. The CXCR3 receptor is also expressed on myeloid dendritic cells, leukemic B-cells, and endothelial cells amongst other cell types [4].

As mentioned previously, chemokine (C-X-C motif) ligand 10 (CXCL10) is an interferon gamma inducible protein and may be referred to as IP-10 [32]. The human CXCL10 gene was initially found and identified as an early response gene in a monocyte-like cell line, U937 cells [26, 33]. The CXCL10 gene is located on chromosome 4, band q21. It has an open reading frame of 1173 bp,
which encodes a 98 amino acid protein and contains 4 exons, and a molecular mass of 10kDa, hence the name CXCL10 [26, 33]. The gene has been shown to regulate chemotactic and mitogenic activities associated with inflammation and cell proliferation. The human CXCL10 amino acid sequence has 63% homology with murine CXCL10 cDNA [33].

CXCL10 is expressed in a variety of cell types including monocytes, fibroblasts, neutrophils, keratinocytes, astrocytes, mesangial cells, and endothelial cells [26]. Functional characteristics of CXCL10 include its pleiotropic capabilities of inducing apoptosis, cell growth, and proliferation, as well as its role in inflammatory and infectious diseases and tumor growth. CXCL10 is a chemoattractant protein for monocytes, Th1 cells, DCs, and NK cells [4, 33, 34]. The expression of CXCL10 drives the recruitment of immune competent cells and the formation of lymphocyte infiltrates in autoimmune inflammation [26]. Th1 cells, for example, are highly recruited by CXCL10 chemokine due to the high amount of IFN-γ that is being secreted by Th1 cells themselves. IFN-γ promotes the secretion of CXCL10 in various cell types, including Th1 cells. This, in turn, causes CXCL10 to recruit more Th1 cells, thus displaying a positive feedback loop [26, 35]. The recurring positive feedback loop leads to both the innate and adaptive immune response and, unfortunately, to the high inflammation that cause tissue damage [33].
**CXCL10 and Diseases**

The manifestations of autoimmune diseases stem from effector mechanisms of the immune system that become directed towards self [5]. Given the role CXCL10 plays in the recruitment of inflammatory cells and the immune response, it is not surprising that the gene is implicated in the pathogenesis of various diseases. Studies have shown that CXCL10 is expressed in many Th1-type inflammatory diseases including type 1 diabetes, psoriasis, multiple sclerosis, atherosclerosis, rheumatoid arthritis (RA), transplant rejection, and **systemic lupus erythematosus (SLE)** [26]. As mentioned previously, SLE is characterized by activation of T and B lymphocytes, autoantibody production, and the development of immune complexes resulting in damage to the tissue and organs [26]. Development of many autoimmune diseases can be contributed to abnormal T helper cell cytokine production, notably, CXCL10, which is especially relevant to SLE and the current study. CD4+/CD8+ T cells that infiltrate the kidney express the CXCR3 receptor, which upon activation causes the production of CXCL10 [28-30]. When overproduced, CXCL10 leads to the recruitment of more Th1 inflammatory cells causing damage to the organs [34]. Evidence linking CXCL10 and SLE has been provided in many recent studies [26]. In one study, researchers saw increased serum levels of CXCL10 in SLE patients [36]. After measuring CXCL10 levels and disease activity in SLE and RA patients they also measured MCP-1 levels as well, since MCP-1 is a known inflammatory chemokine and an early marker for other inflammatory diseases. MCP-1 elevation was detected in high levels of disease activity in both groups,
RA and SLE. However, researchers concluded that the elevated levels of MCP-1 were generated from increased inflammation and were not necessarily dependent upon disease activity [36]. Alternately, the investigators concluded that CXCL10 serum levels increased in relation to severe disease activity after measuring anti-DNA antibodies [36]. Those findings suggest that CXCL10 is an indicator of SLE activity [36]. In recent plasma chemokine studies, researchers observed increased levels of CXCL10 produced by peripheral blood mononuclear cells (PBMC) from SLE patients [37]. The objective was to determine whether there was chemotactic preference for Th1 chemokines or Th2 chemokines or both in SLE patients. Elevated levels of CXCL10, detected from the PBMCs, were significantly higher in SLE patients versus healthy patients [37]. Other evidence linking CXCL10 and SLE are the increased levels of CXCL10 seen in the cerebrospinal fluid of SLE patients. The exact role of CXCL10 within the pathogenesis of SLE has yet to be identified [38]. Previous reports demonstrated that NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) transcription factor is involved in regulating expression of CXCL10 [39].

**Fli-1, SLE, and CXCL10**

CXCL10 is a major macrophage and T-cell chemotactic cytokine, and our lab observed significantly reduced macrophage and T-cell infiltration into the kidney from Fli-1<sup>+/−</sup> NZM2410 mice with reduced expression of Fli-1 compared to that from wild-type controls [16]. Furthermore, another study found that the
kidney CXCL10 concentrations from Fli-1+/− MRL/lpr mice were significantly lower compared to these from wild-type MRL/lpr controls [28]. There were significantly fewer T cells in the kidney, significantly reduced T cells expressing CXCR3, and decreased CXCR3 expression in the kidneys, all of which supports Fli-1’s role in regulating CXCL10-CXCR3 recruitment of T cells to the kidney. Taking all information into account, this suggests that Fli-1 transcriptionally controls the expression of CXCL10. In addition, we have preliminary data demonstrating that endothelial cells transfected with specific Fli-1 siRNA had markedly reduced expression of Fli-1 protein and produced significantly decreased amounts of CXCL10 after stimulation with TLR4 ligand, lipopolysaccharide (LPS), compared with endothelial cells transfected with control siRNA. Analyzing the promoter region of CXCL10 revealed there are many potential Ets DNA-binding sites in the CXCL10 promoter. This leads us to our hypothesis that Fli-1 is a critical regulator in directly modulating the expression of CXCL10, which is associated with inflammation in autoimmune diseases and other inflammatory disease development. The below study was designed to test this hypothesis by determining if Fli-1 regulates the expression of CXCL10 and identifying the mechanism behind this regulation.
Chapter 2 – Hypothesis

Fli-1 is a critical regulator in directly modulating the expression of CXCL10, which is associated with inflammation in autoimmune diseases and other inflammatory disease development.

Specific Aims

**Specific Aim 1:** To confirm that Fli-1 regulates the expression of CXCL10.

**Specific Aim 2:** To investigate the mechanism(s) of how Fli-1 regulates the expression of CXCL10.
Chapter 3 – Materials and Methods

Cells

Endothelial cells (EC) are a cell type of choice for multiple reasons. As mentioned previously ECs contain the CXCR3 receptor and it also expresses CXCL10 [4]. Previous studies have demonstrated that Fli-1 is highly expressed in ECs [40]. MS1 cells, a murine pancreatic islet endothelial cell line, were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained with DMEM containing 5% fetal bovine serum (FBS). Human Umbilical Vein Endothelial cells (HUVECs) were purchased from Lonza (Basal, Switzerland) and maintained in EBM-2 Basal Medium supplemented with EGM-2 SingleQuot Kit growth factors. NIH3T3 cells were purchased from ATCC and maintained with DMEM with 10% fetal bovine serum and 1% penicillin/streptomycin. All cells were grown at 37°C in 5% CO₂.

siRNA Transfections

MS1 cells and HUVECs were cultured to confluence in sterile T-75 Falcon culture flask with complete growth media. To remove cells from the flasks, they were treated with 0.05% Trypsin (MS1) or TrypLE (HUVECs) and incubated at room temperature for 1-2 minutes until cells came off the plate. Both MS1 cells and HUVECs were neutralized with growth media and counted using a hemocytometer. Once plated in a 6-well plate at 0.5 x 10⁵ cells/well with each well containing 2 ml of media, the cells were incubated at 37°C, 5% CO₂ for 24
hours in preparation for transfection. After the 24hr incubation, the media was removed from the cells, replaced with fresh media, and placed back into the incubator for one hour to equilibrate. An RNAi duplex was prepared by combining 5nmol Stealth Silencer Human Fli-1 siRNA (Ambion, Waltham, MA) oligo with Opti-MEM medium (Thermo Fisher Scientific, Waltham, MA) for Fli-1 transfection and siRNA Negative Control, Med GC (Thermo Fisher Scientific) and Opti-MEM medium for the control. An RNAi Max solution was prepared containing 25pmol of lipofectamine as the transfection reagent and combined with Opti-MEM medium. Two solutions of RNAi Max were prepared for the Fli-1 transfection and the control, respectively. The RNAi Max and the RNAi duplex were combined, equal amounts of the mixture were added to the control only 6-well plate and the Fli-1 only 6-well plate. The plates incubated overnight.

**Lipopolysaccharide (LPS) Stimulation**

LPS was used to activate the TLR signaling pathway in cells to observe CXCL10 production. The cells were cultured in a T-75 flask to 80% confluence and plated onto a 6-well plate at a concentration of $1 \times 10^5$ cells/well, each well containing 2 ml of media and incubated at 37°C, 5% CO$_2$ for 24hrs. The media was changed the next day. Cells were stimulated with TLR4 ligand, lipopolysaccharide (LPS) to induce CXCL10 production. Cells were stimulated with 1μg/mL of LPS, and samples were collected at the 0, 2, 6 and 24-hour time points. A dosage response assay was performed to ensure the appropriate amount of LPS was
being administered to the cells. We stimulated the cells with 0.25\(\mu\)g/ml, 0.5\(\mu\)g/ml, 1\(\mu\)g/ml, 5\(\mu\)g/ml, and 10\(\mu\)g/ml of LPS. The supernatants were collected at 2 hours, 6 hours and 24 hours after stimulation and stored at -80°C.

**Immunoblot Analysis**

Immunoblots were performed to monitor the efficiency of siRNA knockdown. Cells were collected in 1X Phosphate Buffer Saline (PBS) and lysed with Radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1% Triton X-100; 0.5 % deoxycholate; 0.1 mM EGTA; 1.0 mM EDTA; 0.1% SDS and protease inhibitor mixture). Equal amounts of each sample were loaded and ran on a 12% Tris Glycine SDS-PAGE gel (Novex, Life Technologies) under reducing conditions. The proteins were transferred onto a polyvinylidene difluoride membrane and blocked for 1 hour in Odyssey Blocking Buffer (LI-COR, Lincoln, Nebraska). The membrane was later probed with 1:100 dilution of specific rabbit polyclonal Ab against Fli-1 and 1:200 dilution of \(\beta\)-actin Rabbit mAb (Cell Signaling Technology). After three washes with PBS containing 0.1% Tween 20 (PBST), the membrane was incubated with donkey anti-rabbit IgG secondary antibody (LI-COR)). Chemiluminescence signals were captured using an Odyssey chemiluminescence and infrared imager (LI-COR). The expression levels were quantified by Ascent densitometry software.
Enzyme-linked Immunosorbent Assay

A 96-well polystyrene microplate was coated with antihuman CXCL10 capture antibody and incubated overnight at room temperature. After 2-3 washes, the plate was blocked with reagent diluent (1% bovine serum albumin in PBS), and samples were added after a second wash. Recombinant human CXCL10 standards were added following a seven point standard curve starting at 2000pg/ml with a 2-fold serial dilution. Standards and samples were sealed and incubated at 4°C overnight. Plates were aspirated, washed, and separately probed with biotinylated goat anti-human CXCL10 detection antibody, streptavidin-HRP, and finally, color substrate solutions, H₂O₂ and Tetramethylbenzidine. Washes were conducted between each step. To stop the reaction, the plate was coated with an acid solution, 2N H₂SO₄. CXL10 levels were measured using a microplate reader at a wavelength of 450nm.

IP-10/CXCL10 Primers for ChIP Assay

To design the primers for CXCL10, we obtained the 5' UTR from the murine CXCL10 sequence using the Ensembl genome database (www.ensembl.com, gene number ENSMUSG00000034855) and identified the promoter region. Next, the sequence was uploaded into the Genomatix MatInspector program to identify potential ETS transcription factor binding sites within the promoter region of CXCL10. Once those sites were identified through the MatInspector program, we further confirmed our findings by using the Clustal X 2.1 program to visualize
the sequence. With this information, we proceeded to design appropriate primers for the promoter region of CXCL10. According to Thermofisher Scientific, the most ideal primers should be low in GC count, with an appropriate length of 15 – 20 nucleotides, melting temperatures around 60°C, and similar Tₘ’s for each primer pair along with other stipulations. An oligonucleotide calculator (http://biotools.nubic.northwestern.edu/OligoCalc.html) was used to ensure these requirements were met. The primers are listed in Table 2.

### ChIP Assay

A Chromatin Immunoprecipitation (ChIP) Assay was performed to determine if Fli-1 directly binds to the promoter region of CXCL10 using the EpiTect ChIP OneDay kit from QIAGEN. Approximately 2-4 million cells were cultured in preparation for the ChIP Assay. Cells were fixed with fresh fixing buffer (1% Formaldehyde in 1X PBS) and washed with 1X PBS before harvesting with cell harvesting buffer containing protease inhibitor cocktail (PIC) in 1X PBS. Cells were then lysed and sonicated to obtain ChIP-ready chromatin. ChIP chromatin was isolated via immunoprecipitation where the anti-Fli-1 rabbit polyclonal antibody was used. A normal rabbit IgG antibody was used for a negative control and the complete IP Fraction as the positive control. DNA elution buffer and ChIP-Grade Proteinase K were added to the ChIP chromatin IP fraction to isolate the targeted DNA fraction. DNA purification was performed through a series of washes and elution buffer to create a viable ChIP fraction DNA sample.
Real Time qPCR

Real Time qPCR was used to quantitate our ChIP assay results and was performed in triplicate. We used our ChIP fraction DNA samples and the primers that were designed for the CXCL10 promoter. The experiment was performed following the basic steps of denaturation, annealing, and extension. Primers, DNA, Water, and Bio-Rad SybrGreen Supermix (contains polymerase, dNTPs, MgCl₂, and dyes) was combined and loaded onto the PCR plate and amplified using a thermocycler. For the control, we used MCP-1 primer. These controls have been published in previous papers by our lab [15, 16, 25]. Once the primers detect the promoter region following IP with anti-Fli-1 antibodies, the RT-pCR machine puts out critical threshold values. Our data is quantitated based on these critical threshold (C_T) measurements. C_T values are defined as changes in fluorescence per PCR cycle number at the set base threshold. To normalize our input, specific antibody was compared to the % input value for each immunoprecipitation represented as ΔC_T (% Input C_T – (specific antibody C_T – Dilution Factor). Genomic DNA was calculated and represented as ΔΔC_T (% Input ΔC_T – Fli-1 specific antibody ΔC_T). C_T (genomic input) and C_T (specific antibody) are the mean threshold cycles of PCR performed. Fold Enrichment (2^{ΔΔC_T}) was determined based on the 2-fold change of ΔΔC_T. The error bar represents standard deviations.
**Reporter & Expression Constructs**

Primers were designed specifically for the promoter region of CXCL10, using the previously mentioned technique. To clone the full length CXCL10 promoter region, -2069bp upstream of the transcription start site, we used the ChIP 1 reverse primer (5' CAC TTG GGT TCA TGG TG 3') and ChIP 14 forward primer (5' GAA TTC GGA GGT CTA CCT 3') that were designed previously for the ChIP Assay. These two primers along with restriction enzymes *Kpn1* and *Sma1* (underlined) were used to obtain the entire 2069bp promoter region through cloning. Genomic DNA isolated from a B6 mouse was amplified using polymerase chain reaction (PCR). DNA concentration was measured after isolation. Although were able to isolate and clone the murine CXCL10 promoter, we were able to obtain the human CXCL10 promoter already sequenced and inserted into the pGL4 vector. The CXCL10 human promoter was used for our DNA transfections. It was obtained from Dr. David Proud (University of Calgary, Alberta, Canada) and initially generated from human genomic DNA [41]. The 972bp promoter construct corresponds to the human CXCL10 gene and its promoter sequence ranges from -875 to +97 in relation to the transcription start site [41]. In comparing, both, the murine and human CXCL10 promoter construct, we found 63% homology within the two sequences. This percentage was calculated based on neighbor joining tree method using ClustalX2.1 program. Taking this information in account, we opted to use the human CXCL10 promoter construct for the DNA transfections.
The following expression constructs were used in the transient transfection assays. The murine Fli-1 expression construct, containing a 5' kozak sequence and a flag tag, was provided to our laboratory by Dr. Dennis Watson (Medical University of South Carolina) [42]. The Fli-1 expression construct was cloned in the pCDNA3.0 vector (Life Technologies), which is under regulation of the CMV (Human cytomegalovirus) promoter. DNA transfections were also performed using the human Fli-1 gene cloned into the pSG5 expression vector (Agilent Technologies, Santa Clara, CA) and a Fli-1 DNA-binding mutant construct inserted into the pSG5 vector. This mutation contains a single amino acid change of tryptophan 321 to arginine preventing DNA binding, which was provided to our laboratory by Dr. Maria Trojanowska (Boston University School of Medicine Arthritis Center, Boston, MA) [42].

**DNA Transfections**

The NIH3T3 cell line, also known as murine embryonic fibroblast cells, contain no endogenous Fli-1 and are a commonly used cell line for DNA transfection. NIH3T3 cells were seeded in a 6 well plate at a concentration of $4 \times 10^5$ cells/well and incubated at 37°C, 5% CO$_2$ overnight in preparation for the transfection. Cells were transfected using Fugene 6 (Promega) as the transfection reagent and 0.1µg of the pGL4/human CXCL10 reporter construct. The Fli-1 expression vector was transfected into the cells in increasing doses (0.025µg, 0.05µg, 0.1µg, 26
0.2μg, 0.25μg, 0.5μg and 1μg) and empty expression constructs (pcDNA or pSG5) was also added so that equal amounts of total DNA were transfected into the cells. A Renilla luciferase construct (pRL/TK) was also transfected into the cells to normalize for transfection efficiency. After transfection, the cells were incubated at 37°C, 5% CO₂ for 48 hours. To harvest the cells, they were washed with sterile 1X phosphate buffer saline and then collected in passive lysis buffer in preparation for the next experiment, the luciferase assay.

**Luciferase Assay**

Using the Dual-Luciferase Reporter Assay System (Promega) and the Luminoskan Ascent Microplate Luminometer (Thermo Fisher Scientific, Waltham, MA), we measured luciferase activity within our NIH3T3 cells. Briefly, 20μl of the cell lysate was placed into white walled wells of a 96-well plate. Using the luminometer, one hundred microliters of the provided Luciferase Assay Reagent II was added to the lysate, a brief waiting period observed and then the luciferase value recorded. Next, 100 μl of Stop and Glo Reagent was added to the samples, a brief waiting period observed and the Renilla luciferase value recorded. We normalized transcription activity to our Renilla luciferase construct pRL/TK (Promega), which was co-transfected into our cells. Fold activation was calculated by dividing normalized luciferase activity over pGL3 basic and presented as mean ± standard error.
Statistics

To determine significance within the data, an unpaired, two-tailed Student’s t-test based on equal or unequal variances was conducted between two groups in the DNA transfection experiments. P values less than 0.05 or 0.01 were determined as statistically significant. Significant differences in CXCL10 production were determined via analysis of standard curve with Prism software from GraphPad (San Diego, CA) and two-tailed Student t-test (Microsoft Excel), which were calculated based on variance.
Chapter 4 – Results

Specific Aim 1- To confirm that Fli-1 regulates the expression of CXCL10

*Inhibiting expression of Fli-1 leads to significantly decreased production of CXCL10*

CXCL10 is a chemoattractant for inflammatory cells and reduced expression of Fli-1 resulted in decreased inflammatory cell infiltration in kidneys in murine model of lupus (see Table 1) [19]. We have found that endothelial cells in glomeruli express high amounts of Fli-1 protein [40]. Given that CXCL10 expression was decreased in Fli-1(+/-) MRL/lpr mice [28], we first chose to determine if inhibiting expression of Fli-1 affects production of CXCL10 in endothelial cells. Both the murine endothelial cell line, MS1, and primary human endothelial cells, HUVECs, were transfected with Fli-1 specific siRNA and negative control siRNA, as described in the Methods section. The cells lysates were collected with RIPA buffer and an immunoblot was performed to measure the Fli-1 protein. As shown in **Figure 2** and **Figure 3**, the expression of Fli-1 protein was inhibited after transfection with Fli-1 specific siRNA compared to the cells transfected with control siRNA. To determine the appropriate concentration of LPS to use on endothelial cells, a dosage response assay was performed on untransfected cells. **Figure 4** shows that increasing the dosage of LPS to murine endothelial cells increases the amount of CXCL10 up until 1μg/ml. At this point, the amount of CXCL10 decreases. Based on these results we chose to treat the cells in future experiments with 1μg/ml LPS.
To determine if the expression of Fli-1 affects production of CXCL10 in endothelial cells, MS1 endothelial cells were transfected with Fli-1 specific siRNA and control siRNA, and stimulated with 1 μg/ml of LPS to induce an inflammatory response. The supernatants were collected at 0, 6 and 24 hours after stimulation and the concentration of the CXCL10 protein measured by ELISA. As shown in Figure 5, the production of CXCL10 in MS1 cells transfected with specific Fli-1 siRNA was significantly reduced compared to the cells transfected with control siRNA at 6 hours and 24 hours after stimulation (mean± SD, 141.8 ± 61.42 pg/ml versus 146.19 ± 80.28 pg/ml before stimulation – 0 hours, and 645.7 ± 167.1 pg/ml versus 336.2 ± 93.26 pg/ml at 6 hours after LPS stimulation, \( P \text{ value} = 0.009967 \); and 1493.75 ± 392.35 pg/ml versus 540.01 ± 187.37 pg/ml at 24 hours after LPS stimulation, \( P \text{ value} = 0.004096 \) \( P < 0.05 \) for each comparison; n = 2 (6 replicates per group). Decreased amounts of CXCL10 in the cells transfected with specific Fli-1 represented more than a 50% reduction compared to the cells transfected with negative control siRNA.
Figure 2. Expression of Fli-1 was inhibited in murine endothelial cells with specific Fli-1 siRNA transfection

Murine endothelial cells were treated with 20nmol Stealth silencer mouse Fli-1 siRNA or siRNA negative control. The cells were collected 24 hours later and lysed with RIPA buffer. Fli-1 was detected by an immunoblot. The Fli-1 protein was indicated at 51kDa in MS1 endothelial cells. Beta actin (42kDa) was used as a control housekeeping gene and ensures equal loading of protein sample.
Figure 3. Expression of Fli-1 was inhibited in human umbilical vein endothelial cells with transfected specific Fli-1 siRNA

Human umbilical vein endothelial (HUVEC) cells were treated with 5nmol stealth silencer human Fli-1 siRNA or siRNA negative control. The cells were collected 24 hours later and lysed with RIPA buffer. Fli-1 was detected by an immunoblot. Fli-1 protein was detected at 51kDa in HUVECs. Beta actin (42kDa) was used as a control housekeeping gene and ensures equal loading of protein sample.
Figure 4. CXCL10 was produced in MS1 cells following LPS stimulation in a dose-dependent way. MS1 cells were stimulated with 0.25µg/ml, 0.5µg/ml, 1µg/ml, 5µg/ml, and 10µg/ml of LPS and supernatants were collected 6 hours and 24 hours after stimulation. CXCL10 concentrations were measured via sandwich ELISA. Experiment was performed in triplicate and values are shown as means ±SEM of N=3 experiments per group. * = P<0.05; ** = P<0.01.
Figure 5. Inhibiting expression of Fli-1 leads to significantly decreased production of CXCL10

Murine endothelial cells were transfected with specific Fli-1 siRNA or control negative siRNA respectively and simulated with 1μg/mL LPS. The supernatants were collected at 6 and 24 hours following the stimulation. CXCL10 concentrations were determined by a sandwich ELISA. The experiment was performed in triplicate and values are shown as means ±SD of N=2 experiments per group. *p<0.05
Specific Aim 2 – To determine the mechanism(s) of how Fli-1 regulates the expression of CXCL10

*Fli-1 binds to the CXCL10 promoter in endothelial cells*

Next, we investigated how Fli-1 regulates the expression of CXCL10. Fli-1 is known to bind the consensus ETS DNA binding motif GGAA/T and in previous studies the Fli-1 transcription factor has been proven to regulate many inflammatory cytokines and chemokines by directly binding to their promoter regions [25] [15]. Using Genomatrix MatInspector software, the potential Fli-1 ETS binding sites in the promoter region of CXCL10 were examined. After thorough examination, 46 putative Fli-1 binding sites were identified as shown in Figure 6. Fourteen primer pairs were designed to cover all 46 Fli-1 binding sites (Table 2). ChIP Assay was performed using specific anti-Fli-1 antibody to determine if Fli-1 binds to the promoter of CXCL10 as described the methods. We determined PCR products from ChIP 3, ChIP 4, ChIP 7, and ChIP 8 were significantly enriched for the Fli-1 antibody compared to normal IgG control (p values less than 0.01) (Figure 7).
Figure 6. Putative ETS/Fli-1 binding to sites on the mouse CXCL10 promoter.

Forty-six putative binding sites were identified on the mouse genomic DNA promoter of CXCL10 using Genomatrix MatInspector. The number above the dash represents the number of potential ETS binding sites within each ChIP primer region (Table 2). TSS – stands for transcription start site of the promoter.
Table 2. ChIP Primers
The 5’ UTR from the murine CXCL10 sequence was obtained and used to identify the promoter region of the mouse CXCL10 genome. Primers pairs were designed to cover the Fli-1 binding sites located along the CXCL10 promoter.
<table>
<thead>
<tr>
<th>#</th>
<th>Primer Name</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Position from TSS</th>
<th>Expected Size</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>ChiP1</td>
<td>5’ GAG TCA TCT CCA AAG TCA G 3’</td>
<td>5’ CAC TTG GCT TCA TGG TG 3’</td>
<td>-2082 to -1922</td>
<td>160bp</td>
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<tr>
<td>2</td>
<td>ChiP2</td>
<td>5’ GAA ACT TAC CTC ACT CG 3’</td>
<td>5’ CTG ACT TTT GAG AGT ACT C 3’</td>
<td>-1941 to -1788</td>
<td>153bp</td>
</tr>
<tr>
<td>3</td>
<td>ChiP3</td>
<td>5’ GAA CCT GAC TTA GAT ATC 3’</td>
<td>5’ CCT CTT GTG CTC TTT TTA 3’</td>
<td>-1830 to -1660</td>
<td>170bp</td>
</tr>
<tr>
<td>4</td>
<td>ChiP4</td>
<td>5’ CTG CTC TAA CTG TTT AC 3’</td>
<td>5’ GTG ATC TAA GTC AGG TTC</td>
<td>-1677 to -1501</td>
<td>176bp</td>
</tr>
<tr>
<td>5</td>
<td>ChiP5</td>
<td>5’ CTG TAA CCA CAC ACT CAC A 3’</td>
<td>5’ GTC AAG AGT TAG AGC AG</td>
<td>-1518 to -1367</td>
<td>151bp</td>
</tr>
<tr>
<td>6</td>
<td>ChiP6</td>
<td>5’ GTT TTG AAG CGG TAC AC 3’</td>
<td>5’ CTT CTT TGT TAC TGT GTG G 3’</td>
<td>-1391 to -1184</td>
<td>207bp</td>
</tr>
<tr>
<td>7</td>
<td>ChiP7</td>
<td>5’ CTT AGC TCT GTT CTA GTC 3’</td>
<td>5’ GTG TGC CCG TCC AAA AC 3’</td>
<td>-1200 to -1027</td>
<td>173bp</td>
</tr>
<tr>
<td>8</td>
<td>ChiP8</td>
<td>5’ CTA TCC TGC AGA AGC AG 3’</td>
<td>5’ GAC TAG AAC AGA GCT AAG 3’</td>
<td>-1027 to -950</td>
<td>168bp</td>
</tr>
<tr>
<td>9</td>
<td>ChiP9</td>
<td>5’ CTG AGA ACT TGT ACA ATA AC</td>
<td>5’ TGC TTC TGC AGA AGA GAC 3’</td>
<td>-873 to -743</td>
<td>148bp</td>
</tr>
<tr>
<td>10</td>
<td>ChiP10</td>
<td>5’ CTC TGG ACT AAG AGA TGC 3’</td>
<td>5’ GTT ATT GTA CAA GTT CTC AG 3’</td>
<td>-753 to -602</td>
<td>151bp</td>
</tr>
<tr>
<td>11</td>
<td>ChiP11</td>
<td>5’ AAG CAG ACA CAG GCA AGT 3’</td>
<td>5’ CCT TAC TGA GGA GAA AGG 3’</td>
<td>-664 to -471</td>
<td>193bp</td>
</tr>
<tr>
<td>12</td>
<td>ChiP12</td>
<td>5’ CTT TGG ACT TCA AAG GG 3’</td>
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<td>156bp</td>
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<tr>
<td>14</td>
<td>ChiP14</td>
<td>5’ GAA TCT GGA GGT CTA CCT 3’</td>
<td>5’ GTT AAT GTC AAA TGA CTA GAG 3’</td>
<td>-156 to -1</td>
<td>155bp</td>
</tr>
</tbody>
</table>

1 Primers are listed based on their distance from the TSS (transcription start site)
2 TSS: transcription start site.
Figure 7. Fli-1 binds to the CXCL10 promoter.

MS1 endothelial cells were cross-linked with formaldehyde, chromatin was isolated from the cells and immunoprecipitated with specific Fli-1 or control IgG antibodies. The genomic fragments associated with the immunoprecipitated DNA were amplified by PCR using the primers designed in Table 2. Fold changes (Fli-1 antibody/control IgG) were calculated by RT-PCR and are shown for each primer pair. Data presented are shown as the mean ± standard deviation where a single asterisk indicates p<0.05. Data are representative of three independent experiments.
Fli-1 drives the transcription of CXCL10

DNA transfection assays were performed to determine whether Fli-1 regulates the expression of CXCL10. Using the human CXCL10 promoter, we amplified it using PCR and genomic DNA and cloned the promoter into the pGL3 basic reporter construct. To determine if Fli-1 drives the expression of CXCL10 we transfected increasing dosages of Fli-1 into NIH3T3 embryonic fibroblast cells, which contain no endogenous Fli-1. Looking at the luciferase data in Figure 8, the results demonstrate that as the dosage of Fli-1 increases so does CXCL10 expression within the promoter.
Figure 8. Fli-1 drives activation of CXCL10.

NIH3T3 cells were transfected with murine Fli-1 expression construct and the human pGL4/CXCL10 reporter construct using the FuGene 6 transfection reagent. Increasing amounts of the Fli-1 expression construct (0.025μg, 0.05μg, 0.1μg, 0.25μg, 0.5μg, and 1μg) were transfected into NIH3T3 cells. Values are the mean ± SEM of 3 replicate experiments (n=3). * = P < 0.05.
Mutation of DNA-binding domain of Fli-1 partially impairs transcriptional activation of the CXCL10 promoter

To determine whether Fli-1 is driving transcription of CXCL10 directly or indirectly, we performed another DNA transfection using a Fli-1 binding mutant. The Fli-1 binding mutant contains a mutation to prevent DNA binding to the promoter region. This mutation consists of a single amino acid change from tryptophan 321 to arginine (W to R) [42]. Figure 9 shows that mutating Fli-1 binding domain results in reduced activation of CXCL10 compared to normal Fli-1.
**Figure 9.** Fli-1 regulates CXCL10 through indirect binding of the promoter and with the help of unknown cofactors. NIH3T3 cells were transfected with human Fli-1 expression construct and pGL4/CXCL10 reporter construct using the FuGene 6 transfection reagent. 1µg of human Fli-1 expression construct and 1µg of human Fli-1 binding mutant were transfected into NIH3T3 cells. Values are the mean + SEM of 3 replicate experiments (n=3). * = P < 0.05.
Chapter 5 – Discussion

The innate immune system is our primary line of defense to protect the host from infection from pathogens [22]. Mammalian cells produce inflammatory cytokines and chemokines in response to innate immune signals and their expression is tightly regulated [20, 22]. IFN-gamma Inducible Protein (IP-10), also known as CXCL10, is an inflammatory chemokine belonging to the CXC chemokine family [26]. CXCL10 is chemotactic for many inflammatory cells including macrophages and dendritic cells [26]. Altered expression of CXCL10 is associated with inflammatory diseases, including lupus nephritis and other autoimmune diseases; its association with numerous autoimmune diseases is what drives interest in determining regulatory factors for this inflammatory cytokine [26]. The Fli-1 transcription factor, a member of the Ets gene family, is known to regulate numerous cellular processes, the immune response being one of interest [10]. Fli-1 has also been associated with the pathogenesis of renal injury and SLE, which has been studied in our lab. Previous studies have shown that Fli-1 heterozygous (Fli-1+/−) NZM2410 mice, with reduced expression of the Fli-1 transcription factor, had a marked reduction in nephritis, necrosis, and proteinuria compared to wild-type NZM2410 mice [19]. The Fli-1+/− NZM2410 mice, also had reduced infiltration of inflammatory cells including macrophages, T cells, B cells, and neutrophils in kidneys and survived significantly longer compared with wild-type NZM2410 controls [19]. Our lab has demonstrated that Fli-1 directly regulates expression of inflammatory chemokines and cytokines including MCP-1, RANTES, and IL-6, all of which are associated with
inflammatory diseases [15, 16, 25]. Because Fli-1+/− NZM2410 had significantly decreased infiltration of inflammatory cells, including dendritic cells and macrophages, and IP-10/CXCL10 is known to attract these cells, our hypothesis was that Fli-1 is a critical regulator in directly modulating the expression of CXCL10. Within this study we initially looked at mouse endothelial cells. As mentioned previously, the overexpression of Fli-1 has been implicated in SLE development and is also found in endothelial cells [16, 19, 43]. After LPS stimulation within these cells, we saw an increase in CXCL10 production over 24 hour time period (Figure 5). With the siRNA knockdown of Fli-1 within the same cell type we see significant reduction of CXCL10 6 and 24 hours after LPS stimulation. Similar results have been seen within the G-SCF study where it was determine that Fli-1 was a direct regulator (Lennard Richard et al., unpublished data). In comparing the current data with previous studies we can conclude from this experiment that the partial absence of Fli-1 leads to reduced CXCL10 expression. These results are consistent with our initial observation and first aim that Fli-1 plays a role in regulation CXCL10.

Looking into the mechanism behind the relationship of Fli-1 and CXCL10 we determined that Fli-1 binds to the promoter region of CXCL10 at several different sites based on results from the ChIP Assay (Fig. 7). We have identified that Fli-1 binds to 6 regions within the murine promoter identified by ChIP assay (ChIP3 [-1830 to -1660], ChIP4 [-1677 to -1501], ChIP7 [-1200 to -1027], ChIP8 [-1027 to -858], ChIP9 [-873 to -733], and ChIP10 [-753 to -602]) (Figure 7). Since ChIP 3 and ChIP 4, ChIP 7 and ChIP 8, and ChIP 9 and ChIP 10 sites are
very close to each other we will need to conduct further analysis within the 
CXCL10 promoter to identify which sites Fli-1 actually bind to. Some proposed 
methods of determining specific sites Fli-1 may bind to would be to insert a 
mutation. A mutation will hopefully hinder the binding of Fli-1 and we can narrow 
down the exact location where Fli-1 is binding within the promoter region.

Upon performing the transient DNA transfection, using human Fli-1 
expression construct, luciferase assay results determined Fli-1 drives CXCL10 
activation starting from $0.025 \mu g$ of Fli-1 to $0.1 \mu g$ of Fli-1 (Fig. 8). These results 
clearly show that Fli-1, not only plays a role in the regulation of CXCL10 but 
through binding of the promoter region.. Based on our data from DNA transient 
transfection of the human Fli-1 binding mutant, the mechanism Fli-1 uses to 
regulate the expression of CXCL10 may be both direct and indirect ways.

Mutation of Fli-1’s DNA binding domain, shown in Figure 9, shows an 18% 
reduction of transcriptional activity. This is reduction is significant when 
compared to the human Fli-1 expression construct ($P = 0.00213$). Since the 
reduction is rather low, these results suggest that Fli-1 maybe driving 
transcription largely indirectly and there may be other factors assisting with the 
transcription of CXCL10. These results are very similar to previously published 
literature looking at Fli-1 regulation of MCP-1 [44]. Our lab provided evidence that 
Fli-1 regulated MCP-1 both directly and indirectly due to fact that Fli-1 was 
binding to the promoter MCP-1 and driving transcription in a dose-dependent 
manner [44]; however when the DNA binding mutant was introduced
transcriptional activity was reduced by only 27%, which led researchers to conclude that Fli-1 regulated MCP-1 largely indirectly [44].

Although we were able to provide viable evidence of Fli-1’s role in CXCL10 expression there are a few more areas that we would like to explore with this project. The majority of our work was conducted in pancreatic murine endothelial cells and or fibroblast cells for our DNA transfections; and although these cells were sufficient enough to use for our current work, our goal is to transition to murine and/or human kidney cells in hopes to obtain similar results and gain a greater insight on Fli-1’s role with CXCL10. This would greatly mimic realistic views on the mechanism that is taking place and introduce more authentic environmental factors that may be assisting Fli-1 in transcription.

In drawing conclusions from this study, Fli-1 has been identified as a critical regulator of IP-10/CXCL10 in endothelial cells. While Fli-1 binds to the CXCL10 promoter, the mechanism of activation is mainly through indirect binding and interactions with the promoter. These findings differ from our original hypothesis of Fli-1 being a direct regulator of CXCL10; however the Fli-1 transcription factor is still a critical regulator. This can be seen through results from our first aim, siRNA knockdown of Fli-1 results in a significant reduction in CXCL10 protein expression, and previous studies where Fli-1 is a major regulator of other inflammatory cytokines and chemokines [15, 16, 25, 44]. There are a number of factors that maybe assisting Fli-1 with the transcriptional regulation of CXCL10, these include post-translational modifications and transcriptional co-activators. Our upcoming goals are to identify the co-factors
and post-transcriptional modifications that may be involved through use of proteomics, specifically the MALDI-TOF mass spectrometer and other molecular biology techniques. Many cytokines such as IFN-α and IFN-β are known to stimulate other cytokine production; we also plan to determine if Fli-1 regulates expression of CXCL10 following stimulation with IFN-α and IFN-β. The primary goal of this project was to establish a relationship between Fli-1 transcription factor and CXCL10 due to the fact of there being evidence of Fli-1 being a major chemokine regulator and CXCL10 playing a role in inflammation in autoimmune diseases. Our studies will add more to the list of chemokines that Fli-1 regulates and contribute to further studies of the transcription factor. Lupus nephritis is the leading cause of death in SLE patients today and our current studies will contribute to the molecular and immunological understanding of the disease. CXCL10 is associated with several other inflammatory diseases and role of Fli-1 in these diseases are needed to be explored.

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