5-2013

Analysis of Macrophage--Derived Inflammatory Response in the Presence of Glycosaminoglycans: A Possible Clue on the Role of Inflammation in the Pathogenesis of Morquio A

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https://doi.org/10.33015/dominican.edu/2013.bio.06

Analysis of Macrophage-Derived Inflammatory Response in the Presence of Glycosaminoglycans:
A Possible Clue on the Role of Inflammation in the Pathogenesis of Morquio A

A thesis submitted to the faculty of

Dominican University of California

&

BioMarin Pharmaceutical Inc.

in partial fulfillment of the requirements

for the degree.

Master of Science

In

Biology

By

Heather Drew

San Rafael, California

May, 2013

CERTIFICATION OF APPROVAL

I certify that I have read Analysis of Macrophage-Derived Inflammatory Response in the Presence of Glycosaminoglycans: A Possible Clue on the Role of Inflammation in the Pathogenesis of Morquio A by Heather Drew, and I approve this thesis to be submitted in partial fulfillment of the requirements for the degree: Master of Sciences in Biology at Dominican University of California and the Buck Institute of Aging.

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TABLE OF CONTENTS

ABSTRACT

Mucopolysaccharidoses is a family of metabolic disorders caused by the absence or irregular functioning of lysosomal enzymes required to degrade glycosaminoglycans. Mucopolysaccharidosis IVA (MPS IVA or Morquio A syndrome) is characterized by the functional loss of the enzyme N-acetylgalactosamine-6-sulfatase (GALNS). The absence of GALNS leads to the chronic accumulation of the glycosaminoglycans keratan sulfate (KS) and possibly chondroitin-6 sulfate (C6S). KS accumulation occurs in the lysosomes of KS-rich tissues such as cartilage, cornea, and heart valve as well as some specific cell types such as macrophages. This suggests that MPS IVA is a systemic disease that may disrupt cellular homeostasis throughout the body.

Recent work on mucopolysaccharidoses and KS has produced two opposing models to explain the repercussions of KS accumulation. Although both theories implicate a dysregulation of immune functioning, specifically in regards to inflammatory response, one theory assumes that KS is (like other GAGs) in that it is pro-inflammatory while the other theory suggests that it is anti-inflammatory. In this study, two macrophage cell lines— the human acute monocytic leukemia cell line THP-1 and human monocyte-derived macrophages (MDMs))— are used to investigate the roll of KS in inflammatory response. Initial findings suggest that human macrophages respond to KS at pathological but not physiological levels. Unlike chondroitin sulfate (CS) and lipopolysaccharide (LPS), elevated levels of KS suppress NFkB activation and do not induce a significant release of inflammatory factors. Though KS does not affect cell viability or promote apoptosis, it does inhibit

mitochondrial metabolic activity and the proliferation/differentiation rate of MDMs. These results indicate that, unlike CS, KS does not appear to induce an increase of inflammatory response in human macrophages. In fact, KS may modulate inflammatory response in an immunosuppressive manner thereby suppressing the immune system of Morquio A patients.

Acknowledgments

The following research would not have been possible without the encouragement and support from many individuals within both Biomarin Pharmaceutical and Dominican University of California. Dr. Sibdas Ghosh receives both high praise as many thanks for inviting me to join the Graduate Program in Biological Sciences in the autumn of 2011 and further encouraging me in my pursuit to be successful in both the classroom and the lab. Dr. Michael Vellard, my mentor and the primary investigator of the Cellular and Molecular Biology Lab of the Research and Development Department of Biomarin as well as adjunct professor at Dominican University was also integral in accepting me into the wonderful CMOB lab as well as inspiring me to make the extra effort in both my work under his supervision and my future pursuits as a researcher. Without Dr. Vellard's mentorship the story of this project might never have been told.

Both Dr. Yiming Zhu and Dr. Andrzej Swistowski are due great praise as they took on the role of supervisors and role models for the scientist that I one-day hope to become. Particularly Dr. Zhu who dedicated many hours to my training and provided great support throughout the Master's program. I would also like to thank all of the members of the CMOB lab, particularly Terrence Satterfield, Terri Christianson, Ben Schooler, Gouri Yogalingam, Shinong Long and Melanie Lo. Their support and guidance throughout the program and during this research project was invaluable and the environment created by such an amazing group will be greatly missed but always remembered. I would also like to thank Vice President of Research & Development at Biomarin Gordon Vehar whose continued support of the collaboration with Dominican University will allow future students to conduct research and train at such a fantastic institute.

I would lastly like to thank my friends and family for their continual support in my academic endeavors, even though they may take me farther from home than they would like me to be. The friendship of Lupe Sierra not only encouraged me to make the leap to California but to also finish the program and make time to have some fun while on the west coast.

And lastly, Go Gators!

ABBREVIATIONS

CS: Chondroitin Sulfate

C6S: Chondroitin-6-Sulfate

DS: Dermatan Sulfate

ECM: Extra Cellular Matrix

ELISA: Enzyme-linked Immunosorbent Assay

ERT: Enzyme Replacement Therapy

FBS: Fetal Bovine Serum

GAGs: Glycosaminoglycans

GALNS: N-acetylgalactosamine-6-sulfatase

GM-CSF: Granulocyte-Macrophage Colony-Stimulating Factor

GROα: Chemokine (C-X-C motif) ligand 1, melanoma growth stimulating activity alpha

IL-(6,8): Interleukin

IL-1Rα: Interleukin 1 Receptor Antagonist

IP 10: Interferon Gamma-Induced Protein 10

KS: Keratan Sulfate

KSPG: Keratan Sulfate Proteoglycan

LPS: Lipopolysaccharide

LSDs: Lysosomal Storage Disorders

MCP1: Monocyte Chemotactic Protein-1

M-CSF: Macrophage Colony-Stimulating Factor

MDMs: Monocyte-Derived Macrophages

MIP 1β: Macrophage Inflammatory Protein-1β

MMP (1, 2, 9, 10): Matrix Metalloproteinase

MPS: Mucopolysaccharidosis

MSD: Meso Scale Discovery

NFkB: Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells

PMA: Phorbol 12-Myristate 13-Acetate

qPCR: Quantitive Real-Time Polymerase Chain Reaction

RANTES: Regulated and Normal T Cell Expressed and Secreted

SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

TARC: Thymus and Activation Regulated Chemokine

TIMP-1: Tissue Inhibitor of Metalloproteinases 1

TLR4: Toll-like Receptor 4

TNFα: Tumor Necrosis Factor Alpha

TUNEL: Termical Deoxynucleotidyl Transferase dUTP Nick End Labeling

WST-1: Water Soluble Tetrazolium

INTRODUCTION

Overview

Mucopolysaccaridoses IVA (MPS IVA or Morquio A syndrome) is an autosomal recessive disorder characterized by the functional loss of the enzyme N-acetylgalactosamine-6-sulfatase (GALNS). Although very little is known about the pathogenesis of Morquio A syndrome, it is characterized by the chronic accumulation of the glycosaminoglycans keratan sulfate (KS) and, to a lesser extent, chondroitin-6 sulfate (C6S). This accumulation is known to be the first step in the pathology of this disease.

Numerous studies (6; 19; 20; 21) have shown that inflammation plays a very important role in the systemic manifestation of many lysosomal storage disorders. Specifically, it has been shown that the accumulation of other GAGs in other MPS disorders (such as dermatan sulfate in Maroteaux-Lamy syndrome (MPS VI) and chondroitin 4,6 sulfate in Sly syndrome (MPS VII)) have led to an increase in inflammation (19). On the other hand, several studies have shown that KS could play the role of an anti-inflammatory agent. One study in particular has shown that KS is a promising candidate for rheumatoid arthritis therapy due to its ability to ameliorate chronic inflammation upon direct injection in affected joints (Hayashi et. al., 2010).

As KS accumulation is the most characteristic phenomena in Morquio A, it is very important to know if KS provokes either pro- or anti-inflammatory responses. In order to analyze the KS-specific modification patterns in inflammation, we have developed two *in vitro* macrophage cell models using the monocytic phorbol 12-

myristate 13-acetate(PMA)- differentiated THP-1 cell line and monocyte-derived macrophages purified from whole human blood. The macrophages, which are known to accumulate KS in Morquio A and modulate many inflammatory pathways *in vivo*, were treated with pathological levels of either KS, aggrecan, CS, or LPS. Both CS and LPS were previously shown to be pro-inflammatory while the proteoglycan aggrecan was chosen for its characteristic KS and CS side chains. In this study, KS exhibited effects opposite to those found with other GAGs and LPS. Unlike CS and LPS, KS suppressed NFkB activation and did not induce a significant release of inflammatory factors. While KS failed to affect cell viability or promote apoptosis, it did significantly inhibit mitochondrial metabolic activity and the proliferation/differentiation rate of MDMs. The GAG-specific results indicate that, unlike CS, KS does not appear to induce an increase of inflammatory response in human macrophages but acts in a more immunosuppressive manner. This further supports the theory that KS acts on a receptor which induces the down-regulation of inflammatory response in macrophages.

Background

Morquio A (MPS IVA)

Morquio A, also known as MPS IVA Syndrome, was first identified and characterized in 1929 (Morquio, 1929). The symptoms exhibited by individuals suffering from this disease included hearing loss, aortic valve malfunction, abnormal bone development, liver enlargement, and even premature death, depending upon the severity of the disease (Northover et al., 1996). Almost 30 years later, the term mucopolysaccharide was coined when liver samples from patients with MPS disorders contained high levels of hexosamine, uronic acid, and sulfate (Brante, 1952). Soon after, keratan sulfate was shown to be at elevated levels in the urine of MPS IVA patients (Pedrini et al., 1962); and the enzyme responsible for metabolizing keratan sulfate, GALNS, was identified and shown to be lacking in the fibroblasts of MPS IVA patients (Matalon et al., 1974). Histochemical studies revealed that MPS IVA is a disease that affects cartilage, resulting in disorganized, irregularly scattered chondrocytes that appear swollen with cytoplasmic vacuolation (Bona et al., 1971). Other symptoms of the disease include aortic valve disorders, loss of hearing, abnormal bone development and liver enlargement followed by death at an early age depending on the severity of the disease although cognitive functions are not impaired (Northover et al., 1996). Initially, diagnosing MPS IVA depended solely on determining keratan sulfate levels in the urine (Kaplan et al., 1968). However, identification of 148 unique genetic mutations contributing to the manifestation of MPS IVA (Morris et al., 1994) has led to more accurate diagnoses. The molecular mechanisms contributing to the systematic pathology of

the disease remains elusive. Although this orphan disease is rare it has a devastating impact on the lives of those who have it.

Glycosaminoglycans and the Accumulation of Keratan Sulfate in MPS IVA

KS is one of the many members of the glycosaminoglycan family, which also includes dermatan sulfate, heparan sulfate, heparin, hyaluronic acid, and chondroitin sulfate. They are important components of proteoglycans (Figure 1) that exist in the extracellular matrix (Jones, 1994).

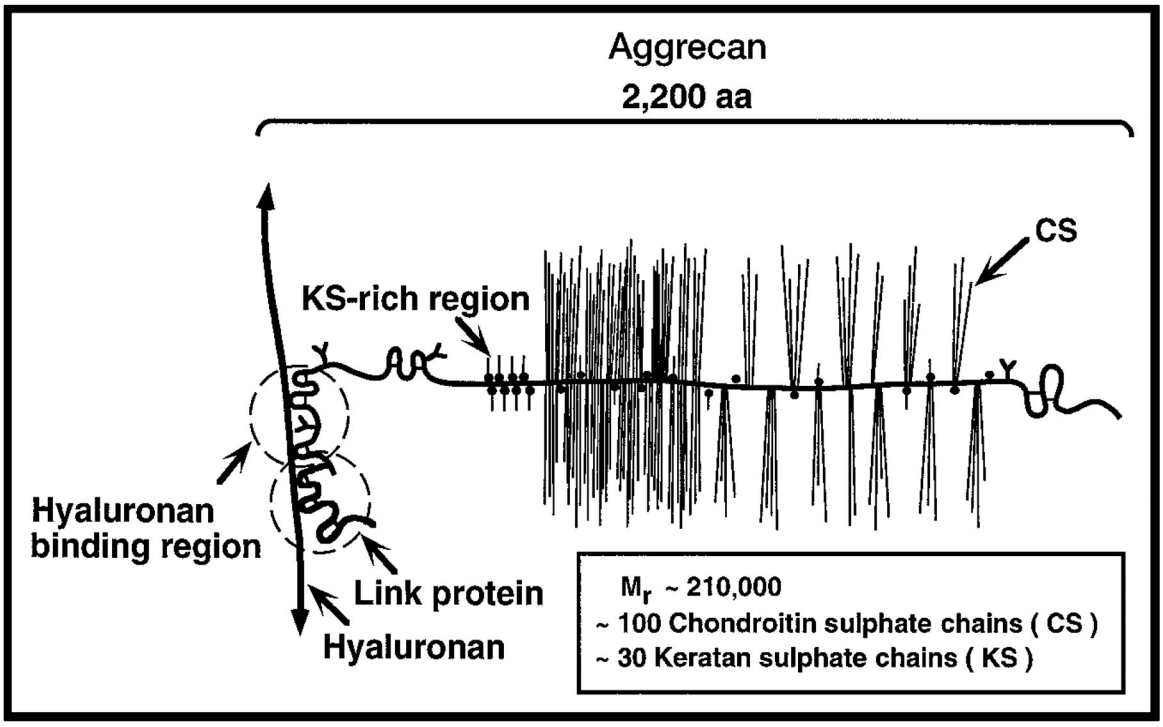


Figure 1: Representation of the proteoglycan aggrecan monomer with keratan sulfate and chondroitin sulfate glycosaminoglycan side chains attached to the protein core. The monomer is attached to nonsulfated glycosaminoglycan hyaluronan and is stabilized at its binding region by link protein. Numerous monomers attach to hyaluronan to form the large protein aggregate *(Figure from Royce PM, Steinmann B, eds. Connective Tissue and Its Heritable Disorders: Molecular, Genetic, and Medical Aspects. New York, New York: Wiley-Liss; 1993:193).*

Glycosaminoglycans (GAGs) are unbranched polymers of repeating disaccharide units composed of a hexosamine (glycosamine) and a non-nitrogenous

sugar. GAGs may be categorized depending upon whether their hexosamine is N-acetylgalatosamine (GalNAc; which is found in chondroitin and dermatan sulfate) or N-acetylglucosamine (GlcNAc; which is found in hyaluronic acid, keratan sulfate, heparan sulfate, and heparin) (Jones, 1994). GAGs are covalently bound to a protein core to form a proteoglycan (Ross & Wojciech, 2006). The individual GAGs extend from the core in a brush-like structure and are connected to either a serine or threonine residue via a uronic acid-trisaccharide-O linkage. Keratan sulfate is unique is that it may also bind to the protein core via an N-asparaginyl bond (Figure 1). GAGs are highly negative charged and yield viscous, mucin-like solutions, hence their original name, mucopolysaccharides (Jones, 1994). This high viscosity is accompanied by a resistance to compressibility and adds rigidity to the matrix, making proteoglycans ideal for lubricating and protecting the joints as well as providing structural integrity to surrounding cells. Proteoglycans compose the ground substance of the ECM and are largely relied on for both their structure and function due to their ability to sequester water within their negative side chains (Ross & Wojciech, 2006).

The proteoglycans lumican (composed of keratan sufate chains) and aggrecan (composed of CS and KS chains) are found in the cornea and in collagenous matrices throughout the body. Several aggrecan proteoglycans form a complex with the nonsulfonated GAG hyaluronan, and this complex is a major component of the articular cartilage. In this study, aggrecan is of greater interest due to its prevalence in the cartilage, its high sulfonation patterns in the KS side chains (which may

regulate the potency of KS), and its unique composition of both CS (a known inducer of inflammation) and KS (Jones, 1994).

MPS IVA is characterized by a deficiency of the enzyme N-acetlygalactosamine-6-sulfatase (GALNS) which is responsible for catalyzing a stepwise degradation of keratan sulfate and chondroitin-6 sulfate (Jones, 1994). When GALNS is absent, keratan sulfate and chondroitin-6 sulfate are not degraded and accumulate in the lysosomes of KS-rich tissues such as cartilage, cornea and heart valve (Northover et al., 1996) and in cell types, like macrophages, which play an important role in systemic inflammation, suggesting that MPS IVA is most likely a systemic disease (Vogler et al., 1987). The continued production and intra-lysosomal storage of GAGs gives rise to a detrimental change in internal pH and a dramatic enlargement of the lysosomes., These eventually lead to cell and tissue dysfunction as well as organomegaly (enlargement of the organs) as the cells continue to occupy an increasing area of the cytoplasm (Jones, 1994; Royce & Steinmann, 1993). Our current investigation of the GAG-driven inflammatory modulation aims to better explain the mechanism behind these symptoms.

Altered Immune Regulation in the Pathogenesis of Mucopolysaccharidoses

The family of metabolic disorders classified as mucopolysaccharidoses are caused by the total or partial functional loss of a lysosomal enzyme required to degrade a specific glycosaminoglycan. Lysosomes, which are frequently described as the “recycling centers” of the cell, process unwanted material and cellular debris into useful substances and nutrients within the cytosol of the cell. These organelles

are responsible for the catabolism of endogenous and exogenous macromolecules, thus performing a particularly important role in maintenance of cellular homeostasis. In all MPS disorders, the disruption of lysosomal functioning leads to systemically progressive and permanent cellular damage with varied degrees of severity. This tissue damage can lead to organomegaly (particularly of the liver, spleen and heart), hernias, joint stiffness and frequent respiratory infections (Simonaro, 2010).

Because many of these symptoms are linked to the deregulation of immunological pathways, much research has been done to further analyze GAG-induced immunological perturbations. Inflammatory factors such as cytokines, matrix metalloproteinases and nitric oxide have been suggested as biomarkers and/or therapeutic targets for MPS bone and joint disease (Coutinho et al., 2011). Outside of MPS disorders, GAGs have been implicated in the altered inflammatory response found in cancer (Afratis et al., 2012), type 1 diabetes (Olga et al., 2012) and disorders of the vascular endothelium (Rix et al., 1996). Antigen-presenting cells, such as macrophages, have been shown to be regulated by high levels of GAGs in their surrounding microenvironment (Wrenshall et al., 1999) as well as accumulate GAGs in their lysosomes (Winmaier et al., 2004). This evidence that keratan sulfate-containing GAGs mediate immunological functioning in many varied and prominent disorders indicates that keratan sulfate is a regulator of the immune system in Morquio A and other diseases.

The Pro-Inflammatory Model

Inflammation, the natural immune response to nocuous stimuli, is an antagonistically pleiotropic process, in that although it is extremely important for an organism's survival, it can also have detrimental effects (Coppé et al., 2012). When inflammatory response is prolonged and cell-derived mediators such as cytokines and chemokines are constantly released into the cellular milieu (as seen in osteoarthritis, for example), surrounding tissue is damaged (Ross & Wojciech, 2006). Phagocytes, such as macrophages, are key mediators in inflammatory responses (Winmaier et al., 2004). Previous studies have shown that patients suffering from inherited metabolic storage disorders such as MPS have an increased incidence (33%) of pulmonary complications as well as increased levels of inflammatory chemokines, including TNF-α, MIP-1α, and G-CSF (Kharbanda et al., 2006). Other work indicates that GAG storage dysfunction found in animal models of MPS stimulate—via inflammatory cytokines and nitric oxide— chondrocyte apoptosis, matrix degradation, elevated proliferation of synoviocytes, increased levels of transforming growth factor-beta (TGF-β) (most likely to counteract the cellular apoptosis and matrix degradation), and altered matrix metalloproteinase (MMP) expression , (Simonaro et al., 2005). In a more focused study, the synovial fibroblasts and fluid of MPS animals showed increased expression of many inflammatory molecules, particularly those related to the signaling networks of lipopolysaccharide (LPS) and the Toll-like receptor 4 (Figure 2) (Simonaro et al., 2008). The Toll-like receptor 4 (TLR-4) plays an integral role in the activation of innate immunity due to its hyporesponsiveness to LPS (a molecule with some

structural components similar to GAGs) and may be a relevant cellular pathway to study in MPS disorders (Takeda & Akira, 2004). With significant evidence that altered inflammatory regulation is a ubiquitous characteristic of MPS disorders, it seems relevant to specifically look at its role in MPS IVA pathology.

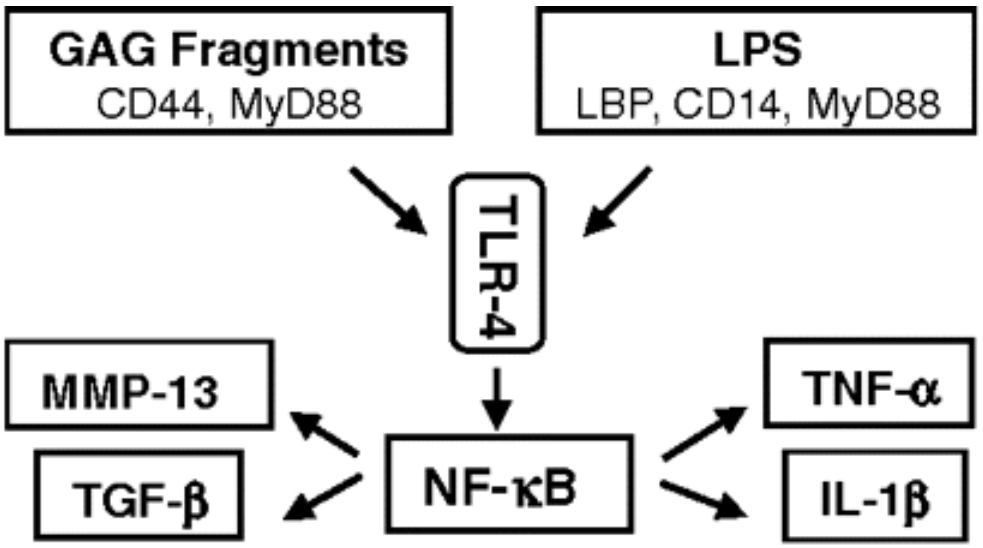


Figure 2: TLR4 signaling can be induced by two pathways, LPS represents the classic pathway and is characterized by interactions with the adaptor protein, myeloid differentiation factor-88 (MyD88), LPS binding protein (LPB), CD14 and CXCR4. The second pathway is activated by GAG fragments via MyD88 and CD44. Both pathways can affect the activation of NF-kB (a mediator of DNA transcription) that, in turn, controls the production of inflammatory-related factors *(Figure from Simonaro CM, Ge Y, Eliyahu E, He X, Jepsen KJ, et al. (2010) Involvement of the Toll-like receptor 4 pathway and use of TNF-alpha antagonists for treatment of the mucopolysaccharidoses. Proc Natl Acad Sci 107: 222–7.)*

The Anti-Inflammatory Hypothesis

While KS is a member of the glycosaminoglycan family that has been shown to induce inflammatory response, it is important to note that KS has yet to be proven inflammatory and that KS is unique compared to other GAGs. This uniqueness is largely due to KS having features common to both GAGs and glycoproteins, the substitution of galactose in place of uronic acid, and high heterogeneity based on variations in chain length and the amount and distribution of sulfate groups

(Morquio, 1929). There are multiple recently published articles that have found KS to exhibit anti-inflammatory properties in such a robust fashion that it may prove to be therapeutic. Two independent studies found that KS suppressed the production of the inflammatory factors IL-12 (Xu, H et. al., 2005) and MMP2 and MMP9 (Isnard N, et. al., 2003). KS has also been shown to attenuate lung inflammation (Yoshida T, et. al., 2010) and suppress cartilage damage as well as ameliorate inflammation in a mouse arthritis model (Hayashi M, et. al., 2011). Another study found that the presence of KS side chains on the proteoglycan aggrecan (which also contains CS side chains) prevents arthritogenic T cell response and protects against the development of arthritis (Glant, et al., 1998). In regards to apoptosis, Nakayama and colleagues have found that in lymphoma cell lines, radiation-induced apoptosis was significantly reduced when KS was present in the microenvironment (Nakayama et al., 2012). Clinical reviews have also found that Morquio A patients are prone to respiratory infections, slow surgery recovery time, and surgery-related complications such as pin site infections and pressure sores under supportive halo body jackets (Tomatsu et al., 2011). These symptoms and secondary infections indicate that immune functioning in patients with Morquio A may be chronically suppressed, thus supporting the hypothesis that KS has anti-inflammatory properties. Although KS-mediated inflammatory dysfunction could be a key component to the pathogenicity of MPS IVA, the biological functions of KS are not well known and little studied, particularly at a pathogenic level. KS-mediated inflammatory dysfunction could be a key component to the pathogenicity of MPS IVA and both pro- and anti- inflammatory theories should be explored.

The Macrophage as a Cell Model

Because of its role in the innate immunological response and its accumulation of glycosaminoglycans (Reuser & Drost, 2006), the macrophage is an ideal cellular model for this study and in fact, may be crucial in the pathogenesis of MPS IVA (Ross & Wojciech, 2006). Two common candidates for a macrophage model system are mature macrophages obtained directly from MPS IVA patients and macrophages differentiated from pluripotent cells originally derived from patient fibroblasts. While these two cell lines may seem ideal as they naturally do not express GALNS, we chose not to use cells displaying the disease phenotype so as to more accurately assess initial cell reactions in the presence of high levels of keratan sulfate. Early cellular response to changes in the extracellular matrix may be difficult to elucidate if the macrophages have the preexisting condition of KS accumulation. Using diseased macrophages would also prove difficult in establishing LPS as a positive control treatment. The cell's reaction to high levels of KS cannot be reliably compared to CS, aggrecan or LPS treatment if these do not accumulate within the macrophage as KS does.

The human acute monocytic leukemia cell line THP-1, however, was an ideal model system for our study as it exhibits macrophage-like characteristics upon differentiation (e.g. phagocytosis), provides a continuous culture when grown in suspension, has an average doubling time of 35 to 50 hours (Tsuchiya et al., 1980), and does not accumulate high levels of KS. Another model system used in our study were macrophage cells derived from human monocytes purified from whole blood. Although the yield of cells may be lower, macrophages differentiated from purified

monocytes are more similar to *in vivo* macrophages than are.THP-1 cells. From each monocytic cell source macrophages were differentiated and cultured (PMA-differentiated THP-1 macrophages and monocyte-derived macrophages (MDMs), respectively) until treatments. Although the cells used in this study still had a functioning GALNS gene, and therefore would not accumulate KS inside the cell, accumulation of KS in the extracellular environment could still allow us to determine the kinetics and pathology of KS-mediated cell response. In fact, robust results seen in normal cells may represent only secondary, and perhaps milder, effects of KS accumulation. This environmental accumulation also allows for a more precise analysis of the initial reaction that macrophages have in the presence of pathological levels of KS. Diseased macrophages with preexisting KS accumulation might not produce clear results of initial alterations to transcriptional pathways, factor release or metabolic functioning as a result of GAG accumulation. For instance, our positive control LPS (a known inducer of inflammatory pathways) might not function as a clear positive control treatment if the macrophages are also naturally accumulating KS. In this way, the THP-1 cell and MDMs provide a comparable and measurable in vitro platform to further understand initial KSPG-mediated cellular response in macrophages.

MATERIALS AND METHODS

Cells and Reagents

The human acute monocytic leukemia cell line THP-1 was purchased from ATCC (TIP-202™) and cultured in RPMI-1640 (ATCC, Manassas, VA) with 10% heat-inactivated fetal bovine serum (Invitrogen, Grand Island, NY). Phorbol 12-myristate 13-acetate (PMA), LPS, bovine CS and bovine aggrecan were purchased from Sigma-Aldrich (St. Louis, MO). Human monocytes (AllCells, LCC, Emeryville, CA) were cultured in RPMI-1640 with 10% fetal bovine serum. Bovine keratan sulfate proteoglycan (KSPG) was purchased from US Biological (Marblehead, MA). LPS, bovine CS and bovine aggrecan were obtained from Sigma Aldrich (St. Louis, MO). Human recombinant M-CSF was purchased from Peprotech (Rock Hill, NJ). The NFkB and pNFkB polyclonal rabbit antibodies for western blotting and cell staining were obtained from Cell Signaling Technology (Danvers, MA).

Human Monocyte Purification

Human whole blood was purchased from AllCells, LCC. Initially, peripheral blood mononuclear cells were obtained by Ficoll gradient centrifugation. Later, monocytes were isolated using Human Monocyte Isolation Kit II (Miltenyi Biotec, Auburn, CA) and AutoMACSpro cell separator per instruction.

Human Monocyte Differentiation and Treatment

Human monocytes were plated at 0.5 x 10^6 per well in a 24-well plate. After seven days of differentiation with 40 ng/ml macrophage colony stimulating factor (M-CSF)

(Peprotech), monocyte-derived macrophages were treated with various doses of KSPG, CS, aggrecan or LPS. After 24 hrs, cell culture media and cell lysates were collected. Chemokine release, cytokine release, and signaling pathways were analyzed by MSD multiplex assays per instruction (Meso Scale Discovery, Gaithersburg, MD), western blotting (iBlot; Invitrogen) and confocal imaging, respectively.

THP-1 Cell Differentiation and Treatment

The THP-1 cells were plated and differentiated at a concentration of $0.3x10^6$ cells/ml with 50 ng/ml PMA for 24hr and rested in growth medium for another 48 hr. Cells were treated with various stimuli. Cell supernatants and lysates were collected and analyzed by MSD multiplex assays per instruction and western blotting.

mRNA Detection Through Quantitative Real-time Reverse Transcriptase PCR (qRT-PCR)

Human MDMs were treated with indicated GAGs at pathological levels. Cells were lysed, and total (?) RNA content was isolated using Qiagen RNeasy Plus mini kit (Valencia, CA). Reverse transcription of 1ug RNA to cDNA was carried out according to instructions using the Superscript Vilo cDNA Synthesis kit (Life Technologies, Grand Island, NY). Quantitative real-time PCR was performed using Applied Biosystem TaqMan assays (Invitrogen) per instruction in conjunction with the LightCycler 480 Real-Time PCR System (Roche, Indianapolis, IN) for *Chemokine (C-*

X-C motif) ligand 1 (CXCL1 or GROα), *Chemokine (C-C motif) ligand 4 (CCL4 or MIP-1β)*, *Tumor necrosis factor*-alpha (TNF-α), *Interleukin 8 (IL-8)*, and *Interleukin 6 (IL-6)* and data was normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

WST-1 Assay of Macrophage and Monocyte Metabolic Functioning

Cells were plated at 0.5 x10^6 per well in 24-well plates and treated as indicated for 24hr. Metabolic rate was then measured using WST-1 assay (Roche) as instructed. Cells were incubated with WST-1 reagent for 30 min at 37°C. Culture medium was then transferred to a 96-well plate and absorbance was measured at 450nm. The WST-1 assay s cellular metabolic activity via NAD(P)H-dependent cellular oxidoreductase enzymes and reflects the mitochondrial activity of viable cells.

Cell Growth Assay of Stimulated Macrophages

Cell growth rate was assessed using the xCELLigence System (Roche). Human monocytes were plated in a 96-well E-plate at 0.4 x10^6 per well. After seven days of differentiation, cells were treated with indicated stimuli. Cell growth was recorded for a period of 24 hr and analyzed by RTCA software (Roche).

TUNEL Staining for Apoptosis

Human monocytes were plated in a 4-well chamber slide (Thermo Scientific, Rochester, NY) at 0.2 x10^6 per well. After seven days of differentiation, cells were treated with the negative control PBS and pathological levels of KSPG and the

positive control staurosporine for 24 hr. TUNEL staining was performed using DeadEnd Fluorometric TUNEL System (Promega, Madison, WI) per manufacturer's protocol. Images were captured by fluorescent microscopy and analyzed using CellProfiler 2.0 (Company, city, State).

RESULTS

The THP-1 Cell Line

Release of Immune Factors in GAG-stimulated THP-1 Cells

Unlike CS and LPS, KSPG fails to induce a pro-inflammatory response in PMA-differentiated THP-1 cells

In order to evaluate the inflammatory response of macrophages in a GAG-rich environment the agent, PMA was used to induce the differentiation of THP-1 cells to a macrophage-like phenotype. After the cells were plated at 0.3 x 10^6 cells/ml and allowed to fully differentiate for 72 hr, they were treated with pathological levels of PBS, KS 10 μg/ml, CS 10 μg/ml, aggrecan 10 μg/ml or LPS 10 ng/ml for a 24-hr period. The media from the cells were then assessed for levels of known inflammatory matrix metalloproteinases (MMPs), chemokines and cytokines (see Table 1). The release of individual factors was compared to the PBS-negative control, and a fold increase or decrease was computed with the warmest colors (red) indicating large fold increase and cooler colors (grey) indicating a negative or no fold increase. With the exception of IL-1Ra, 10 μg/ml KSPG resulted in little to no release of immune factors (as indicated by the numerous grey cells in Table 1. The significant fold increase of IL-1Ra (an IL-1 receptor antagonist and anti-inflammatory factor) over the PBS control treatment (a 18.9 fold increase) was not seen in cells treated with CS, LPS or aggrecan. A significant but slight increase in tissue inhibitor of metalloproteinase 1 (TIMP-1) and a significant but slight decrease in chemokine (C-X-C motif) ligand 1 (GROa) were also seen in KSPG-treated. This

contrasts with the effects seen in LPS treated macrophages, wherein the expression of numerous immune factors showed a ≥50 fold increase. The increase in production of IL-1Ra is of interest as it is the only factor to have a high fold increase in KS-treated cells and has been shown to have clear anti-inflammatory characteristics. Unlike KSPG, CS induced a robust inflammatory multi-factor response with some similarities to that of the positive control LPS. As seen in Table 1, aggrecan (which has a mixture of CS and KS) induced a moderate response in the cells. These early findings indicate that KS-macrophage interactions may be unique

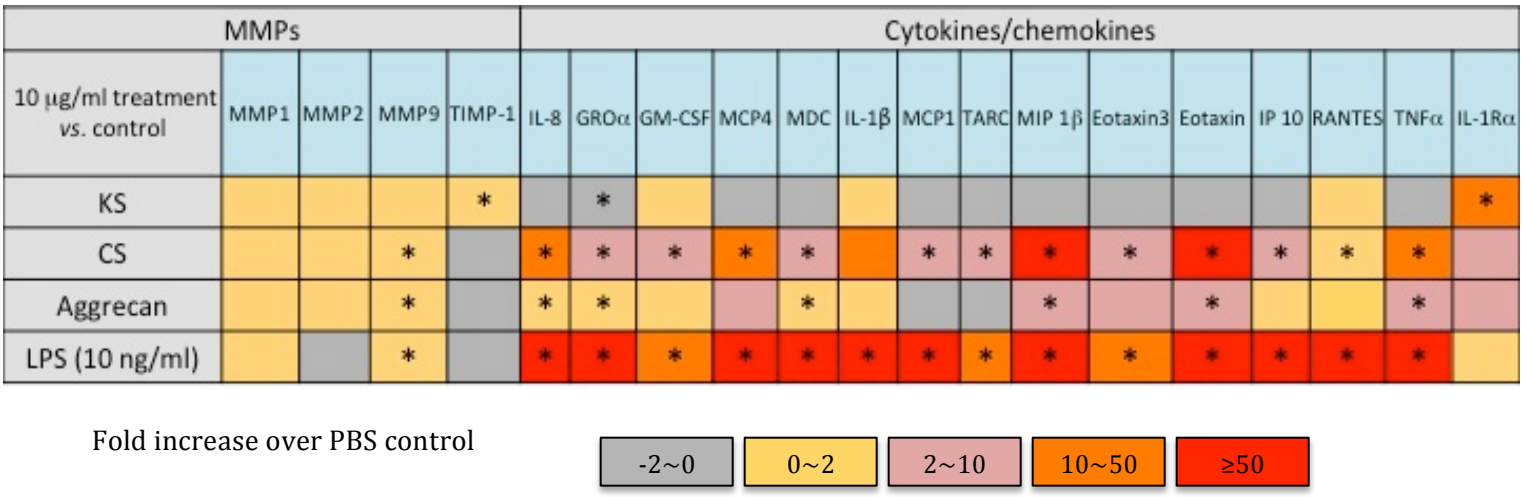


Table 1: Release of immune factors in PMA-differentiated THP-1 cells in response to KSPG, CS, aggrecan and LPS. Cells were treated with indicated stimuli for 24hr. Cell medium was collected and analyzed for indicated factors by MSD multiplex assays, *p<0.05

to the effects of other GAGs when under similar conditions.

KSPG Regulation of NFkB Activation in PMA-differentiated THP-1 Cells

KSPG and CS have Opposing Effects in Time-Dependent NFkB Activation

We next assessed the effect that high levels of KS have on the activation of NFkB— a downstream signaling molecule of the TLR4 pathway and a known transcription

factor of inflammatory response. NFkB was also previously shown to be activated by other GAGs, particularly CS and LPS (Simonaro, 2010). In a time course experiment, there was significant activation of NFkB with acute exposure (30 min) of either CS, aggrecan or LPS (Figure 3A and 3B), which is consistent with previous reports (Simonaro, 2010; Simonaro et al., 2010; Eliyahu et. al., 2011).

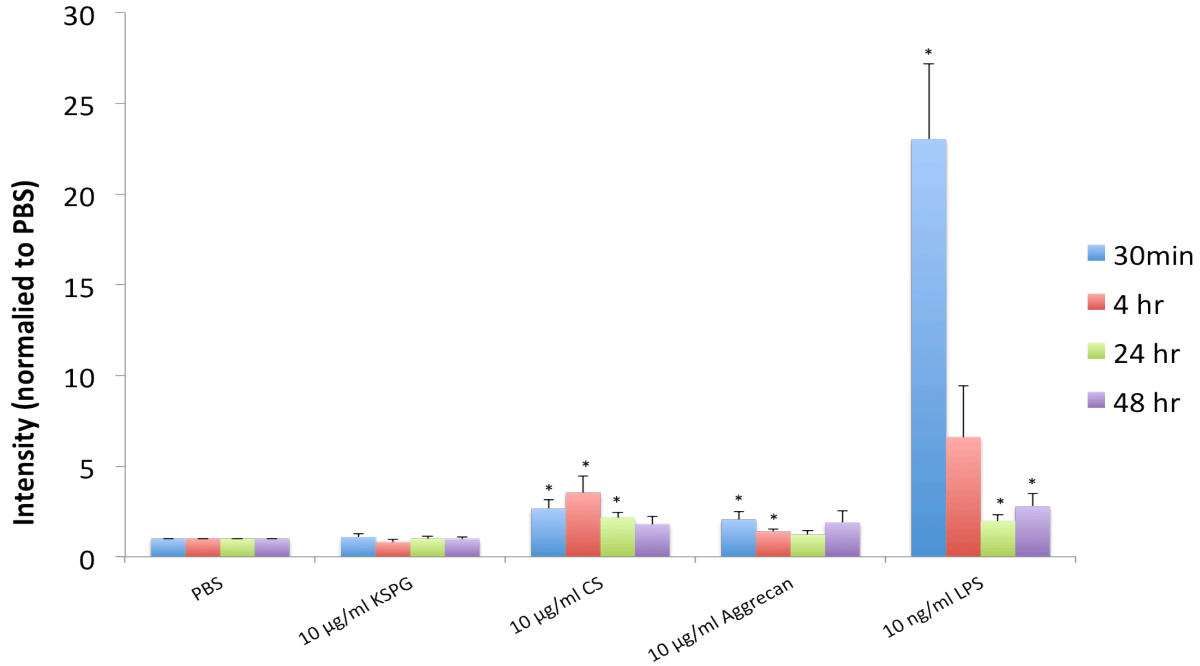


Figure 3A: Time-dependent activation of NFkB in response to CS, aggrecan and LPS but not KSPG. PMA-differentiated THP-1 were treated with indicated stimulus for up to 48 hr. Activation of NFkB was analyzed in cell lysates using antibodies for phosphor-NFkB and total NFkB (loading control) by immunoblotting. Band intensity was normalized to corresponding loading control and presented at fold of PBS treatment. N=4, *p<0.05

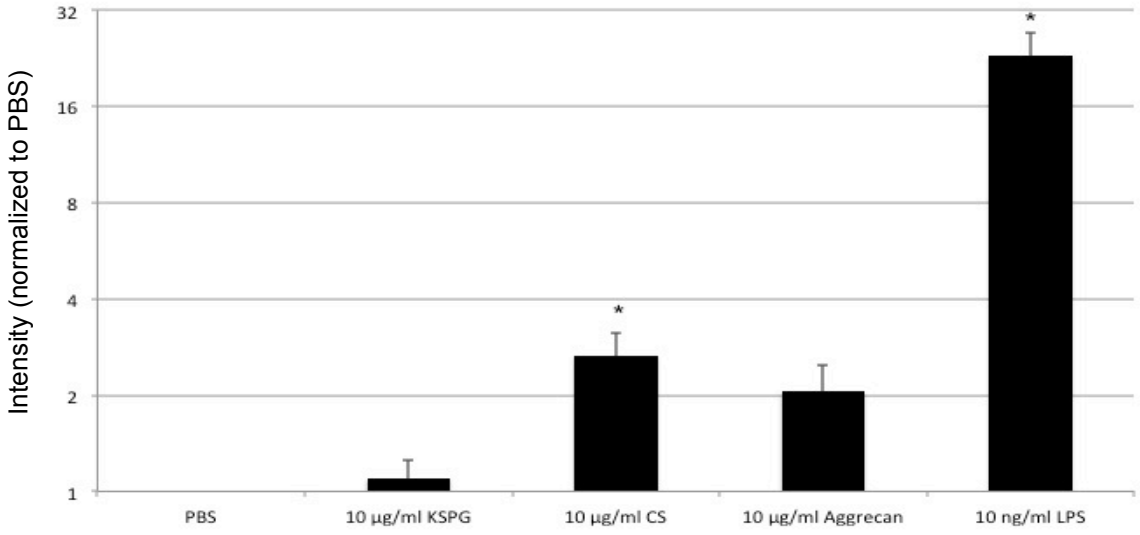


Figure 3B: Time-dependent activation of NFkB in response to CS, aggrecan and LPS but not KSPG. PMA-differentiated THP-1 were treated with indicated stimulus for 30 minutes. Activation of NFkB was analyzed in cell lysates using antibodies for phosphor-NFkB and total NFkB (loading control) by immunoblotting. Band intensity was normalized to corresponding loading control and presented at fold of PBS treatment. N=4, *p<0.05

As expected, the most robust activation of NFkB was observed with LPS, which induced a peak response within the first 30 minutes. Activation of NFkB in response to CS was slower than that of LPS, with maximal activity observed at 4 hours. Aggrecan, with both CS and KS chains attached to its protein core, also activated NFkB but with weaker response when compared to CS. KSPG showed minimal effects on NFkB activation within the 48 hr exposure when compared to the other GAGs. The robust activation of NFkB by CS, aggrecan and LPS, but not KSPG, correlated positively with the release of downstream pro-inflammatory factors (Table 1).

Pathological Doses of KSPG Do Not Affect Cell Viability

In order to assess the effects that high levels of KS have on macrophage viability, mitochondrial functioning was analyzed using a WST-1 assay. After a 24-hr GAG treatment, no significant cytotoxicity was observed in THP-1 cells treated with pathological levels of KSPG, CS or LPS (Figure 4). While a slight increase in mitochondrial functioning was seen in LPS and CS treated cells, there was no indication that any of the treatments significantly affected viability.

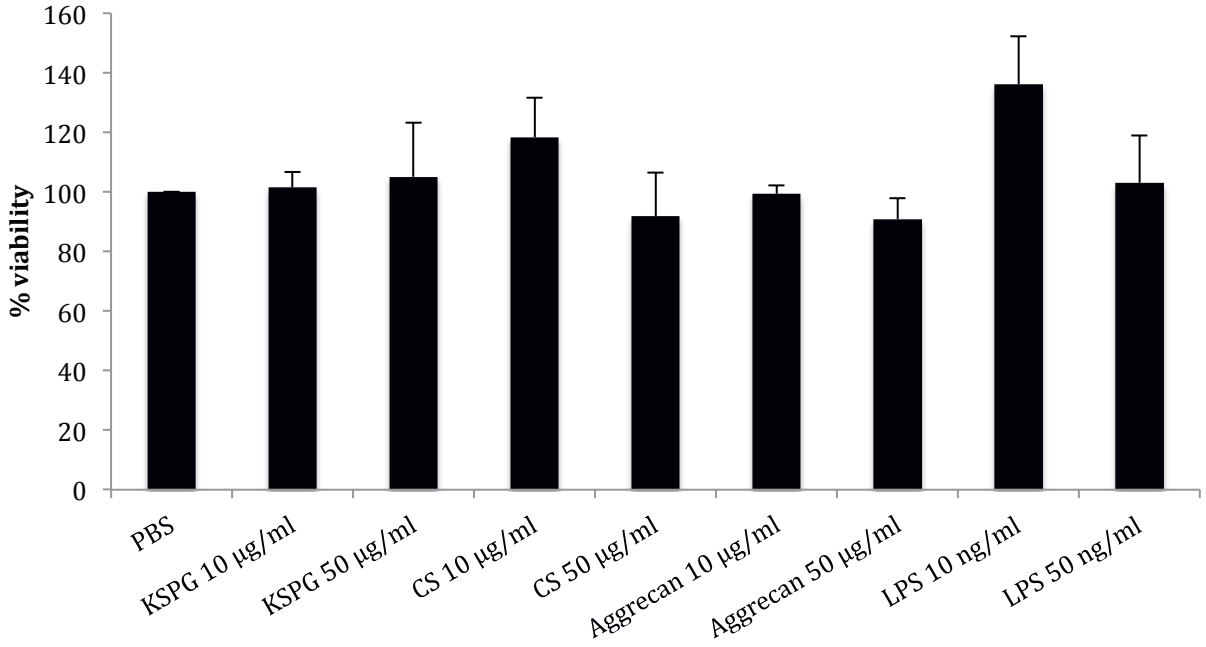


Figure 4: KSPG, CS, aggrecan and LPS did not affect viability of PMA-differentiated THP-1 cells. THP-1 cells were treated with indicated stimuli for 24 hr. Viability was assessed by WST-1 assay. Data was normalized to PBS treatment. N=6, *p<0.05

Human Monocyte-Differentiated Macrophages

Release of Immune Factors in GAG-Stimulated MDMs

KSPG, CS and LPS Induce Different Inflammatory Responses in MDMs

Although there were some significant results found using the THP-1 cell line, we wanted to verify these results in another model system. The new macrophage cell model was constructed using fresh human monocytes purified from whole blood that were further differentiated into macrophages (MDMs). As with the THP-1 cells, the release of specific inflammatory and remodeling factors was analyzed after MDMs were treated with pathological levels of KSPG, CS, aggrecan or LPS for a 24 hr period (see Table 2). MDMs were plated at $0.5x10^6$ cells/ml and differentiated with

MCSF for 7 days prior to treatment. As with the THP-1 cell line, the fold increase over the PBS negative control was assessed and fold levels were color-categorized according to intensity (yellow indicating little to no increase and red indicating an increase of at least 50 fold). KSPG failed to induce an overall pro-inflammatory response, as indicated by the multiple yellow-labeled cells in Table 2. A small number of chemokines (IL-8, GROα, IL-6 and MIP-1β) did show a significant increase when compared with the PBS control. Unlike KSPG, CS induced a robust release of multiple inflammatory and remodeling factors in human MDMs at a level

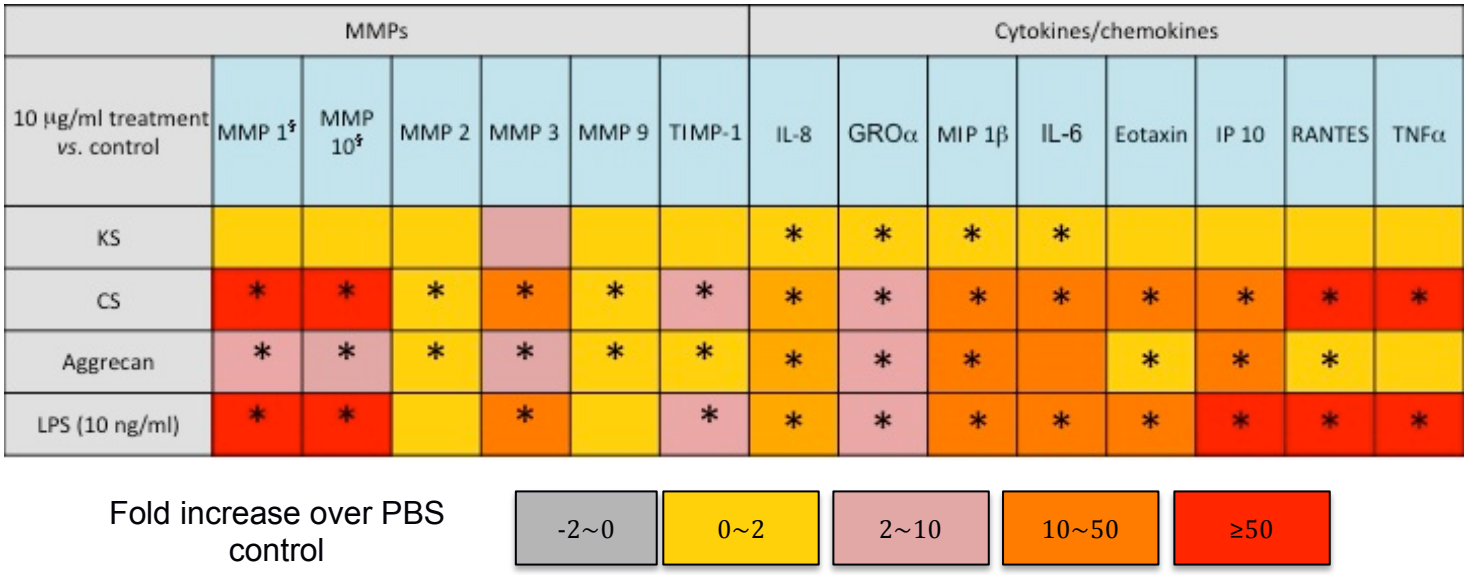


Table 2: Release of immune factors in human MDMs in response to KS, CS, aggrecan and LPS. Human MDMs were stimulated with indicated stimuli for 24 hr. Cell medium was collected and analyzed for indicated factors by MSD multiplex assays. N=6, *p<0.05

similar to cells treated with LPS. For instance, where there was a high increase in MMP1 release in cells treated with LPS and CS, the opposite result was seen in cells treated with KS, and only a mild increase was found in cells treated with aggrecan. As seen in the THP-1 cells, aggrecan had an overall mild inflammatory response in

human MDMs when compared to CS and LPS. Overall, these results were consistent with the data previously obtained with PMA-differentiated THP-1 cells (Table 1).

Dose-Dependent Release of Chemokines in MDMs treated with KSPG

To further understand the specific kinetics of chemokine release in response to pathological levels of GAGs, human MDMs were treated with increasing doses of KSPG for 24 hr. Levels of IL-8, GROα, IL-6,MIP-1β (which were found to be activated by KSPG in Table 2) and TNFα were assessed. Dose-dependent responses of IL-8, GROα, IL-6, TNFα and MIP-1β were observed in cells treated with KSPG at pathological levels of 10 μg/ml and 20 μg/ml (Figure 5A). Concentrations weren't significantly affected by KSPG. While the release of the chemokine MIP-1β demonstrated a positive correlation with increasing concentrations of KSPG, IL-8, GROα and IL-6 appeared to peak at 10 μg/ml of KSPG. The increase in GROα was expected due to its anti-inflammatory characteristics, but the increase in the other pro-inflammatory chemokines shown in Table 2 and Figure 5A was not. However, this increase may be the result of altered cellular homeostasis within the macrophage. As an integral component of the immune system, the macrophage regulates pro- and anti-inflammatory signaling and drastic increase or decrease to any of these signaling mechanisms may trigger a compensatory response. This slight increase seen in pro-inflammatory chemokine release may be caused by the high levels of anti-inflammatory KS accumulation as the macrophage attempts to ameliorate this imbalance in homeostasis. In order to confirm the increased expression of the chemokines IL-8, GROα, IL-6, and MIP-1β, the dose dependent

experiment was repeated and transcriptional expression levels were determined via quantitative RT-PCR. Consistent with previously found MSD data, a dose-dependent increase of IL-8, GROα, IL-6, and MIP-1β mRNAs were observed (Figure 5B). The effects of KSPG on some chemokines were significant yet mild, compared to the LPS-treated cells. These data reveal that although KSPG has mild effects on macrophage activation at lower concentrations, pathological concentrations (≥10 μg/ml) of KSPG do yield low levels of transcriptional up-regulation and subsequent release of certain inflammatory factors in human MDMs. This also confirms earlier findings that KS may provoke both anti-inflammatory and pro-inflammatory responses within the macrophage.

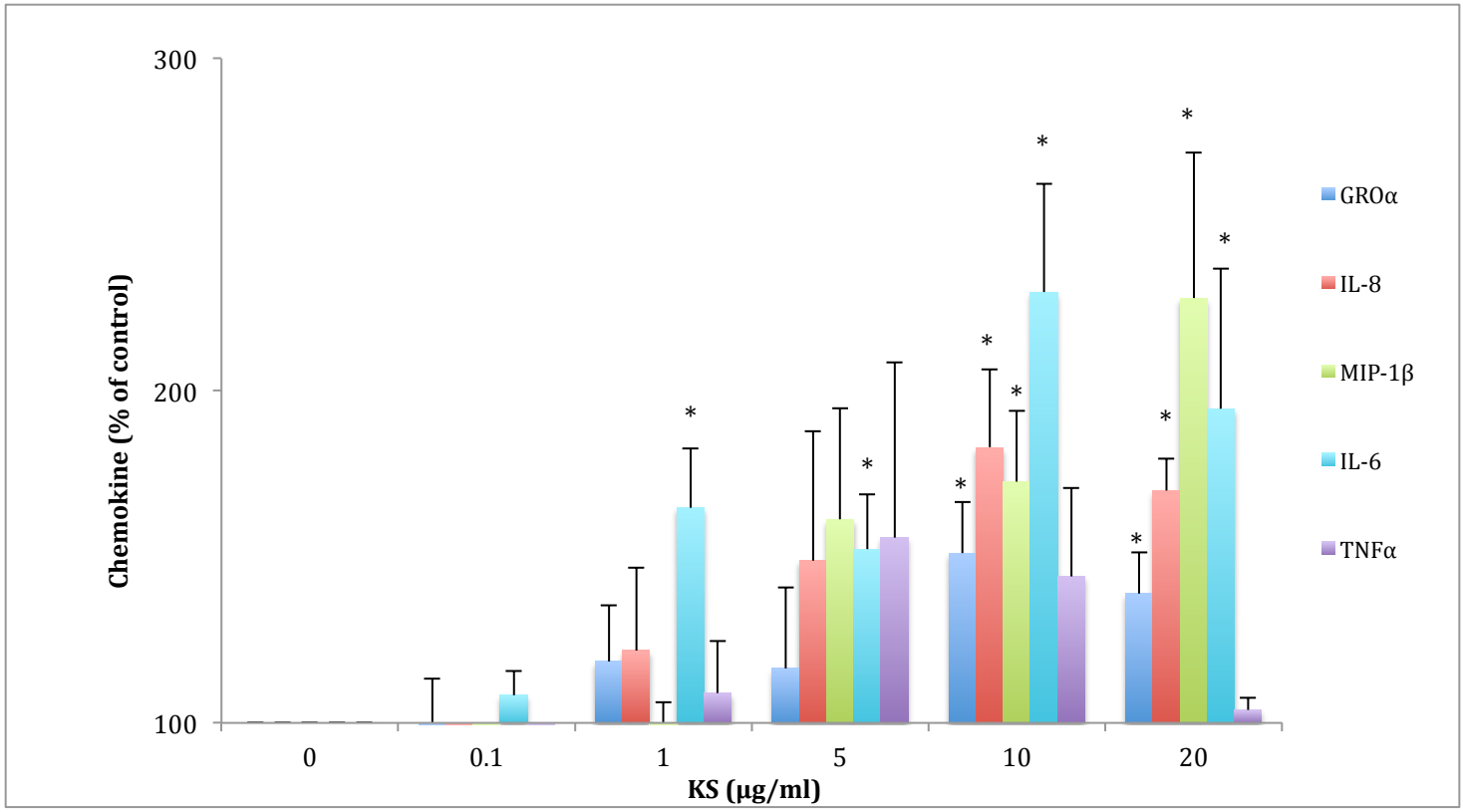


Figure 5A: Dose-dependent release of GROa, IL-8, MIP-1b, IL-6 in KSPG-treated human MDMs. Cells are treated with KSPG for 24 hr and levels of indicated chemokines are analyzed by MSD multiplex assays. N=6, *p<0.05

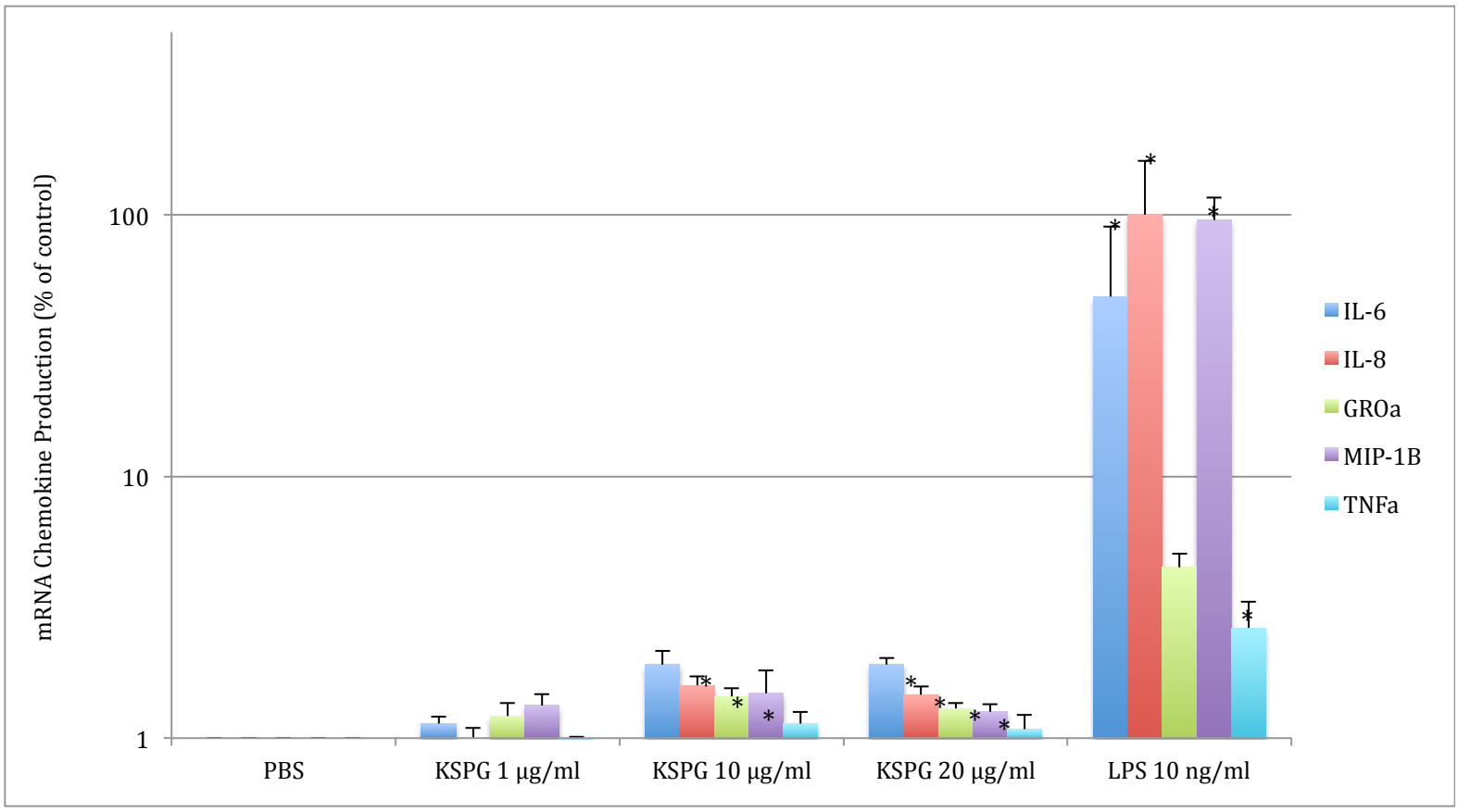


Figure 5B: Dose-dependent release of mRNA of GROa, IL-8, MIP-1b, IL-6 in KSPG-treated human MDMs. Cells are treated with KSPG for 24 hr and levels of mRNA are analyzed RT-PCR. N=6, *p<0.05

KSPG Inhibition of NFkB Activation

KSPG and CS have Opposing Effects on NFkB Activation

To confirm that KSPG inhibits the release of NFkB in macrophages, as seen previously in the PMA-differentiated THP-1 cell line (Figure 1A), human MDMs were treated with pathological levels of KSPG, CS, aggrecan or LPS for 24 hours and then analyzed for NFkB expression. Consistent with THP-1 cell line data, KSPG significantly inhibited NFkB activation while LPS and CS both significantly increased its activation (Figure 6). Aggrecan, showed a slight, but not significant, release of NFkB. The opposing NFkB activation of KSPG and CS found in both the THP-1 cell line as well as MDMs reveals a consistent pattern between the two GAGs and points to KSPG as an anti-inflammatory agent rather than a pro-inflammatory agent such as CS or LPS.

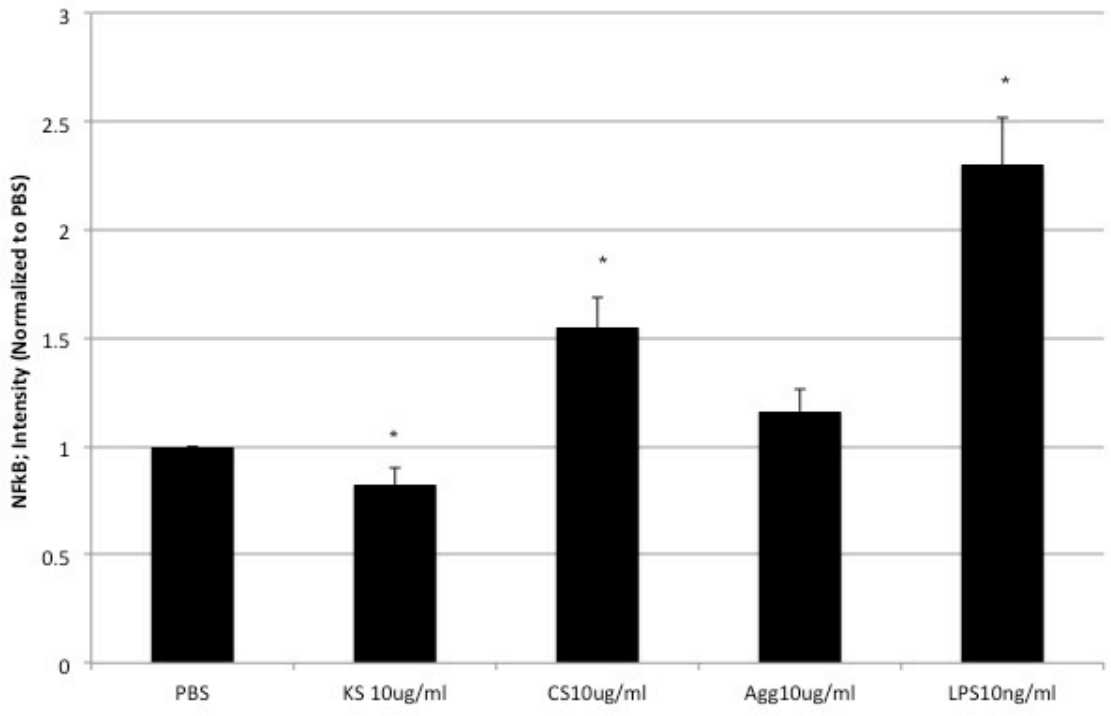
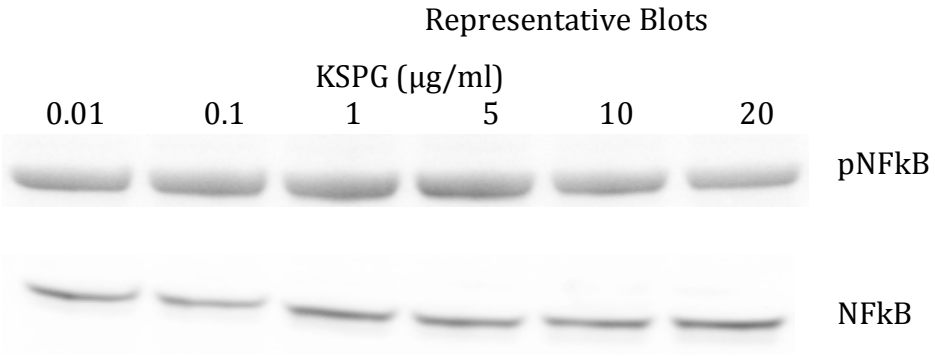


Figure 6: NFKB is activated in human MDMs in response to CS and LPS but not to KSPG. Cells are treated with indicated stimuli for 24 hr. Cell lysates are subjected to SDS-PAGE and immunoblotting. Activation of NFkB is analyzed by using antibodies for phosphor-NFkB and total NFkB. Band intensity was normalized to corresponding total protein. Data presented as fold of PBS treatment. N=6, *p<0.05

KSPG Inhibits NFkB Activation in a Dose-Dependent Manner

Human MDMs were treated over a 24-hr period with increasing doses of KSPG. When NFkB activation was assessed by subjecting the cell lysates to western blotting, it was observed that NFkB decreased as doses of KSPG increased (Figure 7). This pattern was consistent with observations found with the THP-1 cell line (Figure 2) as well as the lack of TNFα release in response to KSPG in both THP-1 cells (Table 1) and MDMs (Figure 5A). These results, along with observations made in the GAG-mediated release of NFkB in human MDMs (Figure 6), suggest that NFkB activation patterns are both consistent among multiple macrophage cell lines as well as specific to individual GAGs.



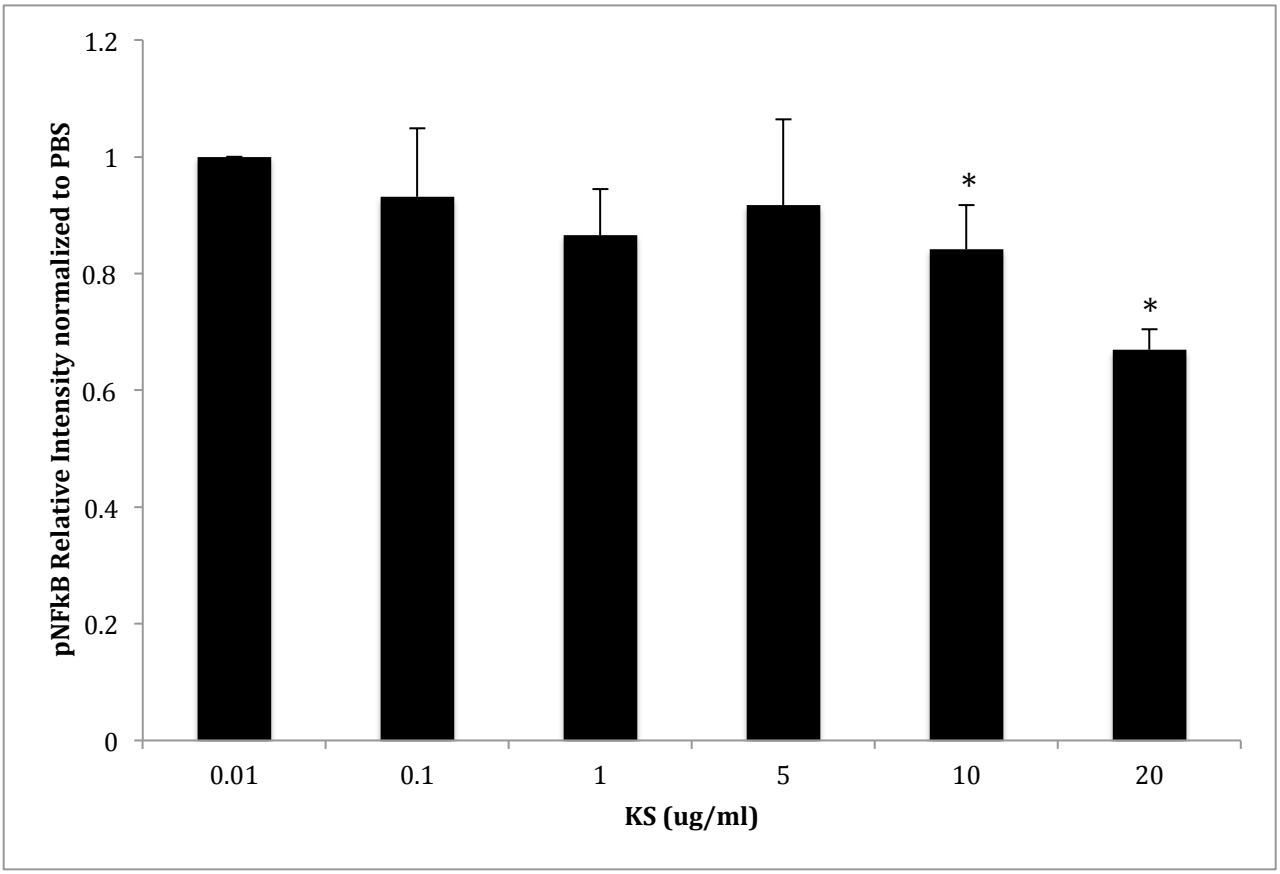


Figure 7: KSPG dose-dependently inhibited NFkB activation in human MDMs. Cells are treated with KSPG at indicated doses for 24 hr. Cell lysates are subjected to SDS-PAGE and immunoblotting. Activation of NFkB is analyzed by using antibodies for phosphor-NFkB and total NFkB. Band intensity is normalized to corresponding total protein. Data presented as fold of time zero. N=6, *p<0.05

Unlike CS, KSPG Does Not Induce Nuclear Translocation of NFkB in MDMs

To further investigate the KSPG-mediated decrease of NFkB activation in human MDMs, the cells were treated for 24 hours with high levels of KSPG (10 μg/ml and 20 μg/ml) and immunostained using the nuclear stain DAPI, the actin stain phalloidin, andphosphor-specific NFkB (pNFkB) antibody (Figure 8). It has been shown that the nuclear translocation of pNFkB is followed by its subsequent activation, allowing for the transcriptional activation of several downstream pro-inflammatory factors (Sun & Andersson, 2002). In human MDMs treated with

pathological levels of KSPG, a no significant level of pNFkB nuclear translocation

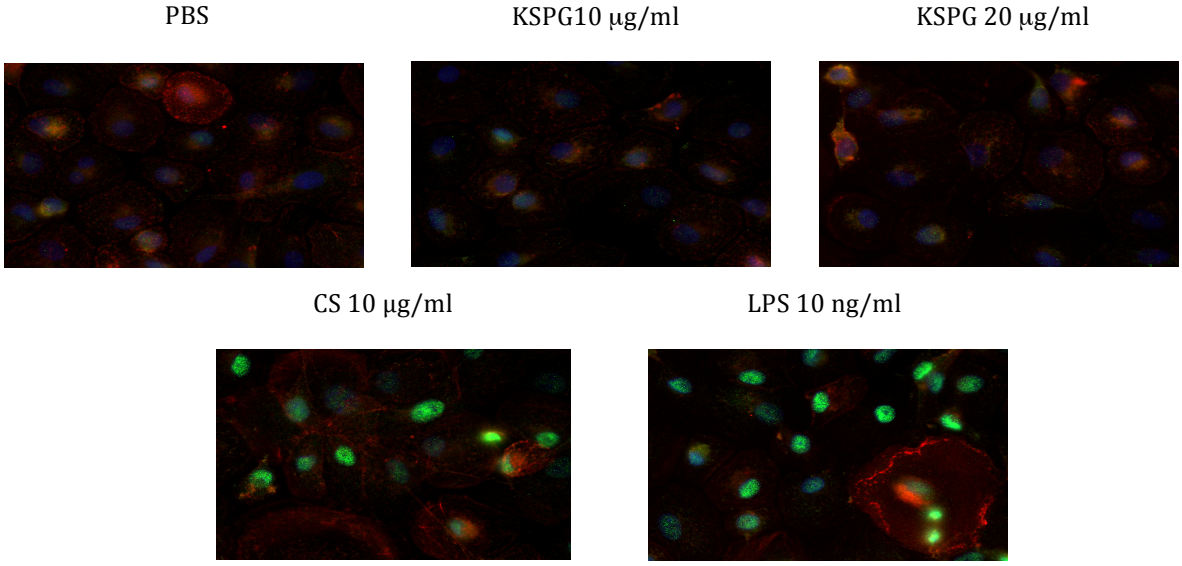


Figure 8: Absence of nuclear translocation of pNFκB in human MDMs in response to KSPG. Cells were treated with indicated stimuli for 24 hr. Subsequently, cells were fixed and permeabilized before staining with antibody to pNFκB (green fluorescence), phalloidin (red fluorescence) and DAPI (blue fluorescence). Images are representative of two independent experiments.

was observed. Unlike KSPG, both CS and LPS induced a robust migration of pNFkB to the nuclear region indicated by the co-localizations of green (pNFkB) and blue (nuclear) fluorescence (Figure 8). These results further demonstrate that KSPG, CS, aggrecan and LPS each have factor-specific mediated signaling patterns in human MDMs and suggest that KSPG has a receptor or method of entry different from that of other GAGs.

Pathological Doses of KSPG Do Not Induce Apoptosis

Macrophage cell viability in response to pathological levels of KSPG in the cellular milieu was assessed in human MDMs using the TUNEL staining system as described in Material and Methods. After a 24-hr exposure of KSPG, only slight DNA

fragmentation (≤ 5%) was detected in cells treated with the highest concentration of KSPG (20 μg/ml) in contrast to the higher levels of fragmentation found in cells treated with the positive control, apoptosis inducing staurosporine (Figure 9). This low level of DNA fragmentation at high levels of KSPG indicates that apoptosis in not

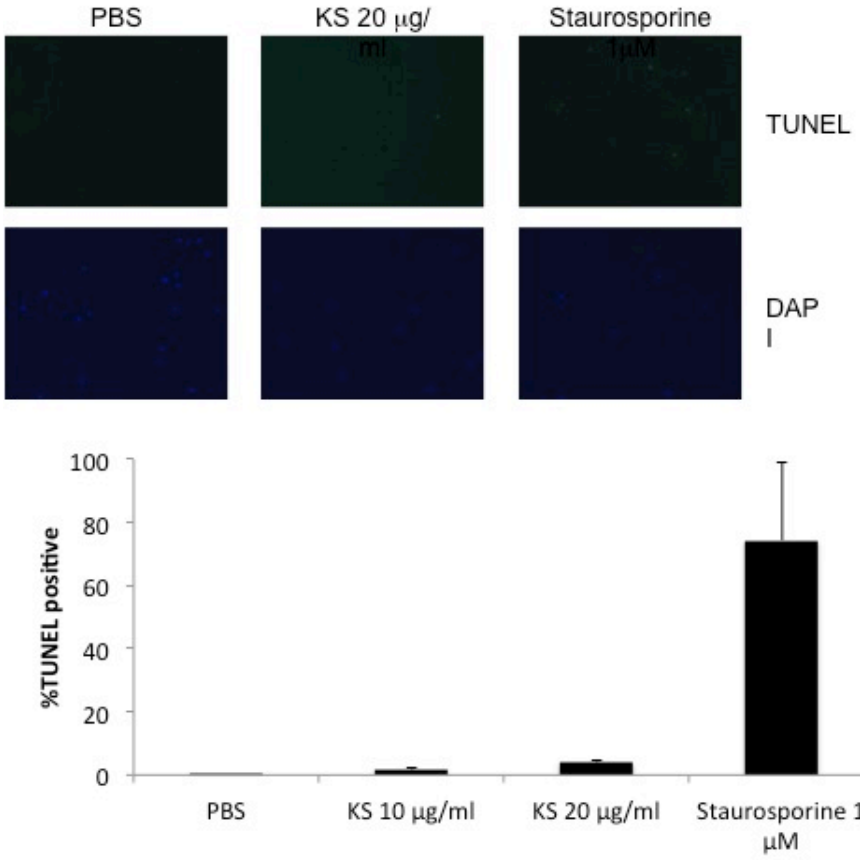


Figure 9: High doses of KS failed to induce apoptosis in human MDMs. Cells were treated with indicated stimuli for 24 hr. TUNEL staining was carried out as mentioned in Materials and Methods.

Upper panel: representative images of three independent experiments
Lower panel: quantification and summary of three independent experiments.

a significant consequence of the KSPG disease state.

Pathological Doses of KSPG Affects Metabolic Activity

KSPG Inhibits Mitochondrial Metabolic Activity in MDMs but not Monocytes

As the disruption of cellular homeostasis is an integral component of the pathogy of many LSDs (Lieberman et al, 2012; Settembre & Ballabio, 2011; Settembre et al., 2011), the metabolic functioning of macrophages might be affected by pathological

levels of KSPG. Therefore, the effects of KSPG on the mitochondrial metabolic rate of MDMs were observed using the WST-1 metabolic assay. After a 24-hour treatment of KSPG at various doses, a significant decrease in mitochondrial metabolic activity was observed only in macrophage cells treated with the pathological levels of 10 and 20 µg/ml KSPG (Figures 10A and 10B). However, undifferentiated monocytic cells were unaffected, even at the highest concentrations (Figure 10A). This inhibitory effect was not seen at lower doses of KSPG or when human peripheral blood monocytes were also treated in a similar manner suggesting that this outcome is specific to pathological levels of KSPG and fully differentiated macrophages.

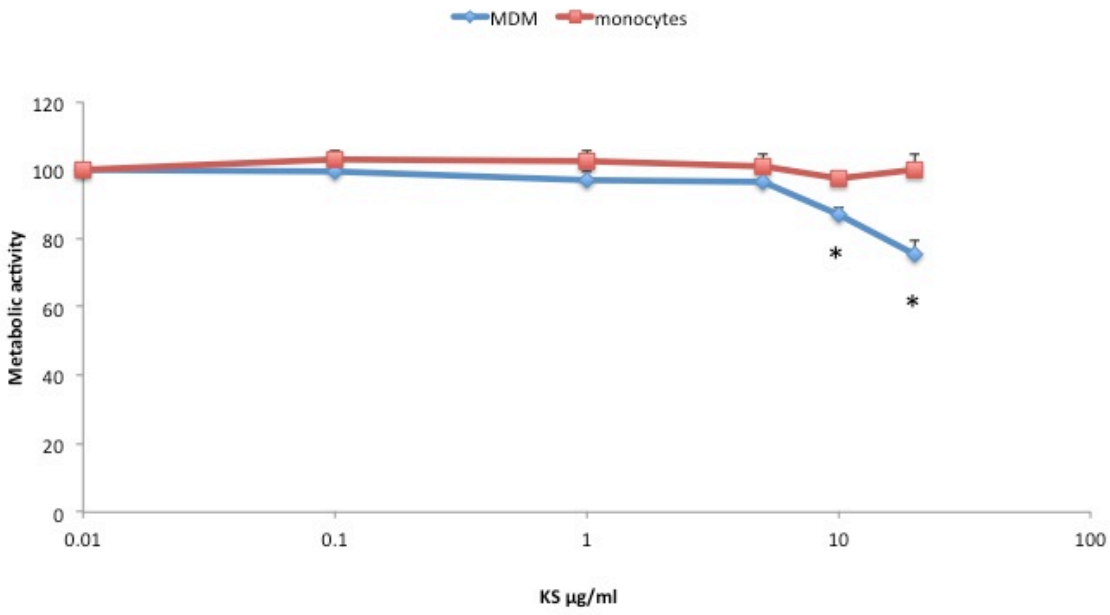


Figure 10A: High doses of KSPG inhibit mitochondria metabolic activity in human MDMs but not monocytes. Cells are treated with KSPG at indicated concentration for 24 hr and metabolic rate is measured by WST-1 assay. N=6, *p<0.05

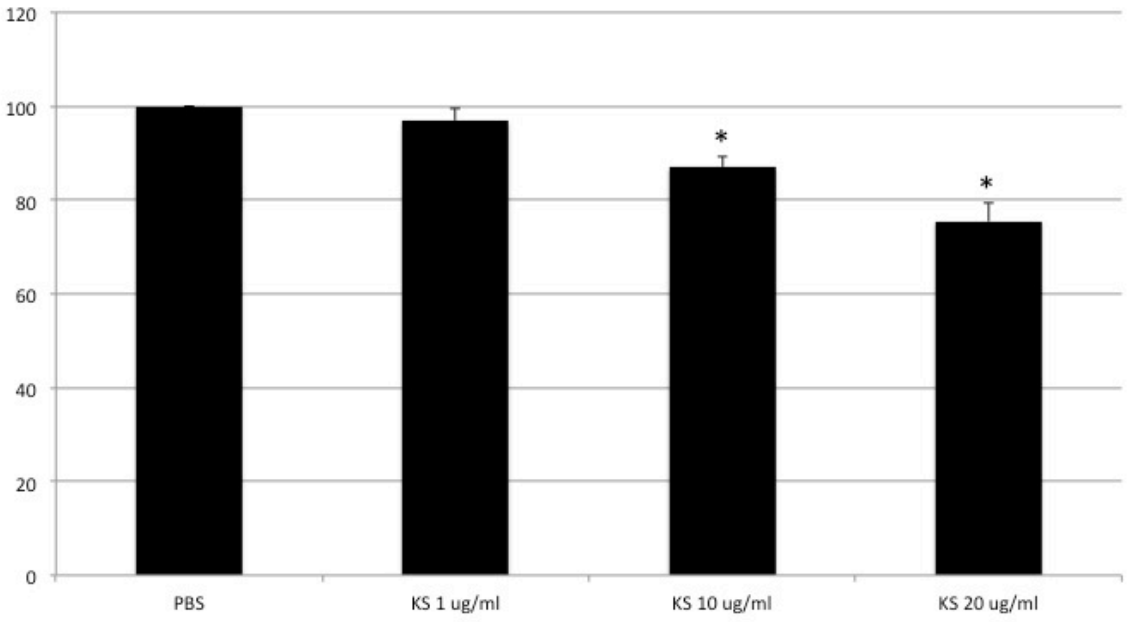


Figure 10B: High doses of KSPG inhibit mitochondria metabolic activity in human MDMs. Cells are treated with KSPG at indicated concentration for 24 hr and metabolic rate is measured by WST-1 assay. N=6, *p<0.05

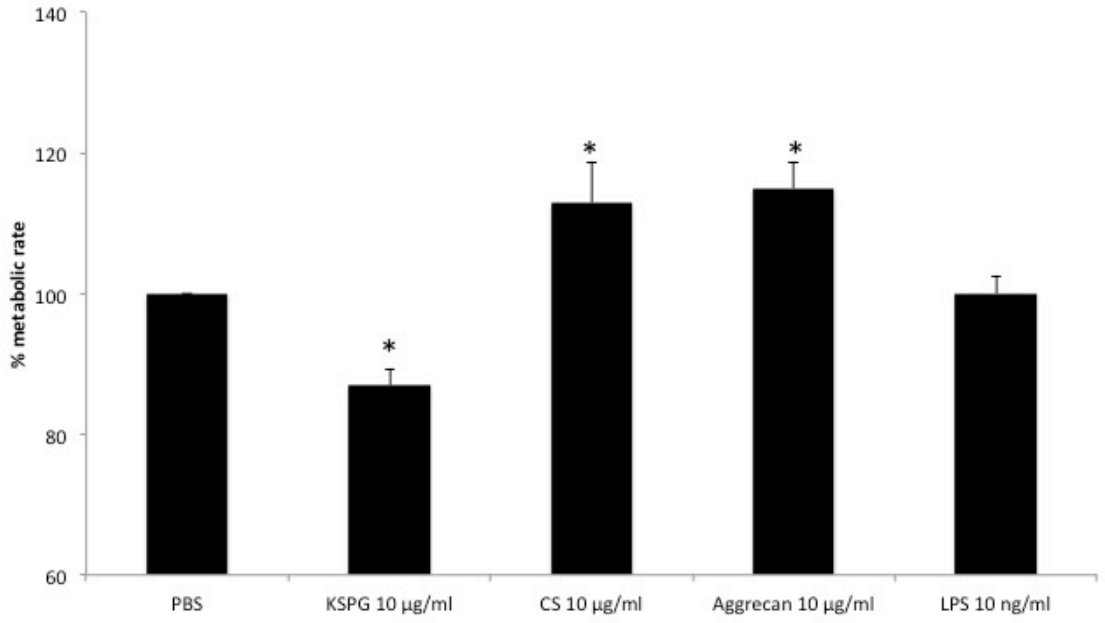


Figure 11: Opposite effects of KSPG and CS/aggrecan on mitochondria metabolic activity in human MDMs. Cells were treated with KSPG at indicated concentration for 24 hr and metabolic rate was measured by WST-1 assay as mentioned in Materials and Methods. N=6, *p<0.05

Metabolic tests were continued with CS, aggrecan and LPS. After a 24-hour treatment, it was found that LPS did not induce a significant change in metabolic rate. which may be due to the acute nature of LPS-induced cellular responses (Figure 11). However, CS and aggrecan both *increased* metabolic rate.

KSPG Inhibits Proliferation and Differentiation of MDMs

The effects that KSPG, CS, aggrecan and LPS have on cell proliferation and differentiation were then analyzed. This was accomplished by using the xCELLigence program to measure the surface area occupied by cells (cell index) to monitor cell growth and proliferation in real-time. Changes in the cell index could be

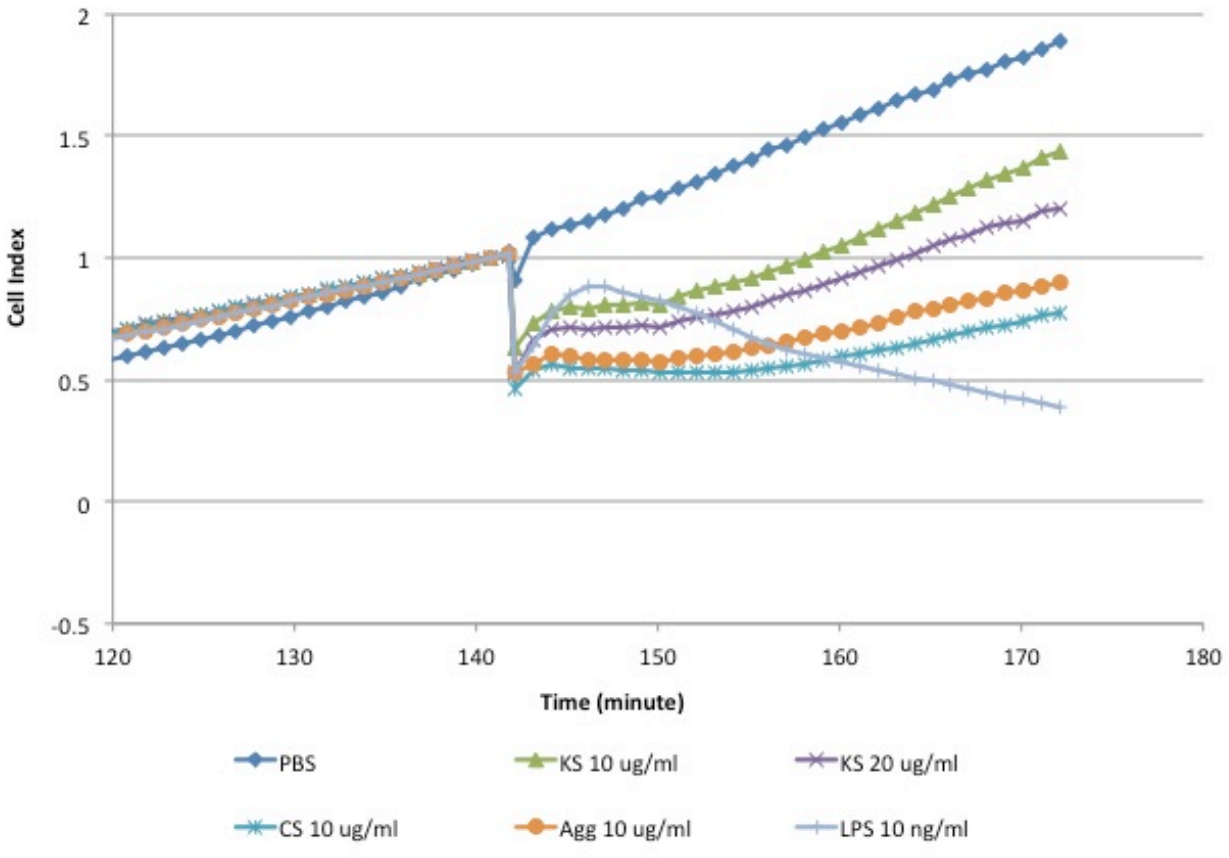


Figure 12A: High doses of KSPG inhibited proliferation/differentiation of human MDMs. After 7-day differentiation, cells are treated with increasing doses of KSPG (at arrow). Cell index is recorded real-time for 24 hr following treatment. N=4, *p<0.05

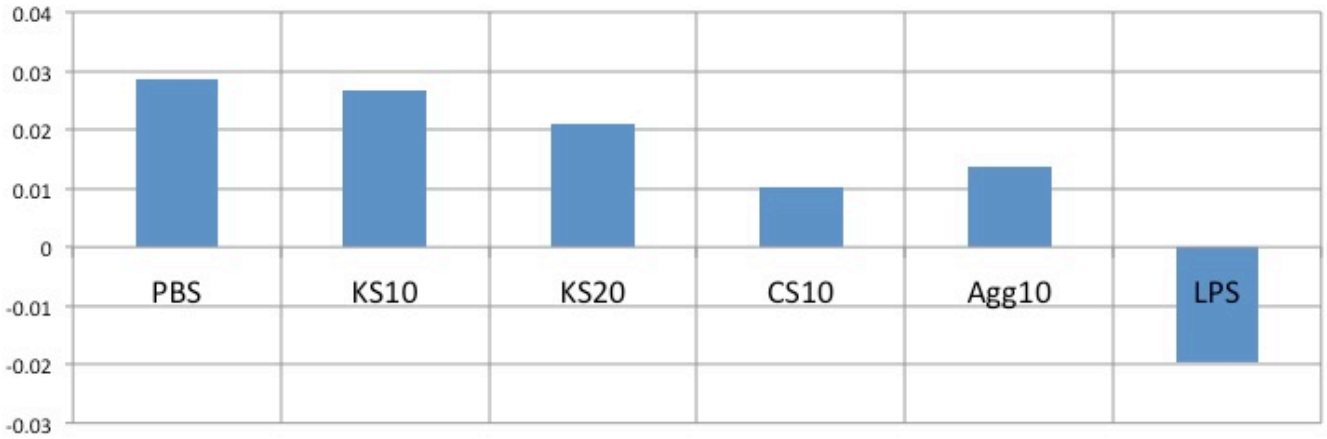


Figure 12B: High doses of KSPG inhibited proliferation/differentiation of human MDMs. Growth slope (1/hr) from left panel was quantified and summarized over four experiments. N=4, *p<0.05

attributed to cell proliferation and/or growth. After a 7-day differentiation period, human MDMs were treated with the indicated GAGs or LPS and the individual cell indexes were monitored for 24 hours (Figure 12A). We observed a decrease in cell index for all treatments, with LPS having the greatest relative decrease when compared to the PBS control (Figure 12B). The pathological concentrations of KSPG significantly impacted the cell index slope, suggesting an inhibitory effect on cell growth and/or differentiation. This decrease in proliferation may be due to inhibition of NFkB, which has been shown to play a key role in cell survival and differentiation in human macrophages and other immune-related cells. These results, along with the data showing that KSPG does not induce apoptosis (Figure 9), suggest that pathological doses of KSPG induces an overall suppressed metabolic phenotype, but not cell death, in human macrophages.

DISCUSSION

This study assessed the effects of KSPG accumulation on monocytic lines and their differentiated counterparts in order to better understand the perturbed inflammatory regulation found in the disease MPS IVA. The results demonstrate that KSPG, CS, aggrecan and LPS each induce specific signaling patterns and kinetics in both PMA-differentiated THP-1 cells and MDMs. This further supports the hypothesis that KS has either separate receptors or forms of entry when compared to CS and LPS. This has been shown by the differential release of inflammatory chemokines and cytokines, activation of the transcription factor NFkB, and decrease of mitochondrial metabolic activity. Specifically, while both CS and LPS induced the release of various inflammatory chemokines and cytokines as well as the activation of NFkB, KSPG had either no effect or suppressed such responses. Further investigation of KSPG demonstrated that it did not induce apoptosis but did suppress cellular metabolism and proliferation/differentiation. These results strongly support the hypothesis that, unlike other GAGs such as CS, KSPG does not induce inflammation but rather suppresses such cellular responses by inhibiting NFkB pathways and the release of inflammatory factors.

The hypothesis that KSPG acts in an immunosuppressive way corroborates the Morquio IVA clinical data previously mentioned (Tomatsu et al., 2011) as well as multiple studies that designate KS as a possible therapy in the treatment of various forms of arthritis (Zhang et al., 1998; Hayashi M, et. al., 2011; Glant, et al., 1998). However, our studies also showed that KSPG can induce the release of pro-inflammatory factors, specifically IL-8, IL-6 and MIP-1β (Figure 5A). While the

release of these chemokines was relatively mild when compared to LPS (Figure 5B), this induction of pro-inflammatory factors indicates a more complex cellular mechanism that takes place when macrophages are in the presence of high levels of KS. An explanation for these seemingly contradictory findings is that KS interrupts the balance of pro-inflammatory and anti-inflammatory activation within the body. In this way, if the cells are chronically in a state of immunosuppression, they may activate pro-inflammatory pathways in order to counteract this imbalance and obtain homeostasis.

This interpretation of the data may also explain why KS and CS (shown to be pro-inflammatory) are both commonly found together on the proteoglycan aggrecan. With both anti- and pro-inflammatory inducing GAGs attached to its core, aggrecan may act as a moderator in the immune system. It is also important to note that the effects seen in our work may be mild in comparison to the disease model due to the use of a keratan sulfate I and keratan sulfate II mixture with unknown sulfonation patterns rather than highly sulfonated KSII alone which is the form most commonly accumulated in the disease. The use of normal macrophages instead of macrophages missing the enzyme GALNS may also produce a milder affect than what is seen when the cells accumulate KS. Further work is needed to help elucidate the biochemical role that KS plays in regulating the immune system, particularly since it appears to function in a way dissimilar to other GAGs.

Future Studies

Evidence that high levels of KS may act in a unique way when compared to other GAGs reveals that our current knowledge of proteoglycans is not complete. These new findings question our understanding of the MPS family of disorders and what methods we use to treat patients with these diseases. Clinical data has already shown that Morquio A patients are prone to secondary infections in the respiratory system and during treatment at injection sites indicating, that patients may be immunosuppressed (Tomatsu et al., 2011). New clinical data reveals that Morquio A patients treated with enzyme replacement therapy (ERT) show an initial increase in pro-inflammatory proteins (such ENRAGE, a pro-inflammatory ligand for the receptor for advanced glycation endproducts receptor) followed by a decrease and subsequent modulation of immune factors (Marteli et al., 2011). This sudden up-regulation in pro-inflammatory factors may be the result of the body's response to the enzyme or to the sudden removal of high levels of KS acting as an immunosuppressant. It is also important to consider that patients are concurrently treated with anti-inflammatory agents which may both compound the anti-inflammatory nature of KS and attenuate an up-regulation of pro-inflammatory factors once the immunosuppressant KS is removed from the cellular matrix. Further review of patient response during ERT and access to patient samples would benefit future research into the mechanisms of action of KS.

Another area of study to be expanded in regards to KS is the sulfonation patterns of individual side chains. Previous work by other groups (Guerassimov et al., 1998; Glant et al., 1998) indicates that the level of sulfonation of KS side chains

may directly influence the potency of KS as an immunosuppressant. This area may be further explored with the development of KS chains with specific sulfate stoichiometry (e.g. low sulfonation verses high sulfonation). Investigation into the heterogeneity of KS chains would also facilitate the identification of KS-specific receptors. As our data illustrated, KS appears to have a receptor or mode of activation different from that of other GAGs. Identification of GAG-specific receptors will allow for a clearer understanding of the molecular mechanisms set in motion when irregular levels of KS are present in the extracellular matrix and what consequences may arise from this imbalance. The results of this research should act as an impetus for further investigate the modulatory action of KS in regards to immunological functioning and highlight the unique characteristics of KS when compared to other GAGs.

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