A KERATIN BIOMATERIAL FOR TREATMENT FOLLOWING SPINAL CORD HEMISECTION INJURY AND INVESTIGATION OF SECONDARY DAMAGE MECHANISMS

BY

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<table>
<thead>
<tr>
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<th>Description</th>
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<tbody>
<tr>
<td>AIS</td>
<td>American Spinal Injury Association Impairment Scale</td>
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<tr>
<td>AM</td>
<td>Astrocyte media</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>BMP-2</td>
<td>Bone morphogenetic protein-2</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>BSB</td>
<td>Blood-spinal cord barrier</td>
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<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
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<tr>
<td>chABC</td>
<td>Chondroitinase ABC</td>
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<tr>
<td>CMAP</td>
<td>Compound motor action potential</td>
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<tr>
<td>CMG</td>
<td>Cystometrogram</td>
</tr>
<tr>
<td>CSPG</td>
<td>Chondroitin sulfate proteoglycan</td>
</tr>
<tr>
<td>CT</td>
<td>Computerized tomography</td>
</tr>
<tr>
<td>C-MCM</td>
<td>Collagen-treated macrophage conditioned media</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>DI</td>
<td>Deionized</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EAA</td>
<td>Extracellular excitatory amino acid</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>EMG</td>
<td>Electromyography</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>FB/FN</td>
<td>Fibronectin/fibrin</td>
</tr>
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<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>GDNF</td>
<td>Glial cell line derived neurotrophic factor</td>
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<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GTPase</td>
<td>Guanosine triphosphatase</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronic acid</td>
</tr>
<tr>
<td>Hr</td>
<td>Human recombinant</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon γ</td>
</tr>
<tr>
<td>IL-</td>
<td>Interleukin</td>
</tr>
<tr>
<td>KAPs</td>
<td>Keratin associated proteins</td>
</tr>
<tr>
<td>KIFs</td>
<td>Keratin intermediate filaments</td>
</tr>
<tr>
<td>K-MCM</td>
<td>Keratin-treated macrophage conditioned media</td>
</tr>
<tr>
<td>LDV</td>
<td>Leucine – aspartic acid – valine</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>M1-MCM</td>
<td>M1 macrophage conditioned media</td>
</tr>
<tr>
<td>M2-MCM</td>
<td>M2 macrophage conditioned media</td>
</tr>
<tr>
<td>MPSS</td>
<td>Methylprednisolone</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cell</td>
</tr>
<tr>
<td>MV</td>
<td>Micturition volume</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>NFkB</td>
<td>Nuclear-factor-kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NG2</td>
<td>Neuron/glial antigen 2</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NIH</td>
<td>National Institutes of Health</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly(lactic acid co-glycolic acid)</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol myristate acetate</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>P/S</td>
<td>Penicillin/Streptomycin</td>
</tr>
<tr>
<td>PPAR-γ</td>
<td>Peroxisome proliferator-activate receptor-γ</td>
</tr>
<tr>
<td>RA</td>
<td>Reactive astrocyte</td>
</tr>
<tr>
<td>RFU</td>
<td>Relative fluorescence units</td>
</tr>
<tr>
<td>ROM</td>
<td>Range of motion</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SCI</td>
<td>Spinal cord injury</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>TCP</td>
<td>Tissue culture-treated plastic</td>
</tr>
<tr>
<td>TDM</td>
<td>THP-1 derived macrophage</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>TE/RM</td>
<td>Tissue engineering/regenerative medicine</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>Th1/Th2</td>
<td>T helper cell type 1/T helper cell type 2</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor-α</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular cellular adhesion molecule</td>
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</table>
Spinal cord injuries (SCI) are a severely debilitating injury that affects about 200,000 people in the United States alone. The only current treatment options include use of steroids, spinal fixation surgery, and rehabilitation, none of which provide any significant recovery of function. SCIs are particularly difficult to treat because of the inhibitory nature of the lesion site and overcoming it to allow for repair and regeneration. There are many contributing factors to secondary damage, including the inflammatory response that follows SCI. Peripheral immune cells are able to infiltrate to the site of injury through the damaged blood-spinal cord barrier (BSB). It is well-established that macrophages play a major role in this process and, in response to SCI, are known to polarize to a proinflammatory phenotype that promotes secondary degeneration. These M1 macrophages dominate at the lesion site with concurrent downregulation of the M2 anti-inflammatory, growth supporting phenotype.

This dissertation work uses a novel biomaterial derived from human hair keratin that has previously demonstrated efficacy in regeneration following peripheral nerve injuries. The objective of these studies was to assess keratin’s ability to promote repair and recovery
following a traumatic spinal cord injury and the degree to which it can prevent further downstream injury. To address this, Aim 1 investigates keratin’s role in vivo in a rat model of SCI, Aim 2 focuses on cell-based assays to demonstrate macrophage activation upon exposure to keratin, and Aim 3 reveals a glial functional response to keratin-derived macrophages.

In a rat hemisection injury model of SCI, a keratin hydrogel applied to the lesion site showed significant recovery of gait and bladder functions, as well as better remodeling of the spinal cord tissue. It is believed that keratin’s effect may lie, at least in part, in its capacity to modulate the inflammatory response to a degree that allows for improved regeneration. Macrophages cultured on keratin coatings display a significant preference for polarizing toward a M2 phenotype, whereas macrophages cultured on collagen coatings exhibited a mixed M1/M2 phenotype that favors a M1 phenotype by later timepoints. Further, astrocytes exposed to the conditioned media from the macrophages on keratin coatings show less reactivity and CSPG production compared to those exposed to conditioned media from macrophages on collagen coatings. These results suggest keratin allows for improved functional recovery because it is able to indirectly support axon regrowth by mediating the macrophage response which can diminish reactive astrocyte activity, both of which are crucial factors in the inhibitory properties of the lesion site. Future work based on these studies will lead to mechanistic investigations of keratin-macrophage interactions and further development of regenerative properties in vivo.
CHAPTER I

INTRODUCTION
KERATIN BIOMATERIALS

The use of keratin in wound healing dates back to the 1500s, when Chinese herbalists used pyrolyzed human hair to promote wound healing and stop bleeding [1]. Recent advances in the extraction and characterization of keratin proteins from human hair has led to the development of keratin-based biomaterials for biomedical applications [2]. Keratin hydrogels have previously demonstrated their efficacy in a mouse model of peripheral nerve injury. Keratin-filled conduits resulted in lower nerve conduction latency, higher compound motor action potential (CMAP), and greater blood vessel cross-sectional area (all reaching significance) compared to saline-filled conduits and autograft control groups [3]. This trend was continued at 3 and 6 months post-injury, including keratin-treated nerves maintaining significantly greater axon density [4]. A similar study confirmed keratin’s ability to promote significantly better recovery of electrophysiological function in a rabbit model of peripheral nerve defect [5]. Additionally, a mechanistic study in a rat model revealed keratin treatment supported more active Schwann cells that were able to phagocytose and clear debris faster, a crucial process for enhanced repair and plasticity and primes the injury site for reinnervation of target muscles [6]. These studies utilized a keratin hydrogel comprised of a complex mixture of alpha, keratin associated proteins (KAPs), gamma, and other keratin derivative compounds, which has also been utilized in the present work.

Keratin is a structural protein that is a major component of human hair, as well as wool, hooves, and nails. Human hair keratins are characterized by their high cystine content and disulfide crosslinking, which must be broken down to isolate the cortical proteins. Keratin proteins can be extracted using oxidative chemistry, resulting in
“keratose” derivatives, and using reductive chemistries, resulting in “kerateine”
derivatives [7]. Data has shown the greatest neuroinductive characteristics lie within the
keratose derivatives [3, 5-6], which are the subject of this work, and will thus be the form
referred to by the word “keratin” in this dissertation.

A typical human hair fiber extract solution contains low sulfur alpha keratins
(MW 48-60kDa), which can be bound to KAPs, high sulfur-containing keratins (MW 10-
15kDa) typically referred to as “gamma” keratins that are purported to arise from the hair
cortical matrix, and fragments of each that result from amide bond hydrolysis [8]. Alpha
keratin proteins make up 50-60% of the human hair fiber and interact to form keratin
intermediate filaments (KIFs). Gamma keratin proteins compose about 20-30% of the
hair fiber and function as a glue-like matrix to maintain cortical structure [8].

Biomaterials constructed from human hair keratins are exceptionally versatile and can be
processed into gels, coatings, films, and sponges [2]. In addition, keratin-based hydrogels
are easily and inexpensively produced due to their readily available source material,
human hair fibers. Because of keratin’s biocompatibility and resistance to proteolytic
degradation [9], it is able to act as a scaffold to support damaged and early infiltrating
cells, yet avoid persisting in the injured lesion and inhibiting axonal repair. Because of
these intrinsic properties, there exists a wide range of possible biomedical applications.
Specifically, previous work in peripheral nerve injury models has given sufficient
evidence to suggest a keratin biomaterial is capable of robust repair and recovery, the
extent of which may translate well to applications in the central nervous system (CNS).
SPINAL CORD INJURIES

Spinal cord injury (SCI) is a traumatic insult resulting in permanent disability and loss of movement and sensation in tissues innervated below the site of damage. Excluding those who die at the scene of the accident, there are approximately 12,000 new cases of SCI each year the United States alone. The majority of SCIs result from motor vehicle crashes (37%), [10]. The majority of those afflicted are young adults between 16 and 30 years old, with more than 80% of cases reported to occur among males. SCI patients experience a range of medical complications, such as breathing problems, blood clots, spasticity, autonomic dysreflexia, bladder and bowel problems, and neurogenic pain. Life expectancies for these patients are significantly lower than average and vary depending on the level and extent of injury, and mortality rates are considerably higher within the year following injury compared to subsequent years [10].

SCIs are classified as being complete or incomplete. A complete injury is associated with loss of voluntary movement of parts innervated by the segment, loss of sensation, and spinal shock. An incomplete injury has some function present below the site of injury and overall has a more favorable prognosis compared to complete injuries [11]. SCIs are evaluated based on the American Spinal Injury Association Impairment Scale (AIS). AIS categorizes injuries into five types, where A is a complete injury (complete absence of sensory and motor function below the level of injury), B-D represent varying degrees of incomplete injuries, and E is normal with all sensory and motor functions present [12]. Tetraplegia occurs when one of the eight cervical segments of the spinal cord are damaged, while paraplegia occurs with injuries to the thoracic,
lumbar, or sacral regions of the spinal cord. The majority of all SCIs, around 41%, are reported as incomplete tetraplegia [12].

Disability after injury to the spinal cord depends on the level at which the trauma occurs since a significant loss of neurologic function will take place caudally. The primary mechanism of SCI is usually due to the direct, mechanical destruction of the tissue. This immediate, primary phase, sometimes lasting up to several hours, is characterized by damage to the soft tissue and vasculature surrounding the injury epicenter. Secondary injury presents as progressive destruction of the tissue surrounding the injury epicenter and is characterized by several mechanisms, including vascular damage due to the breakdown of the blood-spinal cord barrier (BSB) as well as the infiltration of peripheral inflammatory cells [13-14], formation of free radicals and nitric oxide (NO), release of proteases[15-17], and an increase in extracellular excitatory amino acid (EAA) concentration resulting in excitotoxicity via excessive amounts of extracellular glutamate and aspartate [18-19]. There is also a strong cellular reaction occurring during the secondary phase and is characterized by a potent inflammatory response, mostly mediated by macrophages, who make up the majority of the macrophage population within the lesion site and are known to persist in the lesion for several months post-SCI [14, 20]. A more in-depth discussion of the role of macrophages is included in a subsequent section of this chapter. Microglia, neutrophils, and T cells also partake in this reaction and the extent of their participation can greatly alter the lesion microenvironment [13-14, 20-23]

Other glial cells, oligodendrocytes and astrocytes, experience acute loss due to apoptosis triggered by damaged axons and oxidative stress within hours at the injury site
and continue a steady decline up to 7 days post-SCI [24]. Astrocytes tend to play a more active role in the inhibitory environment within the injured spinal cord and undergo a process known as reactive astrogliosis, where changes include increases in proliferation, hypertrophy, and immunoreactivity of glial fibrillary acid protein (GFAP) [25-26]. Reactive astrocytes produce various inflammatory cytokines, reactive oxygen species (ROS), and several growth inhibitory components [27-28]. Due to the direct mechanical damage of the SCI and the various processes carried out by infiltrating immune cells and damaged glial cells, neurons are especially vulnerable to loss, the mode of which appears to be mainly necrosis [14]. Neurons experience extensive damage and death due to over-stimulation from excitotoxicity, free radicals and other cytotoxic compounds being released from supporting cells, and axon demyelination as a result of apoptotic oligodendrocytes [29-30].

The chronic, late phase of SCI occurs over weeks to months and even years post-injury. This phase is distinguished by Wallerian degeneration, development of cysts, and glial scar formation. The astroglial scar is the end-stage of reactive astrogliosis and is made up primarily of tightly interwoven astrocyte processes. The growth-deterring environment found in the glial scar is believed to serve a purpose of seclusion of the injury site from the surviving healthy tissue in an effort to prevent further uncontrolled tissue damage [31]. A principle component of the glial scar, chondroitin sulfate proteoglycans (CSPGs) are produced by glial cells in the CNS and form a relatively large family including aggrecan, brevican, neurocan, neural/glial antigen 2 (NG2), versican, and phosphacan [32]. Reactive astrocytes have a well-documented contribution to the glial scar not only through physical entanglement, but also from their significant
production of CSPGs. Extensive studies utilizing chondroitinase ABC (chABC), an enzyme that specifically removes the GAG chain of the CSPG, suggest the GAG portion is inhibitory to neurite extension and the ablation of the sugar moiety and subsequent remainder of the protein core and carbohydrate stub results in considerably enhanced functional recovery [33-36]. Although, it is important to note that the residual core proteins and sugar stubs still exhibit inhibitory properties [36-37]. It is believed that this function of reactive astrocytes in the glial scar plays a critical role in failure of repair mechanisms.

Current treatment options offer some protection from secondary damage-mediated functional loss. Spinal fixation surgery is a common procedure to stabilize the spine and prevent further injury from unstable bony elements. However, there is no current evidence to suggest it offers advantages in functional outcomes [38-39]. Despite the widespread use of the steroid methylprednisolone (MPSS) to reduce swelling in the spinal cord, it has been found to offer relatively little, if any, improvements in recovery and has been associated with severe side effects [40-41]. Rehabilitation programs combining physical and occupational therapy can offer improvements in muscle strengthening, fine motor skills, and coping strategies for associated problems like spasticity, autonomic dysreflexia, and neurogenic pain [12]. Rehabilitation typically involves multiple phases and for most patients, beginning as an inpatient where goals focus on restoring independence in daily tasks such as bed mobility, wheelchair and other equipment management, and caregiver training and education [42]. Typical physical therapy activities include mobility training, range of motion/stretching exercises, balance exercises, and strengthening and endurance exercises. The use of neuromuscular
electrical stimulation can aid in the relaxation of muscle spasms, prevention or delay of muscle atrophy, and increasing local blood circulation. Robotic gait orthosis in combination with a body weight support system and treadmill can improve patients’ locomotion, metabolic performance, and increased activity in the cerebellum [43-44]. Thus, current treatment options afford some relief from long-term concerns, particularly as more progressive rehabilitation becomes widely available.

ROLE OF MACROPHAGES IN SPINAL CORD INJURY

Previously there was a long debate as to the role of macrophages/microglia in SCI and other CNS injuries, and it was believed they caused more damage than repair. Emerging research suggests the opposite however, where brain macrophages, if able to maintain a delicate balance, can play a major part in resolving the inflammatory reactions and thus prevent further secondary injury. Extensive studies have shown that macrophages can promote both secondary injury and repair following SCI [45-51]. In particular, several groups have demonstrated the neuroprotective nature and promotion of functional recovery when macrophages are inhibited or depleted [46-48, 52]. It is now believed that these divergent results can be attributed to macrophage phenotype. Kigerl, et al. demonstrated proinflammatory M1 macrophages dominate the site of SCI (especially areas of Wallerian degeneration) and persist for several weeks following injury, while the SCI milieu downregulates the M2 macrophage phenotype [53]. Because macrophage polarization is a dynamic process, it has been suggested that following SCI, as well as in other inflammatory-mediated injury and disease states (e.g. multiple sclerosis, wound healing, atherosclerosis), there arises a dysfunctional state of M1 or M2
phenotype control and a failure to switch from one to the other, which perpetuates an aberrant immune response. In SCI, the proinflammatory signaling mechanisms persist indefinitely in the lesion, creating a vicious cycle of M1 macrophage-mediated neurotoxic events where the inflammatory reaction is never able to resolve [53-54]. Accordingly, attention has been shifted to how to regulate this process, rather than prevent it all together.

MACROPHAGE FUNCTIONS IN TISSUE REPAIR AND REMODELLING

Biomaterials represent a promising approach to tissue engineering/regenerative medicine (TE/RM) applications. Within the context of TE/RM studies, biomaterial design strategies have recently shifted to focus on the macrophage response that is elicited following implantation. Macrophages are mononuclear phagocytes that continuously mature from circulating monocytes and migrate into tissues throughout the body. Monocytes recognize sites of inflammation near blood vessel walls through adhesion molecules and chemokines bound to the vascular endothelium, which they utilize to migrate across the endothelium into the surrounding tissue [55]. At this point, the monocyte undergoes differentiation into a macrophage, which proceeds to move through the tissue according to cytokine and chemokine signals toward the site of trauma or infection. Macrophages respond to these different signals through a process of reprogramming, resulting in a spectrum of distinct functional phenotypes that mimic the T helper cell types 1 and 2 (Th1/Th2) nomenclature [56]. So-called “M1” macrophages are the classically activated phenotype and are known to be induced by interferon-γ (IFNγ) and/or microbial products like lipopolysaccharide (LPS) [56]. “M2” macrophages
are the alternatively activated phenotype and are induced by several cytokines including interleukin-4 (IL-4) and interleukin-13 (IL-13) [57]. The functional states of M1 and M2 macrophages represent extremes of a continuum, where M1 macrophages release high levels of oxidative metabolites (e.g., NO), proinflammatory cytokines, and contribute to tissue destruction, while M2 macrophages release anti-inflammatory cytokines and support angiogenesis, matrix remodeling, and wound healing [54, 58-59]. In general, M1 macrophages are IL-12\textsuperscript{high} IL-10\textsuperscript{low} and are efficient producers of ROS and inflammatory cytokines, such as IL-1\(\beta\), tumor necrosis factor (TNF), and IL-6, participate in Th1 responses, and mediate resistance against virus and mycobacteria infections and tumors. M2 macrophages take part in Th2 responses, parasite clearance, dampening of inflammation, and retain an IL-10\textsuperscript{high} IL-12\textsuperscript{low} phenotype [56, 59]. Based on subtleties of different signaling molecules and effector functions, these distinct macrophage phenotypes can be subdivided further into M1a, M2a, M2b, and M2c [60]. This more detailed classification scheme is not as well established as the classically activated M1 and alternatively activated M2 phenotypes, but is beginning to emerge as an important method for distinguishing some of the nuances among polarized macrophages. One of the essential roles of activated macrophages is their ability to switch phenotypes, particularly based on cues within the tissue microenvironment [54]. Thus, an important feature of macrophage polarization is the plasticity of activation states, which constantly responds to minute changes.

Macrophages are essential contributors toward the resolution of inflammation, which is a highly regulated, dynamic process. In terms of infection, a phenotypic switch from M1 to M2 macrophage phenotypes generally corresponds to the transition from
early to chronic phases. But in models of wound healing, this balance or switch to a resolving phenotype is often disrupted. M1 polarized macrophages appear first and mediate tissue damage and initiate inflammatory responses, and during the early stages of this healing response, the newly recruited, infiltrating macrophages express a M2 phenotype [61-62], indicating a temporal switch from mediating the inflammatory response to resolving it. Furthermore, their depletion inhibited the formation of a highly vascularized, cellular granulation tissue [63]. There is some evidence supporting the idea that macrophages interact with progenitor and stem cells and that such interplay may contribute to repair and remodeling. Mesenchymal stem cells (MSCs) are a popular cell therapy candidate for the promotion of tissue repair or immunoregulation [64] and are known to engage in a bidirectional interaction with cells of the monocyte-macrophage lineage [56, 64]. M2 macrophages and their mediators can promote growth of human MSCs [65] and stimulate MSC motility [66]. Conversely, MSCs can greatly influence the function of macrophages and have been reported to induce an IL-10^{high} IL-12^{low} alternative (M2) activation phenotype in macrophages [67].

In a peritoneal model of inflammation, macrophages involved in the resolution phase expressed a mixed M1/M2 phenotype and it was shown that cyclic adenosine monophosphate (cAMP) is essential in impeding M1 activation [68]. In a mouse model of ischaemia, induction of the nuclear factor kappa B (NF-κB) pathway in macrophages promoted their polarization to the M2 phenotype and resulted pro-angiogenic functions [69]. A macrophage phenotypic switch has also been observed in models of ischaemic heart and kidney disease, where monocytes recruited to the tissue undergo dynamic changes in their activation state, shifting from a predominantly M1 phenotype to a
predominantly M2 phenotype [70-74]. These studies suggest this phenotypic switch is a general feature of the natural progression of repair processes.

There are some examples of disease and injury states characterized by chronic inflammatory conditions caused by a failure to switch macrophage phenotypes. Chronic venous ulcers are not able to resolve chronic inflammatory conditions and a mouse model has shown that the infiltrating macrophages fail to switch from M1 to the M2 phenotype and prolonged, excessive M1 activation state was associated with ROS-mediated cell damage, fibroblast cellular senescence, and defective tissue repair [75]. It is known that a similar peak in M1 macrophages is observed in Duchenne muscular dystrophy, where M1 phenotypes are greatly elevated in areas of muscle damage and mouse studies have shown that by decreasing M1 polarizing mediators (e.g. IFNγ) and increasing M2 polarizing cytokines (e.g. IL-10), increased muscle cell proliferation and improved motor function are observed [76-77].

Implication of M1 macrophages in tissue destruction is often contributed to their production of lipid mediators and other inflammatory signals like ROS. Lipid mediators play a key role in the regulation of inflammation [78-79] and M1 macrophage-produced ROS can react with cell membrane lipids causing the peroxidation of cellular lipids that release arachidonic acid and generate additional proinflammatory mediators such as prostaglandins, thromboxanes, leukotrienes, and cytotoxic peroxides [80]. Differential regulation of arachidonate-related enzymes has been described in M1- and M2-polarized macrophages, where M1 macrophages show strong induction of cyclooxygenase 2 (COX2) with downregulation of COX1 while M2 macrophages demonstrate upregulation of COX1 [81]. It has also been shown that M1 macrophage signals, like LPS, induce
higher levels of prostaglandins, while M2 macrophage signals, like IL-4 and IL-13, down-regulate inflammatory prostaglandin synthesis by macrophages [81]. Thus, conditions of a polarized inflammatory response involve macrophages that can profoundly alter their lipid profile and production of lipid mediators, which plays a major role in the induction, regulation, and resolution of inflammation. Elucidating the signaling pathways involved in this process represents an important area of investigation that refers to a wide range of pathologies. M1 macrophage-derived ROS has been associated with liver injury induced by hepatotoxicants like acetaminophen and examination of macrophages accumulating in toxicity-induced liver damage are known to release excessive quantities of ROS and other oxidants that exacerbate injury [82-87]. Furthermore, the stimulation of these macrophages to increase production oxidants worsens injury [82, 85].

Another mediator of M1 macrophage-induced tissue damage is from proinflammatory cytokines, such as TNFα, IL-1, and IL-6. TNFα, in particular, has been implicated in the pathogenesis of septic shock and inflammatory tissue injury, as well as the regulation of apoptosis, acute-phase inflammatory gene expression, and cytochrome P450 activity [88-90]. TNFα also promotes the stimulation of the release of cytotoxic and immunoregulatory mediators including IL-1, IL-6, colony-stimulating factors, prostaglandins, and ROS from macrophages and neutrophils, all of which can augment tissue injury and perpetuate inflammation [91-93]. Moreover, in the case of liver injury, tissue damage is attenuated with the administration of antibodies that functionally block TNFα or TNF receptor antagonists [94-96] and is suppressed in mice lacking TNFα or
TNF receptors [97-99]. Such findings suggest that TNFα is a critical mediator of M1 macrophage-induced damage in liver injury.

Under normal conditions of inflammation and the initiation of wound repair, there is a phenotypic switch from the M1 phenotype to a resolving M2 macrophage polarized state, where M2 macrophages accumulate at the sites of injury. M2 macrophages can restore homeostasis by down-regulating M1 macrophage response, cytotoxic inflammatory mediators, and stimulating tissue repair. M2 macrophages have been shown to be immunosuppressive in animal models of multiple sclerosis, rheumatoid arthritis, and lung inflammation [100-102]. Furthermore, depletion or blockage of activation or recruitment of M2 macrophages into inflammatory sites in the liver delays repair and/or exacerbates injury and the development of fibrosis [103-106]. Similar to M1 macrophages, M2 macrophages exert their functions by way of cytokine secretion, such as IL-4, IL-10, and IL-13. The upregulation of these inflammatory mediators protects against tissue damage, while their inhibition or ablation results in an exaggerated inflammatory response [76-77, 107-108]. In another mouse model of liver injury and inflammation, administration of IL-13 protects mice from endotoxemia, while treatment of animals with IL-13 antibodies exacerbates damage and reduces survival [108-109]. Thus, many states of inflammation and tissue repair are characterized by a perpetuation of the M1 macrophage response with concomitant suppression of the M2 macrophage response. It is important to note, however, that these polarization states should not be viewed as having opposing actions at inflammatory sites; rather, there is a complex interplay between the two and both are required for an appropriate response. Activated macrophages are crucial for proper immune defense and wound repair and an imbalance
in polarized states contributes to tissue damage. The extent of this contribution is likely
due to the balance of phenotypic functions and the timing of their appearance at sites of
injury and inflammation.

MACROPHAGE POLARIZATION RESPONSE TO BIOMATERIALS

Macrophages are the primary cell type responsible for the degree of foreign body
reaction around biomaterial implants. They are known to interact with biomaterials
through integrin-mediated mechanisms that provide intracellular signals, including
cytoskeletal remodeling, gene transcription, motility, and proliferation, which are crucial
in regulating the ultimate functions of macrophages [110-111]. Traditionally,
macrophages were considered deleterious and exerted mostly destructive functions
toward implanted biomaterials because of their fusion into multi-nucleated cells and the
fibrous encapsulation of the material [110]. But the macrophage response is a vital one
and is necessary for proper resolution of any insult and, ultimately, is a deciding factor in
the incorporation or rejection of a biomaterial. In fact, in macrophage depleted rats, it was
proposed that the absence of macrophages resulted in inhibited scaffold degradation, thus
hindering an improved remodeling process [112].

Biomaterials with similar properties to the one utilized in this study, keratin, (i.e.
naturally sourced, highly porous structure, easily modifiable, and biodegradable) have
been shown to reduce inflammation to some extent [113-117]. For example, hyaluronic
acid (HA) has been shown to block the downstream effects of IL-1 [116-117], IL-6 [118],
and TNF [119]. In addition, the biocompatibility of collagen is generally considered
acceptable and its use in biomedical applications is considerable, including drug delivery,
prosthetic implants, and tissue engineered organs [113-114, 119]. One study demonstrated that collagen combined with a highly sulfated HA can reduce early inflammatory mediators and support an increased but non-significant expression of M2 macrophage markers at later time points [115]. The same study also showed that collagen alone can modulate a monocyte to macrophage transition, but exhibits a mixed cytokine profile and significantly higher levels of only an M1 marker (similar to the results of the present study, as discussed further in Chapter 3). Recent studies that more closely examine this dynamic have shown that beneficial outcomes can be obtained if macrophage polarization is biased toward an M2 phenotype through some activity of the biomaterial. For example, in an abdominal wall defect model, there was a strong correlation found between favorable tissue remodeling and increasing numbers of M2 macrophages after implantation of an ECM-derived scaffold [120]. It was also demonstrated that M2 macrophages have an enhanced ability to recruit progenitor-like cells to sites of remodeling. Additional studies utilizing collagen biomaterials showed that collagen induced low amounts of both pro- and anti-inflammatory proteins, indicating a more neutral reaction where it is not necessarily promoting M1 inflammatory-produced localized destruction, but is also not promoting an M2 response [121]. A second study using collagen showed similar effects where it was able to encourage differentiation to a degree, but ultimately elicited a mild reaction or maintained a higher M1:M2 ratio [115].

Synthetic biomaterial strategies for the modulation of macrophage phenotype are also being investigated to influence regeneration and healing. One study showed that, when compared to small size particle coatings, macrophages cultured on large size
particle polyvinylidene fluoride induced a mixed activation state characteristic of both M1 and M2 macrophages [122]. More importantly, a gene array analysis indicated 180 genes were upregulated by the surface structure and 50 genes were found to be specifically regulated by the polyvinylidene fluoride structures (and not cell-secreted mediators present), suggesting that macrophage response to biomaterial surfaces may be mediated through different cellular processes and that regulation of the macrophage polarization state is possible with biomaterials. Similarly, Garg, et al demonstrated that macrophages cultured on polydioxanone scaffolds with larger diameter fibers and pore sizes increase expression of M2 macrophage markers while decreasing M1 macrophage markers [123]. Furthermore, it has been shown that differing surface chemistries of biomaterials can have significant effects on leukocyte cytokine messenger ribonucleic acid (mRNA) responses in mice [124].

Still, there remain relatively few synthetic biomaterial studies that have been published advocating strategies to bias macrophage polarization for purposes of constructive tissue remodeling or regenerative medicine. A recent review suggested that such biomaterial engineering may be possible, and certainly desirable, but has yet to occur in the mainstream [125]. This is likely due to an inadequate understanding of the underlying mechanism and hence, limited synthetic strategies to engineer predictable immune responses with regard to M1/M2 macrophages. In fact, the modification of natural biomaterials and scaffolds with chemical crosslinkers, which has been shown to delay or prevent macrophage-mediated degradation and is a typical strategy to improve mechanical properties, actually prevents initiation of the M2 response and ultimately results in weakened remodeling and downstream scar tissue formation [112, 126-127].
This finding suggests that there may be an interaction occurring between host cells and ligands present on the biomaterial surfaces, the detailed investigation of which may lead to targeted strategies to directly influence macrophage phenotype with biomaterial design. Thus, the interaction between macrophages and biomaterials often depends on the material’s source and degree of modification, but it is now recognized that there is a necessity to bias the macrophage response to an M2 phenotype.

The following dissertation work investigates the ability of a keratin biomaterial to modulate inflammation and remodelling. The first aim examines this in the context of a spinal cord hemisection injury in rats and the extent to which keratin’s immunomodulatory properties can influence functional recovery. The second aim elucidates the macrophage polarization response to keratin in vitro. Lastly, the third aim investigates how keratin-polarized macrophages can manipulate reactive astrocytes and their production of CSPGs and GFAP expression. Keratin may offer several advantages over traditional biomaterials because it does not rely on complex techniques such as chemical cross-linking, genetic modification to append cell binding domains, and requiring administration with cells, growth factors, and other adjuncts. Previous studies have demonstrated its inherent biologic activity and excellent biocompatibility [2-6, 128].

The goal of this work was to take a more broad approach to understanding keratin’s potential mechanism in inflammatory-mediated states of injury and damage.


TREATMENT OF A SPINAL CORD HEMITRANSECTION INJURY WITH KERATIN BIOMATERIAL HYDROGEL ELICITS RECOVERY AND TISSUE REPAIR

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ABSTRACT

Medical care costs can reach an estimated $4 billion for spinal cord injuries (SCI) each year in the United States alone. With no viable treatment options available, care remains palliative and aims to minimize lifelong disabilities and complications, such as immobility, bladder and bowel dysfunction, breathing problems, and blood clots. Human hair keratin biomaterials have demonstrated efficacy in peripheral nerve injury models and was shown to improve conduction delay and increase axon number and density. In this study, a keratin hydrogel was tested in a central nervous system (CNS) application of spinal cord hemisection injury. Keratin-treated rats showed increased survival rates as well as a better functional recovery of gait properties and bladder function. Histological results demonstrated reduced glial scar formation with keratin treatment, and suggest a greater degree of beneficial remodeling and cellular influx. The data provided in this pilot study suggest the possibility of using a keratin-based treatment for SCI and warrant further investigation.
1. **INTRODUCTION**

More than 2.5 million people worldwide are effected by spinal cord injuries (SCI), with approximately 12,000 new cases each year in the United States alone [1]. The majority of these cases are caused by motor vehicle accidents (36.5%) and falls (28.5%), and predominantly affect young males (80.7%) [1]. Depending on the extent of the injury, survivors can experience severe lifelong disabilities, including immobility, pressure sores, autonomic dysreflexia, neurogenic pain, and breathing problems. Most SCIs will interrupt bladder and bowel functions because the nerves controlling these processes originate in the lower segments of the spinal cord and are subsequently cut off from brain input. In the case of the bladder, loss of neural input causes voiding to become abnormal and thus requires the chronic use of catheters. SCI injury has an even greater impact when lifetime costs are considered, which easily reach into the millions per patient. Medical care costs alone reach an estimated $4 billion per year in the United States [2]. Taking into account the fact that the majority of patients are young, active people who are now relegated to long-term palliative care, these numbers increase even more when considering loss of productivity and income wages [1].

Treatment options after SCI are limited, and life expectancies have not improved for patients since the 1980s. The use of the steroid methylprednisolone sodium succinate (MPSS) has fallen out of favor for many clinicians because of the lack neurological improvements and serious side effects [3-5]. Spinal stabilization surgery is performed if the spinal cord is being compressed by a blood clot or herniated disc, and is effective in terms of stabilizing the spine, relieving pressure, and preventing further deformities.
However, it does nothing to address the damage that has already been done. Moreover, there is no current evidence to suggest it offers advantages in functional outcomes [6-7]. Physical therapy can offer some improvements, but the standard of care remains palliative.

After the initial injury, inflammation, cell death, and limited axon sprouting occur before a glial scar seals the site of injury. However, the lesion continues to expand due to secondary damage [8-9]. The pathophysiological development of secondary tissue damage in SCIs occurs through a complicated progression of cellular and molecular events [2]. Following the immediate mechanical damage to neural and other soft tissue, there is ischemic cellular death and edema, along with the infiltration of inflammatory cells, demyelination, and scarring and cyst formation at the lesion site. Because the injury compromises the blood-spinal cord barrier, immune cells migrate from the periphery and elicit an inflammatory response. The infiltration of neutrophils, macrophages, and lymphocytes helps fight infection and clear debris, but it also sets off the release of dozens of pro-inflammatory cytokines and chemokines that have been shown to negatively influence glial and neuronal cells. Another consequence is the production of reactive astrocytes, which in addition to producing inflammatory cytokines, ultimately participate in the formation of scar tissue. All of these processes eventually lead to loss of neuronal function.

Therapeutic emphasis in clinical and preclinical studies has been on cellular and molecular therapeutic interventions, and include transplantation or administration of a
variety of cell types, neuro-protective agents, and growth factors [8, 10-12]. But many are associated with several limitations, including the need for immunosuppressive drugs and a mode of continuous infusion [13-14], which naturally raises the question of translation to patient care and how easily such a treatment could be administered to encumbered patients.

Keratin biomaterials have unique chemical, physical, and biological properties and have shown a significant ability for nerve repair in peripheral nerve injuries that surpasses conventional biomaterials, yet do not rely on some of the more complex technologies needed to enhance other therapies, such as the addition of cells, growth factors, and/or gene constructs [13-18]. These studies using other biomaterial fillers such as hyaluronic acid, collagen and fibrin gels and scaffolds, as well as numerous tubular devices made from synthetic and natural polymers have shown promise, primarily in rodent studies, but relatively few have translated clinical and no filler materials are current approved for human use by the US Food and Drug Administration. Studies using keratin biomaterial hydrogel fillers improved peripheral nerve regeneration by promoting Schwann cell activity via chemotaxis, increasing attachment and proliferation, and the up-regulation of genes that contribute to cell proliferation and differentiation [19]. However, later studies demonstrated only a modest increase in cellular infiltration into the conduit at early time points [20]. Pace et al. found that while keratin hydrogel fulfills primarily a structural role, increased myelin debris clearance in the distal nerve stump and reduced inflammatory cell influx also may also influence the regenerative process in peripheral nerve regeneration.
Keratin biomaterials constructed from human hair are particularly versatile and can be processed into hydrogels, coatings, films, and sponges. The purpose of the current study was to investigate the potential use of a keratin hydrogel in SCI following a hemisection injury. Based on our earlier work in peripheral nerves, we postulated that keratin would be able to overcome the deterrent environment normally caused by SCI and provide a permissive milieu that supports repair and regeneration, as well as functional recovery. To investigate this, a rat hemisection model was used. This study addressed the critical question of assessing functional outcomes, which some previous studies have neglected. We hypothesized that treatment with a keratin hydrogel would encourage restoration of the ability to walk as well as void, the two most common disabilities present in persons with SCI.

2. MATERIALS AND METHODS

2.1 Keratin Hydrogel Preparation

Isolation of the crude keratin material was achieved using previously described methods [19, 21]. Briefly, human hair was oxidized using peracetic acid followed by washing with deionized (DI) water to remove the residual oxidant. The soluble keratin proteins were extracted in tris(hydroxymethyl)aminomethane (Tris) base and DI water. The pooled solution was then dialyzed and neutralized (pH 7.4) with NaOH, lyophilized, and ground into powder. The keratin powder was sterilized using a 25 kGy dose of \( \gamma \)-irradiation. To form hydrogels, sterile lyophilized keratin was aseptically reconstituted to 15\% (w/v) solution in phosphate buffered saline (PBS) and left to shake at 37°C overnight.
2.2 Rat Spinal Cord Hemisection Injury

25 male Lewis rats were randomly divided into two treatment groups, keratin hydrogel and saline treatment. All animal procedures were approved by the Wake Forest University Animal Care and Use Committee and conducted according to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. All procedures were performed under sterile conditions. Rats were anesthetized using isoflurane (1.5%-2%) and oxygen. A ~2cm incision was made along the thoracic vertebral level. The laminae were resected and the dura mater incised to expose the spinal cord. A left lateral hemisection at T8-T9 was made by creating a cut along the midline of the cord. Immediately after, 100µl of keratin hydrogel or saline was placed into the gap. Dura mater and skin were closed using 4-0 Vicryl suture (Ethicon). Buprenorphine (0.01mg.kg) was administered after closing and as needed for analgesia. Cefazolin (250mg/kg) was administered after closing and prophylactically for 2 weeks. Rats were given lower abdominal and bladder massages 3-4 times daily to aid digestion until they were able to void independently.

2.3 Cystometric Analysis and Bladder Weight

Cystometric studies were conducted at 3 weeks and 6 weeks post-hemisection injury in awake, moving rats, as previously described [22-24]. Briefly, the implanted bladder catheter was attached to a two-way valve that was connected to a pressure transducer and infusion pump. An ETH 400 (CD Sciences) transducer amplifier and MacLab/8e (Analog Digital Instruments) was connected to the pressure transducer. Saline was introduced at
10mL/h and micturition volumes (MVs) measured using a force-displacement transducer. Analyses were conducted over a 25 minute time period. After cystometric analysis, animals were sacrificed and bladders immediately harvested and individually weighed.

2.4 Evaluation of Hind Limb Locomotor Function

Gait properties were evaluated using a treadmill and the TreadScan® software system. Prior to the hemisection injury, rats were introduced to a treadmill to acclimate them and obtain normal baseline levels of gait parameters. It has been previously shown that increased exposure to the treadmill preceding injury can lead to adverse training effects [25], so animals were given minimal exposure. The recording and analysis procedure has been previously described [25]. Briefly, a high-speed digital camera recorded the rats from underneath as they walked across a clear treadmill. The TreadScan® software identifies each footprint and analyzes it for numerous gait parameters. All analyses were done using data from the lower right leg (per lateral hemisection injury).

2.5 Histology/Tissue Preparation

Rats were sacrifice at 3 weeks and 6 weeks post-hemisection injury. Animals were anesthetized with a ketamine/xylazine (100mg/ml, 20mg/ml, respectively) solution and trans-cardially perfused with 4% paraformaldehyde. Spinal cords were dissected and removed and stored in 30% sucrose for 24 hours at 4°C. Cords were then cut into 2cm segments, embedded in O.C.T compound tissue freezing medium (Sakura), flash frozen using liquid nitrogen and stored at -80°C. Serial sections were cut longitudinally using a cryostat at 15µm thick. Masson’s trichrome staining was done to elucidate scar formation
and tissue organization. Images were captured using a Zeiss Axio Vert.A1 (Carl Zeiss Microscopy).

### 2.6 Statistical Analyses

Comparisons between treatment groups were analyzed for statistically significant differences using Prism v5.0 (GraphPad Software, Inc.). All data are reported as mean ± standard deviation (SD). Survival curves were derived by the Kaplan-Meier method and compared by log-rank test. One-way analysis of variance (ANOVA) was used to determine significant differences between groups with \( n \geq 3 \) for bladder weights. Repeated measures ANOVA with Tukey’s post hoc were run on the baseline averages to assess any significant differences due to time or treatment in gait characteristics. \( p < 0.05 \) was considered statistically significant.

### 3. RESULTS

#### 3.1 Effect of Keratin Treatment on Survival

Kaplan-Meier survival curves demonstrate that treatment with keratin hydrogel improved survival rates compared to saline treatment following hemisection injury (Figure 1). However, a high mortality rate in the saline group, particularly at long time points, contributed to the non-significance of this outcome.

#### 3.2 Cystometry and Recovery of Bladder Function

Representative cystometrograms (CMGs) at 6 weeks post-hemisection of the treatment groups and age-matched controls show distinct recovery of voiding abilities in rats that
have been treated with keratin (Figure 2). Voiding pattern variability was observed within all groups at 3 weeks and 6 weeks, making a quantitative analysis difficult. However, trends in voiding pattern were observed. A typical saline-treated rat’s bladder presented spontaneous uninhibited contractions not coupled with definite voids, which is indicative of an incontinent bladder (Fig. 2B). Although the keratin-treated rat’s bladder has some instability during the filling phase (Fig 2C), it is much closer to a normal micturition pattern (Fig. 2A), as it shows a regular pattern of definite voids coupled with pressure spikes and uninhibited contractions between voids (Fig. 2C). This is representative of a functioning yet neurogenic bladder.

Average bladder weights at 6 weeks post-hemisection demonstrates the keratin group had significantly smaller bladders than the saline group overall. Bladders from the saline group were significantly larger than normal bladders from the uninjured age-matched control group, indicating a greater degree of hypertrophy. There is no significant difference between the keratin group and the uninjured age-matched controls.

3.3 Effects of Keratin Treatment on Hind Limb Motor Function
Keratin treatment was shown to significantly improve recovery of several gait properties (Figure 4). Stance time, which is the time elapsed while the foot is still contact with the tread in its stance phase, was significantly higher in the keratin group at 3 weeks post-hemisection (Fig. 4A). Although the saline group showed increased recovery at 6 weeks, the keratin group still maintained its significance. Stride length, which is the distance the tread has moved while the foot is still in contact with it, also revealed a significant
increase in the keratin-treated group by 6 weeks post-hemisection (Fig. 4B). Range of motion (ROM), or the distance between the farthest point and the closest point during each stride, is typically a good indication of front-to-back motion. Again, by 6 weeks following hemisection injury, keratin treatment significantly improves recovery and is closer to the uninjured baseline level (Fig. 4C).

3.4 Histologic Analyses

By 3 weeks post-hemisection, both keratin and saline-treated rats show degeneration as well as scar formation at the lesion epicenter (Figure 5). However, by 6 weeks the keratin group displayed remarkable recovery to a near-normal anatomy. Of particular note is the lessening of the glial scar, where it is almost completely absent and limited to the lesioned half of the cord. Spinal cords from the saline-treated group had especially severe degeneration at 6 weeks post-hemisection.

4. DISCUSSION

SCIs are particularly difficult to treat and there are many experimental approaches being pursued, including the use of stem cells, small molecule therapies, and various autologous cell and tissue grafts. While some promising results have been noted, a recent stem cell clinical trial by Geron Corporation was halted, indicating that a clinical treatment using these complex approaches may still be many years away. This study has taken a simpler, biomaterials-based approach using a keratin hydrogel. Keratin biomaterials have previously demonstrated efficacy in promoting repair following injury in a peripheral nerve model in mouse, rat, rabbit, and, most recently, non-human primate
models [19-20, 26-27]. Given the significance of those results, keratin was considered a viable candidate for use in the central nervous system. Moreover, hydrogels are especially well-suited for SCIs because of intrinsic properties such as softness, highly porous three-dimensional structure, mimicry of the extracellular matrix (ECM), and a reduced foreign body response [28]. In addition to embodying these classic characteristics, studies have shown that keratin hydrogels are able to avoid any major fibrous capsule response, can support cellular and vascular infiltration, can evade foreign-body giant cell formation and graft rejection, and because of the absence of genetic elements, is non-antigenic and non-immunogenic [21, 29-30].

The purpose of this study was to investigate keratin’s potential role in promoting repair following SCI. Due to the exploratory nature of this pilot study and the high mortality rate, the experiments were underpowered. Survival rates for those rats treated with keratin was markedly greater than for those treated with saline (Figure 1), but due to the lack of surviving animals in general, and in the saline group in particular, especially at the 6 week time point, statistical significance in this particular outcome was not achieved. The attrition rates observed in this study were higher than expected, although this type of data are typically not published and difficult to obtain. Nevertheless, all animals were given the exact same post-operative care and treatment, so within the present study it is difficult to ignore the effects observed.

It is well known in SCI that urinary bladder control is one of the first problems patients encounter. The normal expectation after spinal cord damage above the level of S2 is an
initial spinal shock where there is complete urinary retention with possible overflow incontinence, followed by the initiation of an automatic bladder, or loss of micturition reflexes, abnormal amounts of residual urine, and involuntary urination within 2-3 weeks. In the present study, the effect of keratin on regeneration in the spinal cord was studied and consequently bladder function was used as an indication of successful recovery. Our postulate was that in rats, more rapid recovery from the spinal shock phase would be observed, and thus our analysis was focused on the 3 week time point. As expected, the saline group showed severe nerve damage with no clear voiding pattern in any of the animals tested (Fig. 2B). Pressure data showed marked uninhibited contractions and irregular voiding pattern that was not correlated with specific spikes in pressure. This pattern indicates loss of upper motor neuron control on the bladder and failure of nerve regeneration at the spinal cord level. In the keratin group however, there was a more robust pattern of voiding in some of the animals tested and there were regular bouts of micturition in 60% (3/5) of animals tested (Fig. 2C). The pressure data shown in animals showed a clear trend toward normalization of micturition, albeit with a sustained increase in uninhibited contractions in between those sections. These data suggest at least some regeneration at the spinal cord level, allowing at least minor upper center control of the bladder to resume.

Another indication of a neurogenic response by the bladder to SCI is the degree of hypertrophy. As a muscle, the bladder will compensate for any voiding problems by enlarging. Measuring the weights of bladders taken from each treatment group provides an indication of how the bladder is responding. Keratin treatment yielded significantly
smaller bladders compared to saline treatment and there was no significant difference between uninjured age-matched controls and the keratin treatment group bladder weights (Fig. 3).

Another substantial functional outcome following SCI is gait analysis. The ability to walk is a good assessment of SCI deficit because it shows definitively that neurite connections have regenerated to an extent to become functional. Moreover, walking is noted to be the highest priority for SCI patients [31-32]. A thoracic hemisection injury, such as the one employed for this study, will cause the animal to lose the use of one hind limb, in this case the right hind limb. The TreadScan® system allows the animal to be recorded from underneath a clear treadmill as they are walking. It provides a highly sensitive and objective output that in most, but not all, cases is more insightful than the classic open-field assessments [25]. Baseline readings taken at Day 0 are pre-SCI and show how a normal response appears (Fig. 4). Keratin treatment promoted a significant recovery of all three properties by 6 weeks. Previous studies using mouse models of SCI have found that injured animals display a phase of initial recovery, which then plateaus by 2-3 weeks [33-35]. This is consistent with trends observed within the saline treatment group, as there was no significant difference between 3 weeks and 6 weeks for all three gait properties measured. In contrast, there was significant restoration of stance time, stride length, and longitudinal ROM to near-normal levels with keratin treatment, thus showing recovery occurring longer and later.
Regeneration of neurons is limited by many factors that contribute to an environment that is impermissible to growth and repair, unlike regeneration of peripheral nerves which can occur at a rate of up to 5mm per day [36]. However, previous studies have shown regeneration is possible if the lesion milieu promotes and supports growth [36-38]. Specifically, the inhibitory lesion site, or glial scar, must be prevented or kept at a minimum to allow CNS axons to grow [36, 38-41]. Spared white matter has been shown to correlate well with functional recovery following SCI [34-35, 42-43]. This study demonstrated consistent findings in this regard, as seen at 6 weeks post-hemisection where the keratin treatment group spinal cords showed greater remodeling (Fig 5), which is also the time point at which the greatest functional recovery appeared to take place (Figs. 2, 4). At 3 weeks post-hemisection, the keratin treatment group’s spinal cords were not visibly different from the saline treatment group, as both displayed degeneration and some scar tissue formation. By 6 weeks post-hemisection, it is apparent how similar the tissue structure and organization of the keratin-treated cord is to the uninjured spinal cord (Fig. 5). There are several potential cellular and molecular mechanisms that could be responsible for regeneration at this level, such as modulation cell-mediated inflammatory cascades and/or promotion of glial cell remodeling activity. However, previous studies would suggest keratin is not acting directly on neurons or axons, but rather as a structural support, providing an environment more conducive to growth, which is achieved in the spinal cord through promoting beneficial cell types like oligodendrocytes, microglia, and macrophages.
5. CONCLUSION

This pilot study suggests the potential of a keratin biomaterial-based treatment in SCI applications. Although the study was underpowered, gait parameters did reach statistical significance and other notable observations were made. In general, keratin treatment seemed to improve survival rates compared to saline treatment. Rats treated with keratin also appeared to have greater recovery of bladder function. Although micturition patterns are shown in qualitative terms, the average weight of bladders was significantly smaller with keratin treatment, indicating less hypertrophy and thus restored innervation of the bladder muscle. There was also a significant improvement in hind limb motor function recovery with keratin treatment. Histological analysis suggests an increased cellular presence with keratin, which may be indicative of several potential mechanisms of action. However, further studies are needed to confirm these findings in a more statistically robust design, as well as explore these initial observations and address mechanistic questions.

6. ACKNOWLEDGEMENTS

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7. CONFLICT OF INTEREST STATEMENT

Author Mark Van Dyke holds stock and is an officer in the company, KeraNetics LLC, who provided partial funding for this research. Wake Forest School of Medicine has a potential financial interest in KeraNetics through licensing agreements.
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Figure 1. Kaplan-Meier Survival Curve of saline-treated rats (n=11) compared to keratin treated rats (n=12).
Figure 2. Representative cystometrograms showing the qualitative trends observed within each group. (A) Normal uninjured bladders show normal micturition pattern. (B) Saline-treated rats displayed uninhibited contractions not coupled with definite voids, while keratin-treated rats (C) showed a closer to normal voiding pattern. Each group includes a pressure curve (0-60cm/H2O) versus a volume curve (0-5mL) over a 25-minute time scale (X axis).
**Figure 3.** Average weight of bladders of animals treated with saline (control), keratin (experimental), and an uninjured age-matched control (positive control).
Figure 4. Gait analysis shows greater recovery of stance time (A) with keratin treatment at both 3 weeks and 6 weeks post-injury. Keratin-treated rats also showed significantly greater stride length (B) and longitudinal range of motion (C) by 6 weeks.
Figure 5. Trichrome staining of recovered spinal cords showed notable restoration of tissue in the keratin treated group compared to saline by 6 weeks. While some scarring is evident, the deterioration that occurred with saline treatment was not evident in keratin treated animals.
IN VITRO RESPONSE OF MACROPHAGE POLARIZATION TO A KERATIN BIOMATERIAL

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ABSTRACT

Macrophage response to biomaterials is emerging as a major focus in tissue repair and wound healing. Macrophages are able to differentiate to two distinct states eliciting divergent effects. The M1 phenotype is considered pro-inflammatory and up-regulates activity related to tissue destruction, while the M2 phenotype is considered anti-inflammatory and supports tissue remodeling. Both are necessary but a fine balance must be maintained as dysregulation of naïve macrophages to M1 or M2 polarization has been implicated in several disease and injury models, which has been suggested as a potential cause for poor outcomes. Keratin biomaterials have been shown to promote regeneration in several tissues using different animal models. A potential common mechanism may be the general capability for keratin biomaterials to elicit beneficial inflammatory responses during the early stages of regeneration. In the present study, a keratin biomaterial was utilized in vitro to examine its effects on polarization toward one of these two macrophage phenotypes and thus, a role in inflammation. Exposure of a monocytic cell line to keratin biomaterial substrates was shown to bias macrophages toward an M2 phenotype, while a collagen control surface produced both M1 and M2 macrophages. Furthermore, keratin treatment was similar to the M2 positive control and was similarly effective at down-regulating the M1 response. Keratin biomaterial influenced greater production of anti-inflammatory cytokines and decreased amounts of pro-inflammatory cytokines. The use of a keratin biomaterial in regenerative medicine may therefore provide additional benefit by regulating a positive remodeling response.

Keywords: Keratin; macrophage; inflammation; polarization; immune response
1. INTRODUCTION

Biomaterials have many current and potential applications, including those in tissue engineering and regenerative medicine (TE/RM). Regardless of their intended use, all biomaterials will elicit a reaction from their host, the foreign body response, which has great influence over the degree of success or failure in TE/RM applications. Prominent participants in the response of the body to an implanted biomaterial are macrophages. Exposure to implanted materials generally causes macrophages to fuse into multinucleated giant cells, which ultimately leads to fibrous encapsulation and scar tissue formation around the implant.[1,2] Multinucleated giant cells are generally associated with chronic inflammation and, depending on signals encountered within the environment, can arise from both ends of the macrophage phenotype continuum.[2-4] Within the context of TE/RM, it has been demonstrated that adherent macrophages on biomaterials (precursors to foreign body giant cells) revealed a profile that was neither M1- nor M2-polarized but somewhere in the middle.[2] While many strategies aim to avoid this process and the host immune response completely, macrophages have recently emerged in a different light as an important component of the innate immune system that can modulate and attenuate tissue remodeling following injury.[5-8] More recently it has been suggested that the key to tissue regeneration approaches may be the concept of regulating the balance between two distinctly different sub-types of macrophages.

The general utility of keratin biomaterials has been described by several investigators for applications such as drug delivery, tissue regeneration, hemostasis, and wound
Three general findings have been reported - excellent biocompatibility, cell adhesion, and improved tissue healing. As early as 1982, scientists reported work on the general biocompatibility of wool-based keratin biomaterials. This Japanese language publication describes the preparation of both oxidized and reduced, solubilized keratin that were used to coat polyester meshes with a glutaraldehyde crosslinked film of keratin biomaterial prior to implantation into the dorsal muscle of dogs and rabbits. After 2, 4 and 6 weeks, the implants were scored for degree of foreign body reaction by examining histological sections. The investigators found that the degree of foreign body reaction was low in all cases, with no apparent distinction between the different forms of keratin biomaterials used in the study. More recently, several authors have expanded on this initial finding of good biocompatibility in papers utilizing a variety of in vitro and in vivo model systems, sometimes by blending keratin with other biomaterials. Cell adhesion to keratin substrates has also been demonstrated by several authors, and tissue healing (i.e. regeneration) studies have included skin, bone, nerve, cornea, and heart, with consistent findings of improved tissue repair and little notable scar formation reported.

Based on our group’s earlier experience in peripheral nerve regeneration, we undertook a pilot study to investigate the potential for a keratin biomaterial hydrogel to facilitate neuronal regeneration in the spinal cord. Along with several observations demonstrating improved functional recovery, the data from this study suggested that downstream tissue damage normally seen due to the inflammatory cascade was mitigated by keratin biomaterial treatment. Interestingly, Kigerl et al. demonstrated
that these secondary injury mechanisms in the spinal cord are dominated by a pro-
inflammatory M1 macrophage phenotype, a response that overpowers the relatively smaller and transient anti-inflammatory M2 macrophage phenotype.[39] Limited staining of the spinal cord tissue from the aforementioned pilot study revealed a strong M2 presence and a notably smaller M1 population. Other studies have shown that a keratin-based implant such as a hydrogel, quickly becomes infiltrated with resident cells, but that a classical foreign body reaction does not ensue, overall cell population decreases, and the relatively small, initial inflammatory response resolves itself quickly.[30,31] These observations suggest that keratin biomaterials may be influencing the cellular response to tissue injury, particularly inflammation. Based on the foregoing research, we postulated that keratin biomaterials may be capable of inducing macrophage polarization at sites of injury, and that this may represent a common mechanism that is partly responsible for the beneficial tissue regeneration reported by different investigators around the world, including our group.

The purpose of the current study was to investigate the role of macrophage response in keratin’s capacity as a regenerative biomaterial. We hypothesized that keratin can contribute to macrophage polarization, and ultimately tissue regeneration, by favoring the growth- and regeneration-promoting M2 phenotype. To examine this, an in vitro culture system employing a human monocytic cell line was used to determine the relative ratio of M1 and M2 macrophage phenotypes that arise at different time points following growth on a keratin biomaterial substrate, as well as cytokines secreted by these cells, compared to cells grown on tissue culture plastic (TCP) and collagen substrates.
2. MATERIALS AND METHODS

2.1 Preparation of Keratin Biomaterial and Coatings

The keratin biomaterial was extracted and prepared as previously described.[11,31,38] Briefly, a 2% peracetic acid solution was used to oxidize human hair fibers. Following washing with deionized (DI) water to remove residual oxidant from the hair fibers, tris(hydroxymethyl)-aminomethane (Tris) base and DI water was used to extract the soluble keratin proteins. The solution was then dialyzed against DI water, neutralized to pH 7.4 with NaOH, lyophilized, and ground into a powder. The keratin powder was sterilized via exposure to a 25kGy dose of \( \gamma \)-irradiation and aseptically reconstituted in phosphate buffered saline (PBS). Keratin and type-I rat tail collagen (\( \geq \)90% purity; BD Biosciences) were diluted to a final concentration of 200\( \mu \)g/ml and 1ml of these respective solutions were added to the wells of glass chamber slides (Nunc, Thermo Fisher Scientific) and incubated for 24 hours at 37°C to form coatings. After incubation, excess solution was removed and the coated surface rinsed with PBS prior to cell seeding.

2.2 Human Macrophage Cell Culture

The THP-1 human monocytic cell line was obtained from ATCC and maintained in RPMI 1640 (Gibco Life Technologies) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 0.05mM 2-mercaptoethanol (Sigma). To generate adherent THP-1-derived macrophages (TDM), \( 1 \times 10^6 \) cells were added to wells in an untreated TCP six-well plate (Becton Dickinson) and treated with 5ng/ml of
phorbol myristate acetate (PMA; Sigma) dissolved in media for 48 hours at 37°C, 5% CO2. Macrophage phenotype (CD14+) was confirmed using flow cytometry (data not shown) (see Appendix 2, figure 2), and for the purposes of this study, will be considered as having an M0 phenotype. TDMs were then washed with PBS, detached using 0.25% trypsin/0.1% EDTA (HyClone), pelleted, and re-suspended in complete media. 1x10^6 TDMs were then plated and reattached on corresponding substrates of the glass chamber slides (Table 1). For control treatments, TDMs were induced to a polarized phenotype by culturing cells with either lipopolysaccharide (LPS; 100ng/ml; Sigma) and human recombinant (hr) interferon gamma (IFNγ; 20ng/ml; Sigma) to produce M1 macrophages, or hr interleukin 4 (IL-4; 20ng/ml; Sigma) to produce M2 macrophages in glass chamber slides.[40] Media, including that of the control treatments that contained cytokines, were changed every 3 days. TDM M0 macrophages were produced by incubating to their respective time points in the presence of complete media only (Table 1, No coating treatment group).

2.3 Immunocytochemistry

All stains were performed at room temperature (RT), manually, using an optimized double immunofluorescence technique. Briefly, macrophages cultured in glass chamber slides were washed with PBS, fixed in 4% paraformaldehyde (PFA) for 20 minutes at room temperature and washed with a buffer containing 0.1% bovine serum albumin (BSA) in 1x PBS. After blocking non-specific staining for 45 minutes (10% BSA), the first primary antibody was added (CD86 for M1 specificity, 10µg/ml; R&D Systems) and incubated for 1 hour at RT. After washing, the secondary antibody was added (NL-557,
1:200; R&D Systems) and incubated in the dark for 1 hour. After rinsing with the wash buffer, the second primary antibody was added to the wells (CD206 for M2 specificity, 15ug/ml; R&D Systems) and incubated for 1 hour. Cells were incubated with the final secondary antibody (NL-493, 1:50; R&D Systems) for an additional hour, washed and the gasket removed from the slide. Slides were mounted with ProLong® Gold Antifade (Life Technologies) mounting media and visualized using a Zeiss LSM510 inverted confocal microscope.

2.4 Macrophage Quantitative Analysis
Quantitative analysis of CD86+ (M1), CD206+ (M2), CD86+/CD206+ (M1/M2; co-expressing phenotype), and CD86-/CD206- (M0) cells for each treatment group at each time point was conducted by selecting 9 random areas per slide at 20x magnification and capturing digital images. The number of each phenotype, or co-expressing phenotype, present in the selected field was manually counted based on the positive staining of the cell. The mean (n=9) for each cell phenotype was calculated for each sample. The percent of total expression was determined for each phenotype by dividing the number of positively staining cells by total cell count (as determined by corresponding digital interference contrast or DIC images). Six independent replicates of each treatment condition were analyzed for each experimental group at each time point (i.e. six separate chamber slides).
2.5 Enzyme-linked Immunosorbent Assay (ELISA)

Culture media supernatants were collected immediately prior to immunostaining and centrifuged for 10 minutes at 1000 x g to remove any cell debris or particulates. Supernatants were aliquoted and stored at -80°C until analysis. To measure cytokine production, a multi-analyte ELISAArray kit (Qiagen) was used to simultaneously detect levels of multiple cytokines. Manufacturer’s directions and standard ELISA techniques were followed. Briefly, using a 96-well microplate coated with a panel of twelve capture antibodies, assay buffer was added to each well followed by experimental and control samples to their corresponding wells and incubated for 2 hours at RT. Wells were washed, the detection antibody solution added and incubated for an additional hour at RT. After another wash, avidin-horseradish peroxidase (HRP) was incubated for 30 minutes in the dark. Finally, a development solution was added for 15 minutes in the dark, followed by a stop solution. Absorbance levels were measured at 450 nm using a Spectramax M5 microplate reader (Molecular Devices) with a 570 nm correction wavelength.

2.6 Statistical Analysis

For macrophage phenotype quantitation, a two-way analysis of variance (ANOVA) with Bonferroni’s post-hoc test was used to determine significant differences due to time or culture conditions. For cytokine secretion data, negative control (i.e. uncoated TCP) values from ELISAs were subtracted from the corrected absorbance readings. Relative absorbance was calculated by normalizing data to the IL-8 absorbance signal (consistent across all time points and treatment groups). First, secretion levels were compared for
significant differences due to time within a treatment group using a two-way ANOVA with a Bonferroni’s post-hoc test, then differences between treatment groups across time were compared for significance for each cytokine using the same method. All analyses were completed using Prism v. 5.0 (GraphPad Software, Inc.) and data presented as mean ± standard error of the mean (SEM) with \( p<0.05 \) being considered statistically significant.

3. RESULTS

3.1 Macrophage Phenotypic Response to a Keratin Biomaterial

Representative confocal fluorescent images of the double-stained treatment groups at their corresponding time points demonstrates M1 and M2 control treatments are effective at maintaining high levels of their respective phenotype across all time points (Figure 1). These images also suggest that keratin treatment increases CD206+ staining (M2 macrophages), especially after 24 hours, whereas collagen treatment shows cells simultaneously expressing both M1 and M2 antigens. Furthermore, cells cultured with no coating did not express high amounts of either CD86 or CD206 (M1 or M2 markers, respectively; Figure 1).

Quantification of the cell counts from the confocal images indicates TDMs can be pushed to an M1 phenotype, with LPS/IFN\( \gamma \) treatment producing the greatest amounts of CD86+ staining cells (Figure 2A). Similarly, the cells can be differentiated to an M2 phenotype with IL-4 treatment, where the highest levels of CD206+ staining cells among treatment groups was observed across all time points (Figure 2B). Both control treatments produced minimal M0 and co-staining M1/M2 phenotypes. TDMs cultured on TCP (i.e.
no coating) display significantly more un-labeled M0 macrophages compared to M1, M2 and M1/M2 macrophages at 3, 7, and 14 days (Figure 2C), suggesting these cells remain un-activated in a more naïve phenotypic state. These untreated cells produced higher levels of the M1 phenotype at 24 hours, but there was no statistical difference compared to the M0 phenotype. Moreover, this observation disappeared by day 3. Also, despite higher levels of M1-staining cells at 24 hours, there was no statistical difference between M1 and M0 percent expression. Collagen coatings demonstrated significantly more concomitant M1/M2-staining cells compared to M1 and M2 phenotypes at 3, 7, and 14 days, as well as significantly greater numbers of M1 than M2 macrophages across all time points (Figure 2D). When cultured on a keratin biomaterial coating, TDM phenotype displays an obvious shift toward a predominantly M2 phenotype, where there is a significantly higher population of M2-staining cells at all time points compared to M1, M0, and M1/M2 phenotypes (Figure 2E). When comparing the keratin-induced cell populations to the collagen-induced populations, keratin-treated TDMs look more similar to the IL-4 (M2)-induced control populations. Moreover, there is no significant difference between M2 populations from keratin and M2 control treatments, except for the 24 hour time point (Figure 3B). Keratin also produces a significantly greater population of M2-staining cells compared to untreated cells, collagen coating, and M1 control treatment (Figure 3B). In contrast, collagen consistently produced significantly more M1 macrophages compared to keratin across all time points, and there was once again no statistical difference between M2 control treatment and keratin (Figure 3B).
3.2 Cytokine Profiles Following Keratin Exposure

Cytokine production by cells in M1 and M2 control groups demonstrate what would be typically considered pro- and anti-inflammatory profiles, respectively (Figures 4A and B). Generally speaking, M1 control macrophages showed higher levels of overall cytokine secretion compared to M2 controls, in particular IL-1β, IL-6, IFNγ, and tissue necrosis factor alpha (TNF-α), which all reached statistical significance compared to their 24 hour values. The M2 control treatment is known to produce cells that secrete higher levels of IL-4 and IL-10, both common anti-inflammatory cytokines, and indeed IL-10 was secreted in significantly higher amounts at 7 and 14 days compared to 24 hours and 3 days. There was a significant increase in IL-1α at 7 days as well, which is sometimes considered pro-inflammatory, but this cytokine has also been shown to play a major role in beneficial tissue remodeling and wound healing.

Collagen coatings demonstrate a cytokine profile that appears dissimilar to either M1 or M2 controls, but the inflammatory cytokines IL-1β and TNF-α are significantly higher compared to 24 hours and 3 days (Figure 4C), though they did not reach levels observed within the M1 control group. This trend seems to correspond to the immunofluorescent staining results where the majority of cells were positive for the CD86 M1 antigen as well as co-staining CD86+/CD206+ for both M1 and M2 antigens. TDMs cultured with no coating, with the exception of IL-1α at 7 days, do not produce significant levels of either pro- or anti-inflammatory cytokines compared to the other time points (Figure 4D). There were elevated levels of IL-6, IL-10, IFNγ, and TNF-α, but those did not reach statistical significance. Generally, this corresponds to the immunofluorescent staining of
these cells, noted as having scarce M1, M2, and M1/M2 staining, suggesting the majority of these cells remain in an un-differentiated, M0 phenotypic state.

Cytokines produced in the presence of keratin appear consistent with an M2 phenotype, where there is a significant shift toward increasing levels of IL-10 with time, as well as low levels of IL-1β and IL-6 with time (Figure 4E). This is more consistent in the M2 control cytokine profile (Figure 4B), and although there is a significant increase in TNF-α, there is no statistical difference between keratin and the M2 control. The only group to produce significantly higher levels of TNF-α was the M1 control group.

Keratin and collagen elicit different effects on TDM cytokine production, particularly when looking more closely at several cytokines that showed more profound responses (Figure 5). The highest levels of IL-1β were produced by the M1 control group across all time points (Figure 5A). However, at 7 days collagen treatment produced significantly more IL-1β compared to keratin and there was no statistical difference between keratin and the M2 control group. Likewise, the M1 control group produced the greatest amounts of IL-6 and there was no statistical difference between collagen and the M1 control group at 24 hours and 3 days (Figure 5B). Collagen treatment also produced significantly more IL-6 compared to keratin at 7 days. Additionally, keratin treatment was responsible for producing significantly higher levels of IL-10 at 7 and 14 days compared to collagen, and there was, again, no statistical difference between the M2 control and keratin (Figure 5C).
4. DISCUSSION

The plasticity of macrophage phenotypes is diverse and dependent upon factors within the local environment.[41,42] M1 macrophages are considered “classically activated,” pro-inflammatory macrophages that secrete cytotoxic compounds such as reactive oxygen and nitric oxygen intermediates, as well as inflammatory cytokines like IL-1β, IL-6, IL-12, and TNF-α. M2 macrophages are defined as “alternatively activated” macrophages that are immunomodulatory and promote wound healing, angiogenesis, and produce anti-inflammatory cytokines such as IL-4, IL-10, and IL-13.[43-45] The underlying mechanisms remain poorly understood, but in vitro experiments have demonstrated that M1 and M2 phenotypes may be induced with various cytokines and microbial products (e.g. LPS and IFNγ, and IL-4, respectively).[6,39,46,47] However, once polarized, the M1 or M2 phenotype is not fixed and studies have shown that M1 and M2 macrophages exposed to the opposing phenotype’s induction signals can be differentiated to express the other’s characteristic genes and cytokines.[41,48] This dynamic and highly regulated plasticity is believed to be a protective mechanism that allows a host response that is pathogen-appropriate, but also one that is able to resolve quickly and restore tissue homeostasis following injury or infection.[6,49] The dysregulation of macrophage polarization and failure to return M1 and M2 phenotypes to a normal balance is known to play a crucial role in chronic inflammation associated with injuries and disease states.[6,39,49] As such, some injury models like skeletal muscle remodeling demonstrate an initial M1-dominant response, which serves to clear debris and secrete cytokines and chemokines that attract muscle progenitor and satellite cells.[50-53] The initial pro-inflammatory phase is followed by a change to an M2
phenotype that serves to resolve the inflammatory response, as well as promote the
differentiation of recruited satellite and progenitor cells.[54-56] In the case of an aberrant
state of macrophage polarization, such as muscular dystrophy, there exists a constant,
mixed M1/M2 cell population with no shift to a resolving M2 phenotype, resulting in
failure of progenitor cells to differentiate.[56-58] Another important example of
dysregulation of the macrophage response related to tissue injury is in the central nervous
system following a spinal cord injury (SCI). It has been shown that post-SCI, a
predominantly M1 macrophage phenotype persists at the lesion site, and despite a small
number of M2 macrophages present at early time points, there was no observed M1 to
M2 phenotypic shift as seen in normal tissue repair.[39]

The use of biomaterials to modulate the inflammatory response is not a new concept, and
as the field of TE/RM grows, it is becoming increasingly important to understand how
biomaterials interact with the immune system. Macrophages are a crucial mediator in
these processes and often facilitate scaffold degradation and thus, remodeling of tissue
constructs.[5,7,8,59-61] The role of M1 and M2 macrophages in regenerative tissue
processes can be substantial. One study showed that neurons cultured in conditioned
media from M2 macrophages displayed increased viability as well as greater neurite
extension, while those cultured with M1 conditioned media showed decreased survival
and neurite length.[39] Similarly, results have been reported wherein M1 macrophages
caused lysis of muscle cells and M2 macrophages supported satellite cell proliferation
and muscle regeneration.[56,57] This, along with data indicating IL-10 and other M2-
specific cytokines specifically down-regulated the pro-inflammatory response.[43]
suggests that M2 polarization promotes constructive tissue remodeling by abrogating the M1 response. However, abnormal polarization of M1 and M2 cell populations toward either extreme may have negative effects.\[39,57,62\]

Thus, it is essential in the design of biomaterials to consider properties that maintain this delicate balance and support tissue remodeling and functional recovery. Some properties of implantable scaffolds and polymers have been shown to elicit primarily an M1 macrophage response. Specifically, those constructed using cross-linking agents,\[5,8\] containing cellular components,\[7\] biomaterials derived from synthetic sources,\[47\] and those of small or nonexistent fiber and pore size,\[46\] have been shown to promote an M1 phenotype. This can correlate to a poor outcome of tissue remodeling and deposition of dense connective tissue and scarring, while an M2 macrophage response results in constructive remodeling.\[5,7,8,63\] An interesting observation in this study is the largely M1 response to collagen, particularly since previous studies using collagen-based extracellular matrix scaffolds have demonstrated constructive tissue remodeling. Perhaps the use of rat tail collagen, an extracted and presumably damaged and denatured form of the protein, in the present study is one reason for these different outcomes.

Considering the robust M2 response elicited by keratin biomaterials in this study, as well as a tunable biodegradation profile that would allow bioresorption of a keratin scaffold to occur at a controlled rate, it is believed that keratin may offer an improved alternative to other biomaterial-mediated macrophage polarization strategies. The immunostaining and cytokine profiling data demonstrate that the M2 phenotypic character of the positive
control and keratin treated cells are statistically comparable. This suggests that keratin biomaterial is as good as the positive control at supporting an M2-dominated response, but it is also important to note that keratin appears to down-regulate differentiation toward an M1 phenotype. This response is more dramatic than an increase in M2 phenotype alone, and is essential in the role of macrophages in wound healing and tissue repair. It is also important to point out that in this study, TDMs were exposed only to a thin coating of keratin biomaterial at the onset of seeding and not re-seeded or otherwise exposed to additional doses of keratin. Therefore, the significant effects noted in the present study were elicited by a modest interaction with the keratin biomaterial and more profound effects may be seen with other cell treatment modalities.

It has been previously established that if an implanted biomaterial is able to support the normal M1 to M2 shift, there will be greater tissue remodeling and more beneficial downstream effects that avoid the fibrotic, scar tissue response.[5,6,8,63] In previous studies, a keratin biomaterial has been shown to tolerate cellular and vascular infiltration, evade foreign-body giant cell formation, chronic inflammation, and graft rejection, as well as elicit a minimal fibrous capsule response.[25,28,30,31] Keratin has also demonstrated versatility in regeneration and tissue repair. In a bone defect model, a keratin scaffold loaded with bone morphogenetic protein 2 (BMP-2) supported greater tissue remodeling and regeneration;[64] a keratin biomaterial hydrogel has been shown to significantly improve nerve repair in tibial and sciatic nerve defect models;[11,14,22,23,65] a keratin hydrogel injected into an infarcted mouse heart was able to improve cardiac function;[17] and keratin biomaterial was shown to prevent
wound enlargement and promote faster wound closure in both thermal and chemical burns.[24] In our own pilot study using the SCI hemisection model in rats, better functional outcomes were observed,[38] as well as a stronger presence of M2 macrophages at the lesion site (unpublished data) (see Appendix 1, figure 1). Taken together, these data suggest one of the potential mechanisms, and possibly a common mechanism, by which keratin biomaterials may promote tissue repair.

While no mechanistic investigation was undertaken here, previous results using keratin biomaterials may suggest a role for integrin signaling through ligand-like activity of the keratin biomaterial itself. Human hair keratins contain the peptide binding motif leucine-aspartic acid-valine (LDV), a cell adhesion sequence recognized by the α4β1 integrin.[66] Prior studies have shown that blockage of the β1 integrin subunit results in decreased cell adhesion to keratin.[67,68] Most leukocytes, including macrophages, express the α4β1 integrin, suggesting a potential ligand-receptor relationship between keratin and macrophage behavior. However, it has also been shown that cells are capable of interacting with keratin substrates even when they are known to lack the α4β1 integrin,[69] suggesting a different cell-matrix relationship such as recognition of intact surface glycans. Furthermore, functional blockage of the β3 integrin subunit has been shown to prevent cell adhesion to keratin.[67] The β3 subunit has been implicated in impaired and aberrant macrophage migration and subsequent polarization.[70] Additional investigation is needed to confirm such potential interactions and a more focused study to elucidate the mechanisms at play.
This study utilized a simplified in vitro culture model system and as such may not be directly comparable to in vivo situations. In particular, M1 and M2 phenotypes were identified based on a limited number of cell surface markers. Though these markers are known to be highly expressed and indicative of their respective polarized phenotypes,[39,71] it would be advantageous in future studies to expand upon this characterization. Moreover, this study focused on the major role of macrophages in the host response to biomaterials. There are undoubtedly more cellular and related molecular players found in the in vivo system that would come to bear in a TE/RM animal model. The present data is also based on an immortalized cell line whose behavior has been analyzed and confirmed in numerous studies,[72] but there is still a potential uncertainty if the observed effects can correlate with primary cells and explain tissue regeneration responses seen with keratin biomaterial in other animal studies. Additional experiments employing primary cells and importantly, animal models that specifically delineate the role of macrophages in tissue regeneration and the influence of keratin biomaterials are needed before any definitive conclusions can be made. However, and despite these potential limitations the present study results may have profound implications for the use of keratin biomaterials in the future and the existence of a common mechanism not seen with other biomaterials.

5. CONCLUSION

In certain cases, it appears that requirements for beneficial macrophage polarization in response to biomaterial scaffolds include a highly porous structure, lack of modifications (e.g. cross-linking agents, cellular components), and the ability to degrade at a rate that
maintains function but avoids a long-term presence that would impede tissue growth. Based on such conditions, and because its efficacy in several injury models showed it to be better than current treatment standards, keratin biomaterials may represent a unique prospect for modulation of macrophage phenotype in TE/RM applications. Even though there are no known mammalian keratinases, keratin is still subject to proteolytic degradation, though its degradation rate is typically slower than other ECM proteins. These characteristics represent strong justification for further investigation of keratin biomaterial systems, with the data generated in this study providing intriguing evidence of a potential common mechanism for keratin’s activity in other TE/RM research.

6. CONFLICT OF INTEREST STATEMENT

Dr. Mark Van Dyke holds stock and is an officer of KeraNetics, LLC, who provided partial funding for this research. Wake Forest University Health Sciences has a potential financial interest in KeraNetics through licensing agreements.

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### TABLES

<table>
<thead>
<tr>
<th>Time Points</th>
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<tr>
<td>24 hours, 3 days, 7 days, 14 days</td>
<td>Keratin coating</td>
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<tr>
<td></td>
<td>Collagen coating</td>
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<tr>
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**Table 1.** Time points and treatment culture conditions for TDMs. n=6 was analyzed for each condition at each time point.
**Figure 1.** Representative confocal fluorescent images of treatment conditions and time points. M1 phenotype is indicated in red, M2 phenotype is indicated in green, mixed M1/M2 phenotype is shown as yellow, and M0 phenotype are cells with absent staining. Keratin-exposed TDMs exhibited a predominantly M2 phenotype across all time points, with a peak M1/M2 co-staining observed at 24 hours. Scale bar = 100µm.
Figure 2. Percent expression of each macrophage phenotype. M1 and M2 control treatments polarize TDMs (A, B). Collagen coatings express mostly M1 and co-staining M1/M2 cells (D). Except for 24h, non-treated TDMs do not express M1 or M2 cell surface markers (A). TDMs seeded on keratin coatings express significantly more M2 macrophage phenotype (E). (see Appendix II for further explanation)
Figure 3. CD86 and CD206 expression over time. Keratin polarizes macrophages to an M2 phenotype significantly better than collagen or no coating (B). Collagen coatings promote an M1 macrophage phenotype significantly more than keratin (A).
**Figure 4.** Cytokine profiles of treatment conditions. M1 and M2 control treatments produce more pro- and anti-inflammatory cytokines, respectively (A, B). TDMs cultured under normal conditions do not produce significant amounts of most cytokines (D), while those cultured on a collagen coating produce higher levels of pro-inflammatory cytokines (C). TDMs exposed to keratin produce a cytokine profile closer to that of the M2 control (E). (see Appendix II)
Figure 5. Individual cytokine production by treatment groups. TDMs produce significantly more IL-1β when cultured on collagen coatings at 7 and 14 days (A) and significantly more IL-6 at 7 days compared to keratin coatings (B). Keratin coatings promote significantly greater production of IL-10 than collagen at 7 and 14 days (C). (see Appendix II)
CHAPTER IV

The Effect of Media Conditioned with Keratin Polarized Macrophages on Activation of Astrocytes In Vitro

Bailey V. Fearing, Mark Van Dyke
ABSTRACT

Chondroitin sulfate proteoglycans (CSPGs) play a crucial role in formation of the glial scar, which takes over the lesion site following spinal cord injury (SCI). Reactive astrocytes also contribute to glial scarring by rapid proliferation and upregulation of GFAP expression and production of CSPGs. This process corresponds to the inflammatory response of macrophages, which polarize toward a dominant M1 phenotype following SCI. The proinflammatory M1 phenotype is known to release various cytotoxic compounds that exacerbate the glial scar, which in turn impedes tissue regeneration. Recent studies have shown M2 macrophages play a role in allowing neurite extensions to occur even across inhibitory substrates and can lessen the degree of secondary damage. Based on earlier results demonstrating that keratin biomaterials may polarize macrophages toward an anti-inflammatory M2 phenotype, we tested the hypothesis that these polarized macrophages would have the potential to indirectly effect astrogliosis. Using an in vitro model of reactive astrogliosis, macrophage-conditioned media from cells that had been cultured with soluble keratin for 24 hours or 7 days appeared to decrease reactivity and associated CSPG production. These results were statistically similar to the M2-macrophage conditioned media. A comparable collagen-conditioned macrophage media did not resolve astrocyte reactivity, while M1 macrophage conditioned media resulted in an in GFAP expression. These data suggest keratin-derived macrophages are more functionally similar to M2 macrophages and may aid in limiting secondary inflammatory-mediated damage.
1. **INTRODUCTION**

One of the more pronounced late-stage outcomes following a spinal cord injury (SCI) is the formation of an astroglial scar, a reactive process that involves glial cells, mostly astrocytes, accumulating and enveloping the injury site [1-3]. In its early stages, the glial scar is beneficially important in attempting to stabilize the defective blood spinal cord barrier (BSB) and minimizing the spread of cellular damage [3-5]. However, its unintended effect is to create a barrier to regenerating axons through physical and chemical interactions. A dense network of glial cells and their processes physically block advancement of the growth cone, and inhibitory molecules and cytotoxic compounds held within the extracellular matrix (ECM) of the scar present chemical barriers to axon growth [6]. Chondroitin sulphate proteoglycans (CSPGs) are structural biomolecules that make up a major class of inhibitory substrates found within the glial scar and a normal component in the central nervous system (CNS). Their role in the CNS appears to be in controlling neuronal differentiation by guiding axons and restricting growth to improper targets, as well as preventing migration of neural crest cells, both crucial steps in neuronal development [7-9]. They have also been shown to regulate neuronal plasticity by forming perineuronal nets around synapses [10-11]. CSPGs are molecules consisting of a core protein with sulfated glycosaminoglycan (GAG) sugar chains covalently attached. The majority of interactions between CSPGs and cell surface receptors are believed to occur through binding sites on GAG chains and, despite the core protein being able to bind substrates as well, many of the functional properties attributed to CSPGs are credited to the sugar side chains [12]. In fact, it has been demonstrated that removal of the GAG chains with the enzyme chondroitinase ABC (chABC) reduces the
inhibitory effects of CSPGs on neurite growth and increases axonal regeneration [13-15]. This suggests CSPGs can inhibitory properties can be attributed GAG chains, though this may only be true at least in part because the remaining “stubs” left after chABC digestion may likely have biological activity [16].

Of the CSPG-producing glial cells, astrocytes possess a more dominant role because of the range of CSPGs produced and their close association with the glial scar. Following injury in the CNS, astrocytes undergo a process known as reactive astrogliosis. The resulting reactive astrocytes contribute considerably to the glial scar and rapidly upregulate production of CSPGs [17-18]. Astrocytes produce a range of CSPGs known to be inhibitory, specifically aggrecan, brevican, neurocan, versican, and phosphacan [3, 19-22]. It is well established that the inflammatory nature of the SCI lesion site contributes greatly to secondary damage, including the infiltration and activation of macrophages and perpetuating reactive astrogliosis. Astrocytes express cell surface receptors for inflammatory cytokines like interleukin (IL)-6, IL-1β, interferon-γ (IFNγ), tumor necrosis factor-α (TNFα), and TGFβ and it has been shown that treating astrocytes with such inflammatory cytokines causes them to become reactive [23]. One of the major sources of such cytokines results from classical activation of macrophages to a M1 phenotype. M1 macrophages dominate the SCI lesion site and are known to be cytotoxic and inhibit neuron growth. M2 macrophages, conversely, promote repair and allow for greater axon growth across inhibitory CSPG substrates, but significantly decline in number by one week post-injury [24]. It is known that the characteristically high presence of reactive astrocytes is associated with the rapid upregulation of transforming growth factor-β
(TGFβ) following CNS injury [25-26]. Such an increase in TGFβ stimulates M2 macrophages to become “deactivated” and subsequently produce higher levels of TGFβ themselves. The overproduction of TGFβ by macrophages encourages an increase in ECM proteins and decrease in matrix turnover, which consequently leads to structural and functional damage, as well as contributes to the fibrotic scar [27]. Interactions between activated macrophages and reactive astrocytes in the lesioned spinal cord are beginning to be understood but research is ongoing. It has been demonstrated that conditioned media from M2 macrophages promotes axon sprouting without concurrent neurotoxicity, unlike conditioned media from M1 macrophages [24]. Additionally, the same study showed that combining M2 macrophage-conditioned media with chABC resulted in three to five fold increases in axon growth across a CSPG gradient. Moreover, M2 macrophage-conditioned media was more effective at supporting and synergizing with chABC to produce such outcomes.

A new intervention being studied for SCI is keratin biomaterials. A keratin hydrogel has demonstrated efficacy in promoting greater recovery of gait and bladder functions following a rat hemisection injury [28]. It has also shown an ability to skew macrophage polarization toward an M2 phenotype and promote significant upregulation of M2-associated cytokines like IL-10, while downregulating M1-associated cytokines like IL-6 [29]. Therefore, we hypothesized that macrophages polarized by treatment with keratin would be capable of paracrine-mediated modulation of astrocyte behavior. In this study, we used an in vitro method to investigate the effects of keratin-treated macrophage
conditioned media (K-MCM) on reactive astrocytes by quantifying their production of CSPGs following treatment.

1. METHODS AND MATERIALS

1.1 Preparation of Keratin Biomaterial and Coatings

Keratin biomaterial was extracted and prepared as previously described [30-31]. Briefly, a 2% solution of peracetic acid was used to oxidize human hair fibers. After rinsing with deionized (DI) water, soluble keratin proteins were extracted with successive treatments of tris(hydroxymethyl)aminomethane (Tris) base and DI water. The extracts were combined and following dialysis, the solution was neutralized to pH 7.4 with NaOH, lyophilized, and ground into a powder. A 25kGy dose of γ-irradiation was used sterilize the keratin powder, which was then reconstituted in phosphate buffered saline (PBS). Keratin and type-1 collagen (BD Biosciences) solutions were diluted to a final concentration of 200μg/ml and 1 ml of corresponding solutions were added to the wells of a glass chamber slide (Nunc, Thermo Fisher Scientific). Coatings were formed by incubating glass chamber slides for 24 hours at 37°C, followed by removal of excess solution and rinsing with PBS prior to cell seeding.

1.2 Macrophage Conditioned Media and Astrocyte Cell Culture

The THP-1 human monocytic cell line was obtained from ATCC and maintained in RPMI 1640 (Gibco Life Technologies) that was supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (P/S), and 0.05mM 2-mercaptoethanol. THP-1-derived macrophages (TDM) were generated by plating 1x10⁶ cells in six-well plates
(Becton Dickinson) and treated with 5ng/ml phorbol myristate acetate (PMA; Sigma) for 48 hours at 37°C in 5% CO₂. TDMs were washed with PBS, trypsinized (0.25% trypsin/0.1% EDTA: HyClone), pelleted, and seeded on respective coatings and treatment groups. Control treatments were conducted using LPS (100ng/ml; Sigma) and human recombinant (hr) IFNγ (20ng/ml; Sigma) to create M1 macrophages. M2 macrophages were produced by treating with hrIL-4 (20ng/ml; Sigma). Media was collected after 24 hours and 7 days, and centrifuged for 10 minutes at 1000 x g to remove any particulate matter. Samples were stored at -80°C until use.

Primary human astrocytes from fetal donors (~22 weeks) were purchased from ScienCell at passage one (fetal brain donor). Cells were maintained on 2µg/cm² poly-l-lysine coated T-75 flasks in complete astrocyte medium (AM) (ScienCell) in 5% CO₂ at 37°C. Medium was changed other day until cell confluence reached 90% when cells were subcultured.

1.3 In vitro Reactive Astrogliosis Assay

Human astrocytes were plated on glass chamber slides (Nunc, Thermo Fisher Scientific) at 5,000 cells/cm² in complete astrocyte media. Cells were treated with 1 µg/ml TGF-β1 (R&D Systems) for 5 days to create reactive astrocytes (RA) [25-26, 32]. They were then washed with PBS and media was replaced with MCM or control treatment for an additional 5 days (Table 1). Media was changed every third day. MCM was chosen
<table>
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<tr>
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<td>Non-conditioned macrophage media *</td>
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<tr>
<td>AM</td>
<td>Astrocyte media*</td>
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**Table 1.** Culture treatment conditions of human astrocytes; * denotes naïve astrocytes that were not treated with TGF-β1.

from two timepoints from a previous study [29]. Media conditioned for 24 hours and media conditioned for 7 days was collected because those timepoints demonstrated the least and greatest effects observed in macrophage polarization, respectively. Media conditioned for 24 hours will hereinafter be referred to as Treatment A and media conditioned for 7 days will be referred to as Treatment B.

### 2.4 Immunocytochemistry

All stains were performed at room temperature (RT) by hand using an optimized immunofluorescence technique. Astrocyte cultures were divided into two staining groups: triple-stained group using aggrecan, versican, and glial fibrillary acidic protein (GFAP), and double-stained group with CS-56 and GFAP. For the triple-staining, astrocytes cultured on glass slides were washed with PBS and treated with 0.02U protease-free chABC (Sigma) for 3 hours at 37°C. Cells were then fixed with 4% paraformaldehyde
(PFA) for 20 minutes at RT. Non-specific staining was blocked with 10% bovine serum albumin (BSA) for 45 minutes at RT. Cultures were washed (0.1% BSA) and the first primary antibody, versican (1:500; Novus Biologics) was added for one hour. After washing, the secondary antibody was added (AlexaFluor® 633, 1:200; Jackson Laboratories) for one hour in the dark. The second primary antibody, GFAP (1:500; Dako), was added after rinsing for an additional hour, followed by the secondary antibody Rhodamine Red® (1:150; Jackson Laboratories). Lastly, FITC-conjugated aggrecan antibody (1:200; Novus Biologics) was added for one hour in the dark. For the double-stained group, astrocytes were washed and fixed with 4% PFA for 20 minutes at RT. No chABC digestion was necessary since CS-56 antibody targets the GAG portion rather than the core protein. Anti-chondroitin sulfate CS-56 (1:200; Sigma) was added for one hour at room temperature. Cells were washed and a secondary AlexaFluor® 488 (Jackson Laboratories) antibody was added (1:200) for one hour in the dark. The second primary antibody, GFAP, was added for one hour in the dark, washed, and its secondary antibody Rhodamine Red® incubated for one hour. Following the triple- and double-staining, gaskets were removed from the chamber slides and slides were mounted with ProLong® Gold Antifade mounting media (Life Technologies) and visualized using a Zeiss LSM510 inverted confocal microscope. Technical controls consisted of stained cells from each treatment group without primary antibodies.

1.4 CSPG Quantitative Analysis

Quantitative analysis of aggrecan, versican, CS-56, and GFAP-positive staining areas was conducted by selecting fields on each slide with 20x magnification. Nine pre-
determined areas were chosen on each sample in order to avoid overlapping areas. These pre-determined fields were consistent and in the same locations on all sample slides. Regions used for quantification were as follows: 3 fields located within the top third of the slide, 3 fields in the middle third of the slide, and 3 fields on the bottom third of the slide. All areas were analyzed for relative fluorescence using ImageJ (NIH) software. Replicates of 3 for each treatment and control group were analyzed. MCM treatments A and B were compared to the corresponding M0-MCM treatments A and B values because M0-MCM represents media conditioned with a mature, but unpolarized macrophage phenotype that can serve as normal levels for an unactivated macrophage.

1.5 Statistical Analysis

Relative fluorescence units (RFU) of control groups and MCM conditions were compared using a one-way analysis of variance (ANOVA) test with Bonferroni’s post-hoc test. For MCM conditions, the average mean value of M0-MCM was calculated and indicated on the graphs as a dashed line. Significant values are compared to the average mean value of M0-MCM from treatment A and treatment B of each CSPG (versican, aggrecan, CS-56) marker and GFAP. All tests were completed using Prism v.5.0 (GraphPad Software Inc.) and data presented as ± standard error of the mean (SEM) with \( p<0.05 \) being considered statistically significant.
2. RESULTS

3.1 Macrophage media does not contribute to reactive astrogliosis

Naïve astrocytes cultured with macrophage media alone (RPMI) did not exhibit any changes in CSPG or GFAP expression (data not shown). When reactive astrocytes were cultured with macrophage media (RA-RPMI) there was no statistical difference in aggrecan, versican, or CS-56 immunoreactivity compared to RA-AM (Figure 3A-C). There was also no change in overall reactivity as indicated by GFAP marker expression (Figure 3D). Macrophage media containing solubilized keratin (K-RPMI) demonstrated a significant decrease in aggrecan (Figure 3B) and GFAP immunoreactivity (Figure 3D).

3.2 Keratin-conditioned media treatments A and B promotes diminished CSPG production

Reactive astrocytes cultured in the presence of keratin-conditioned media (K-MCM) produce significantly less versican, aggrecan, and CS-56 when compared to M0-MCM treatments A (Figures 4A-C) and B (Figures 5A-C). A similar trend was observed with M2 positive control conditioned media (M2-MCM) treatments A and B showing a significant decline in versican (Figures 4A and 5A), aggrecan (Figures 4B and 5B), and CS-56 (Figures 4C and 4B). GFAP expression was significantly decreased with K-MCM and M2-MCM for both treatments A (Figure 4D) and B (Figure 5D) compared to M0-MCM. There was no statistical difference observed between any M2-MCM and K-MCM.
3.3 Collagen-conditioned media has no effect on reactive astrocytes

Reactive astrocytes cultured in the presence of collagen-conditioned macrophage media (C-MCM) showed no statistical difference in aggrecan, versican, and CS-56 immunoreactivity for both treatments A (Figures 4A-C) and B (Figures 5A-C) compared to M0-MCM. There was also no change in GFAP expression from C-MCM treatments A (Figure 4D) and B (Figure 5D). M1-MCM treatment A resulted in a significant increase in GFAP expression (Figure 4D), while promoting a significant increase in CS-56 immunoreactivity (Figure 4C) and no difference in versican and aggrecan production compared to M0-MCM treatment A (Figures 4A, B). M1-MCM treatment B caused no change in versican and CS-56 expression (Figures 5A, C) compared to that of M0-MCM, while promoting a significant increase in GFAP (Figure 5D) and a significant decrease in aggrecan (Figure 5B).

3. DISCUSSION

It is well known that the glial scar that forms at the lesion site following SCI is greatly responsible for the inhibitory environment preventing axon repair. Major components of the glial scar are reactive astrocytes and their associated CSPGs. Several studies have shown that by diminishing CSPGs, regeneration and functional outcomes improve [13-14, 16]. The present study shows that media conditioned in the presence of macrophages skewed to different phenotypes has an effect on reactive astrogliosis in vitro (Figures 1 and 2). Furthermore, activated macrophages may have a direct role in reactive astrogliosis (Figures 4 and 5). Several groups have demonstrated the relationship between activated (M1 and M2) macrophage-conditioned media on neurons, as well as that of
reactive astrocytes on neurons [23-24, 33]. However, the role of M1 and M2 macrophages in reactive astrogliosis is not well-characterized. M1 and M2 macrophages release an array of cytokines and chemokines that are well-established as having deleterious and growth-promoting effects (respectively) in the SCI lesion milieu. An early presence of M1 and M2 related cytokines in the lesion can rapidly increase the amount of surrounding reactive astrocytes [34], indicative of pro- and anti-inflammatory mechanisms that correlate with findings showing a mixed M1/M2 phenotype during the first week following SCI [24]. It was also shown that by later timepoints, especially when M1-macrophage bias occurs, that increased production of proinflammatory cytokines, such as IL-6, leads to more inflammatory reactive astrocytes.

Astrocytes express cell surface receptors to common inflammatory cytokines like IL-6, IL-1β, IFNγ, and TNFα [23]. A previous study demonstrated a significant increase in IL-6 produced by macrophages grown on a collagen coating and those cultured in the presence of M1-driving cytokines (i.e. LPS and IFNγ) [29]. This finding corresponds to the current study where the same collagen-conditioned macrophage media produced no differences in reactive astrocyte-associated CSPG and GFAP expression (Figures 4 and 5) and M1-conditioned media produced significant increases in GFAP (Figs.4 and 5). Furthermore, it has been established that exposure to inflammatory cytokines such as IFNγ, causes astrocytes to become reactive and further skews already-reactive astrocytes to a highly proinflammatory phenotype, leading to a greater production of proinflammatory cytokines [23, 35-36]. Conversely, by altering the cytokine profile presented to reactive astrocytes, their reactivity can be lessened. One group demonstrated
that administration of IL-10 attenuates astroglial reactivity but has no direct effect on naïve astrocytes [37]. This also correlates our previous study, where there was an increase in IL-10 production in the presence of keratin coatings and IL-4 (M2 control) treatment [29] with the current one, where the same keratin and M2 conditioned media yield significantly less reactive (GFAP+) astrocytes and CSPGs (Figs.4 and 5). This was also evidenced by diminished staining (Figs.1G-J and 2G-J). In this study, it is important to note that treatment B, a media solution from the 7 day timepoint, actually represents media from cells that had been in culture for 7 days, but the accumulation of cell secretion factors was only for 24 hours, due to the need to change the media every third day. This may suggest some responses are being underestimated because there may be some factors that are present in different concentrations than would be truly representative of 7 days.

It has long been understood that astrocytes respond to cytokines and inflammatory mediators by altering functional and transcriptome profiles [35-36, 38]. Based on the rapid upregulation of TGFβ following CNS injury and its close association with inducing reactive astrogliosis and regulating the ECM, it is also known that treating astrocytes with TGFβ will cause them to become reactive (i.e. hypertrophy, increased expression of GFAP and ECM proteins) [25-26, 39-40]. This presents a potential problem in vitro because of the presence of serum, an intrinsic source of TGFβ. Some studies have shown that the TGFβ present in serum exists only in its latent form and thus should have no direct effect on astrocyte reactivity [41-42]. Nonetheless, this potential confounding effect was considered in the design of the controls for this study. It was effectively shown
that the macrophage media alone does not induce reactive astrogliosis (data not shown) and no labeling was detected from cells not activated. This discounts most possibilities that the serum may be interfering with the results. It was further shown that not only do astrocytes cultured in RPMI have no statistical difference from the AM naïve astrocyte group, but the macrophage media also does not contribute to changes in reactivity (Fig. 3).

Interestingly, we observed with K-RPMI a significant decrease in aggrecan production (Fig. 3B) and GFAP expression (Fig. 3D). The solubilized keratin added to the same macrophage media should indicate if there is any effect due to the possibility of keratin coatings dispersing into the media. Thus it appears keratin may have a direct effect on reactive astrocytes as indicated by the reduction in GFAP and aggrecan production in macrophage media with solubilized keratin (K-RPMI) compared to macrophage media alone (RA-RPMI) (Figure 3). There also appears to be less GFAP expression in controls than would be expected even with naïve astrocytes, as GFAP is generally accepted as a pan-astrocyte marker. But this marker has some limitations as far as recognizing non-reactive astrocytes and it has been demonstrated that mature, non-reactive astrocytes sometimes are capable of expressing very low or undetectable levels of GFAP [43] and that its expression depends on intra- and inter-cellular signaling molecules with regional and local variability [5]. Astrocytes display a remarkable degree of heterogeneity in their morphology and function and GFAP expression can vary considerably depending on the region of the CNS [44]; for instance only about 15-20% of astrocytes from the cortex of mature animals express GFAP [45].
One of the limitations of this study was the absence of an intermediate timepoint. Treatment A represents media that had been cultured, or conditioned, for 24 hours in the presence of macrophages and a corresponding coating or treatment, while treatment B represents media that had been cultured for 7 days. These were chosen because of the response observed in macrophage polarization at these timepoints from a previous study from our group [29]. It is possible that the addition of another conditioned media (from a different timepoint) could result in a dose-like response of CSPG and GFAP marker expression, although there was no statistical difference between K-MCM treatment A and K-MCM treatment B. It is important to note, though, that different CSPGs have different temporal expression patterns, so future studies should include a range of conditioned medias to give a more comprehensive representation of reactive astrocyte response.

The role of M1 and M2 macrophages in SCI is becoming a crucial focal point for therapeutic interventions. In this study we demonstrate a functional outcome of macrophages activated in response to a keratin biomaterial. We have previously shown that keratin skews macrophages toward an M2 phenotype significantly greater than a collagen biomaterial and promotes an anti-inflammatory cytokine profile [29]. The conditioned media collected from these macrophages reveals a potential new role for keratin in SCI, where it can bias macrophages toward an M2 phenotype which then can downregulate production of CSPGs and overall reactivity resulting from reactive astrogliosis. While studies on the effects of activated macrophages on reactive astrocytes are ongoing, the reaction of macrophages in response to changes in astrocytes has been
demonstrated. One study has shown that viral vector delivery of chABC post-SCI promoted a greater remodelling of CSPGs that was closely associated with CD206+ macrophages, a specific M2 marker [46]. The same study also demonstrated in vitro chABC treatment supported a significant increase in CD206 expression in monocytes. Similarly, macrophages can polarize to opposing phenotypes based on injured neurons, which seem to bias microglia and macrophages to a M1 phenotype [47]. Thus, the glial scar environment is multi-faceted where there is a “vicious cycle” at play that perpetuates the strongly inhibitory milieu. The interplay between infiltrating macrophages, resident glia such as astrocytes, and neurons likely involves a paracrine loop that enables the inflammatory reaction following SCI to become increasingly aggressive and difficult to overcome.

4. CONCLUSION

Reactive astrogliosis is heavily implicated in the development and propagation of the glial scar forming at the site of SCI. Aside from their physical entanglement of processes, there is a marked upregulation in CSPGs that further complicate the inhibitory effects on axon outgrowth. The inflammatory response involving the activation of macrophages is known to contribute to this process, where modulating this response to preferentially polarize macrophages to a M2 phenotype has been shown to promote axon regeneration across the lesion site. Here we establish a keratin biomaterial that has previously been shown to polarize macrophages to a M2 phenotype can further diminish astrocyte reactivity and CSPG production through conditioned media from macrophages. Keratin likely has an indirect role, in part, in attenuating reactive astrogliosis through its robust
effects on macrophage phenotype, although a direct role cannot be ruled out as keratin in
the absence of macrophage conditioning was able to significantly decrease aggrecan and
GFAP expression in reactive astrocytes.

5. FIGURES and TABLES

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**Table 2.** Antibody details from those utilized in this study.
Figure 1. Immunofluorescent images of treatment groups showing CS-56 (green) and GFAP (red) expression. M0-MCM: no coating (M0)-macrophage conditioned media; M1-MCM: M1-macrophage conditioned media; C-MCM: collagen-macrophage conditioned media; M2-MCM: M2-macrophage conditioned media; K-MCM: keratin-macrophage conditioned media; Scale bar =100µm.
Figure 2. Immunofluorescent images of treatment groups showing versican (purple), aggregan (green), and GFAP (red) expression. Yellow arrows are indicating areas of red (D), purple (E), and green (F). M0-MCM: no coating (M0)-macrophage conditioned media; M1-MCM: M1-macrophage conditioned media; C-MCM: collagen-macrophage conditioned media; M2-MCM: M2-macrophage conditioned media; K-MCM: keratin-macrophage conditioned media; Scale bar =100µm.
Figure 3. Control treatments show macrophage media does not influence reactive astrogliosis. There was no statistical difference in versican (A), aggrecan (B), CS-56 (C) or GFAP (D) expression in the presence of macrophage media compared to astrocyte media. There was a significant decrease in aggrecan (B) and GFAP (D) promoted by keratin solubilized in macrophage media (K-RPMI). RA-AM: reactive astrocytes-astrocyte media; RA-RPMI: reactive astrocytes-RPMI (macrophage) media; K-RPMI: keratin (solubilized)-RPMI media. \( p < 0.05 \), 1-way ANOVA (Bonferroni’s post hoc).
Figure 4. CSPG and GFAP expression by reactive astrocytes cultured in treatment A macrophage conditioned medium. There is a significant decrease in versican (A), aggrecan (B), CS-56 (C), and GFAP (D) in the M2-MCM and K-MCM groups compared to the M0-MCM average mean of treatment A. There was no statistical difference between C-MCM and M0-MCM treatment A in versican (A), aggrecan (B), CS-56 (C), and GFAP (D) immunofluorescence. GFAP expression significantly increased with M1-MCM treatment A compared to M0-MCM (D), while CS-56 expression significantly decreased with M1-MCM compared to M0-MCM (C). M0-MCM average mean of Treatment A (M0-MCM Tx A mean values in RFU: versican, 30864.5; aggrecan, 6599.26; CS-56, 25048.67; GFAP, 33789.26). M1-MCM: M1-macrophage conditioned media; C-MCM: collagen-macrophage conditioned media; M2-MCM: M2-macrophage conditioned media; K-MCM: keratin-macrophage conditioned media.*p<0.05, **p<0.01, 1-way ANOVA (Bonferroni’s post-hoc).
Figure 5. CSPG and GFAP expression by reactive astrocytes cultured in treatment B macrophage conditioned medium. There is a significant decrease in versican (A), aggrecan (B), CS-56 (C), and GFAP (D) in the M2-MCM and K-MCM groups compared to the M0-MCM average mean of treatment B. There was no statistical difference between C-MCM and M0-MCM treatment B in versican (A), aggrecan (B), CS-56 (C), and GFAP (D) immunofluorescence. GFAP expression significantly increased with M1-MCM treatment B compared to M0-MCM (D), while aggrecan production significantly decreased with M1-MCM compared to M0-MCM (B). M0-MCM average mean of Treatment B (M0-MCM Tx B mean values in RFU: versican, 33947.67; aggrecan, 6974.93; CS-56, 25075.7; GFAP, 33789.26). M1-MCM: M1-macrophage conditioned media; C-MCM: collagen-macrophage conditioned media; M2-MCM: M2-macrophage conditioned media; K-MCM: keratin-macrophage conditioned media. *p<0.05, **p<0.01, 1-way ANOVA (Bonferonni’s post-hoc).
REFERENCES


CHAPTER V

SUMMARY, CONCLUSIONS, AND LIMITATIONS

Bailey V. Fearing
The goal of this dissertation work was to investigate the potential of a keratin biomaterial in treating traumatic SCI and its role in secondary damage mechanisms. Although previous studies have revealed keratin’s efficacy in peripheral nerve injury models, this is the first study to investigate its capacity to treat CNS injuries. While keratin’s biological activity is larger in scope than could be investigated here, findings from this work may be able to be applied to future mechanistic and clinical studies that utilize keratin biomaterials. Characterization of keratin’s interactions with cells and tissues suggest it is far more than just a scaffold for regenerating tissue. Thus, it was proposed that keratin can promote functional recovery following SCI due to its ability to modulate the inhibitory environment present in the lesion site. The first aim of this project, Chapter II, entailed a pilot study demonstrating rats treated with a keratin biomaterial hydrogel following a thoracic hemisection injury had better gait and bladder outcomes than those treated with saline [1]. Following SCI, infiltrating immune cells as well as activated glial cells play a critical role in exacerbating degeneration and contribute largely to downstream secondary injury. Therefore, the robust recovery observed in keratin-treated rats and histology indicating an increase in cellular infiltration suggested modulation of the inflammatory processes. The second aim of this project, Chapter III, presents findings showing that keratin is able to bias macrophage polarization to a beneficial, repair-promoting M2 phenotype that produces similar cytokines as the M2 macrophage control group [2]. Finally, the third aim, Chapter IV, demonstrates conditioned media from keratin-treated M2 macrophages can lessen the degree of reactive astrogliosis, suggesting a cascading effect of keratin treatment that first affects macrophages, which in turn affect
glial cell behavior. This is the first account of keratin biomaterial’s ability to affect the process of inflammation in damaged tissue.

Due to the exploratory nature of the rat hemisection study, some human errors must be taken into account. A higher than expected attrition rate was experienced and thus these observations are presented as preliminary pilot data only. It is possible that surgical error resulting in a more severe injury contributed to mortality. If the hemisection depth was more complete than intended, this also could have caused more variability between animals. The lower survival rates were also a drawback for the cystometry studies, which made statistical analysis difficult. A follow-up animal study would give more insight into these observed effects, particularly if there was more emphasis on bladder function with a higher number of subjects. Proper statistical analysis would then be possible and could provide more evidence of regeneration of the injured spinal cord. In future hemisection studies, it would be prudent to ensure consistency of the lesion by demarcating the spinal cord midline to serve as a boundary for the hemisection and repeating cuts until the ventral surface of the spinal canal was visible to verify completeness of the injury. Additionally, animals must be excluded from the study if they exhibit weight-bearing movement in the ipsilateral hind limb at 1 day post-injury (indicating hemisection depth was not sufficient) or if they exhibit paralysis in both hind limbs (indicating hemisection depth was too severe).

The adult rat represents an appropriate model because there is evidence supporting the theory that there is secondary injury to the spinal cord as a consequence of the initial
insult [3]. The most significant drawback to the hemisection model is that of clinical relevance as most SCIs observed clinically are contusion injuries, which may present different damage and degeneration mechanisms at the cellular level. However, the rat hemisection model is a commonly accepted one for SCI, if not for its clinical relevance, then for its distinct ability to delineate between sprouting/spared axons and regenerated ones, as axons emerging from the lesion have presumably regenerated [4]. It is important to note that the hemisection model’s strengths are that it avoids the variable incomplete injury and difficulty using tracer methods (such as that found in compression models). Thus, the hemisection model is a good foundation for providing proof-of-concept to support future research of tissue engineering approaches targeting axon regeneration [5].

The pilot study showed an increase in cellular infiltration into the lesion site and a smaller scale study that was repeated by Dr. Dena Howland’s group (see Appendix I, Figures 1 and 2) showed a higher presence of M2 macrophages in keratin-treated rat spinal cords. It was also shown that there was a lesser degree of degenerating axonal bodies, and scar tissue was limited to the lesioned side of the spinal cord with keratin treatment. A more targeted approach to determining regeneration of neuronal pathways could be undertaken through techniques such as retrograde neuronal tracing, which is a particularly useful method in determining the extent to which spared descending axons reach the rostral lumbar enlargement.

Additionally, future work should include expanding on in vivo models, particularly a delayed treatment model. This would provide a more clinically relevant modality as most
SCI patients will experience a lag time between injury and acute care. If keratin is able to maintain its effects after a delayed treatment, translation to clinical studies will become even more important. Another possible model is to alter the delivery mechanism of the keratin. Intrathecal or intravenous injections present two possible modes of delivering keratin that could enhance its benefits. This study indicates a strong possibility that keratin hydrogels are capable of being combined with other treatments to act as a scaffold for repairing cells and tissues. Specifically, other studies have shown that keratin biomaterial is capable of sustained antibiotic release [6], thus it may be possible to combine it with drugs that have shown efficacy in attenuating SCI secondary damage, such as chABC or rolipram. This could even be applied to incorporation and delivery of growth factors and other small molecules. These studies could potentially demonstrate a more enhanced recovery than observed in the current work.

Based upon the preliminary results from Chapter II and the important role macrophages are known to play in the inflammatory response following SCI, an in vitro study of macrophage activation was performed to assess keratin’s influence on polarization to a M1 or M2 phenotype compared to collagen. Leukocyte infiltration is consistently observed in human and experimental SCI, of which macrophages represent the cell type present in the highest quantities and for the longest duration. Macrophages elicit different responses based on signaling cues within the injured spinal cord, which drives polarization of distinct activation states. These activation states, M1 and M2 macrophage phenotypes, have divergent and opposite effects in SCI where the former causes further tissue destruction and production of cytotoxic compounds and the latter promotes
improved axon repair and ability to overcome the inhibitory environment of the lesion site. Evaluation of human THP-1 derived macrophages (TDMs) cultured on keratin coatings for 24 hours, 3, 7, and 14 days revealed a significant increase in CD206+ macrophages, a M2-specific marker. There was no significant difference between the M2 positive control treatment and keratin TDMs, suggesting keratin is statistically as good as the positive control at preferentially polarizing macrophages to a M2 phenotype. TDMs cultured on collagen coatings for the same timepoints expressed a mixed population of CD86+/CD206+ macrophages at early (24 hours, 3 days) timepoints but by 7 and 14 days exhibited a predominantly M1 (CD86+) phenotype. When compared to keratin TDMs, collagen TDMs showed a significant decrease in CD206+ cells with a significant increase in CD86+ cells. These activated macrophage phenotypes were further confirmed in cytokine profile analysis that showed keratin TDMs produced cytokines more similar to the M2 positive control, whereas the collagen TDMs produced cytokines indicative of both M1 and M2 macrophages, that again by later timepoints more closely resembled a proinflammatory, M1-like cytokine profile. This is in contrast to some studies that have shown extracellular matrix (ECM)-derived matrices promote M2-mediated tissue regeneration, but decellularized matrices are vastly different from the soluble collagen used in the present study. These results do suggest, however, that further study of the immune response when using biomaterials as scaffolds, particularly ubiquitous materials such as collagen, should be undertaken when clinical translation is a major goal.

This work and other studies conducted by our group as well as others, have demonstrated strong evidence in support of cell adhesion to keratin biomaterials [7-8]. However, little
is known about cellular recognition and signal transduction mechanisms that may be responsible for such observations. At least in part, biomaterials are believed to interact with their environment through integrins, a family of transmembrane receptors involved in cell-matrix and cell-cell communications [9]. Integrins are vital regulators of cellular and host responses to the implantation of biomaterials and other tissue-engineered constructs [10-11]. Integrin binding at the biomaterial interface may activate specific signaling pathways and other activities that yield cellular and host responses.

Examination of keratin’s primary amino acid structure reveals the presence of binding motifs like leucine-aspartic acid-valine (LDV), which acts as a ligand for the α4β1 integrin receptor [7]. Leukocytes express the α4β1 integrin and based on previous studies demonstrating blockage of the β1 subunit decreases cell adhesion to keratin [12-13], keratin may have a direct effect on macrophage behavior. Previous work in our group using Schwann cells has shown an increase in β1 integrin subunits on cells cultured on keratin coatings. Thus, keratin may be able to influence its own adhesiveness [14]. It has also been shown that cells are capable of interacting with keratin substrates when they are known to lack the α4β1 integrin [15], suggesting a different cell-matrix relationship, such as recognition of intact surface glycans. Additionally, the β3 integrin subunit, which has been implicated in a model of muscle regeneration where depletion of the integrin subunit β3 results in worsened outcomes due partially to impaired and aberrant macrophage migration and subsequent polarization [16], has been shown to prevent cell adhesion to keratin when it has been functionally blocked [12]. Further studies evaluating the role of integrins in macrophage polarization response to keratin are necessary to determine if this is a potential mechanism.
It is also possible that keratin is able to influence macrophage polarization through mechanotransduction mechanisms. Once macrophages adhere to the keratin substrate through possible integrin (as discussed above) or glycan ligands, physically-coupled cytoskeletal filament networks that link to nuclear scaffolds, chromatin, and deoxyribonucleic acid (DNA) inside the nucleus can promote structural rearrangements deep within the cytoplasm and nucleus. Mechanical strain caused by tight adherence to keratin (as was observed in this study) can be sensed by cytoplasmic microtubules, that can respond by releasing reactive oxygen species and/or activating signaling molecules like nuclear-factor-kappa-light-chain-enhancer of activated B cells (NFκB) and vascular cellular adhesion markers (VCAM) all of which can modulate the inflammatory response [17].

One limitation of the macrophage activation study is the use of an immortalized human cell line. Steps were taken to reduce the selection of unwanted subpopulations such as limiting cells used for experiments to passages 3-8 and subculturing when confluence reached ~80%. The advantage of using a cell line is the genotypic and phenotypic uniformity of the cell population, which lends itself well to results that are consistent and reproducible. However, these methods are not always the best way to achieve a good representation of what may be occurring in vivo, even though studies have shown similarities in the behavior of the THP-1 cell line and primary monocytes [18]. Nevertheless, primary cells also have drawbacks as they have a limited life span, can proliferate slowly, and vary from donor to donor. This is an important consideration for
future studies but regardless, confirming these results in primary human monocytes/macrophages will be necessary. Results from such experiments will likely be more variable than those shown here due to differences among cell sources, but will also be more representative of the variability observed in patients. Additionally, the TDMs used for this study were not isolated from the spinal cord nor had any association with the CNS, making them less applicable to a SCI study. Future work should include more relevant sources such as blood-derived or brain-derived primary macrophages.

Another limitation is due to the highly-controlled *in vitro* environment that excludes many important factors that may influence macrophage polarization *in vivo*. In the context of a spinal cord injury, this includes other cell types like neurons, oligodendrocytes, and astrocytes, as well as any secreted factors from native cells such as cytokines, chemokines, and ECM proteins. It is likely these components will alter the responses observed here and conclusions taken from this study are not entirely representative of potential mechanisms.

It is also important to note that the ELISAs used in this aim are similar to an array analysis and, as such, do not allow for standard curve interpretation and are limited to indicating semi-quantitative data. Thus, relative absorbance values are specified and calculated by subtracting the observed absorbance by the absorbance of the negative control for each antigen in order to obtain the corrected absorbance values. Any absorbance values greater than 2.5 are not within the linear range of this assay and were
excluded. Values were normalized to IL-8 levels because those were consistent across all timepoints and all treatment groups.

In addition to replicating these results in primary cells, further studies are needed to examine different approaches to presenting keratin to the cells. The *in vivo* use of keratin in a hydrogel form represents several different ways in which cells and tissue may interact with the biomaterial. It is likely a combination of mechanisms, but to mimic this *in vitro* it is necessary to control the ways in which keratin is exposed to the cells, i.e. coatings, films, gels, and solubilized. The keratin coatings used in this study corresponds to how cells may adhere *in vivo*, but testing this system with a film or gel would give a better indication of how they interact in a three-dimensional environment. Expansion on these mechanisms was not undertaken in this study because the manipulation required to create films and gels (e.g. use of chemical crosslinkers) with the form of keratin used in this work may alter cellular responses. Additional work may also be performed to better characterize the keratin biomaterial utilized. The isolated keratin proteins contain several subtypes with different chemical properties than can be further isolated [18]. Varying the ratios of these keratin subfractions greatly alters its physical characteristics, which can alter keratin’s performance in biological systems [20] and may ultimately yield a more effective keratin biomaterial for SCI.

The final aim of this dissertation, Chapter IV, was focused on evaluating the influence of conditioned media from keratin TDMs on reactive astrocytes. Astrocytes play a major part in SCI, and because the dysfunction or dysregulation of astrocytes (i.e. reactive
astrocytes) occurs in response to CNS injury or trauma, this makes studies involving reactive astrocytes relevant to the injured spinal cord. Because of the prominent roles macrophages and astrocytes play in formation of the glial scar, the ability of the M2-biased macrophage population induced by keratin to have an effect on attenuating reactive astrogliosis and associated CSPG production was examined. Results showed a significant decrease in versican and aggrecan, as well as GFAP expression, when reactive astrocytes were cultured with conditioned media from keratin TDMs (K-MCM). There was no statistical difference in versican and aggrecan production from the C-MCM(24h) group compared to the positive control, as well as versican production for C-MCM(7d). However, C-MCM(7d) produced significantly more aggrecan compared to M1-MCM(7d). C-MCM(24h) and C-MCM(7d) promoted significantly more GFAP immunoreactivity compared to the keratin group, but less than M1-MCM(24h) and M1-MCM(7d).

It can be difficult to analyze CSPGs in vitro due to their large mass, charge, and tendency to aggregate. One of the more reliable means to detect CSPGs is by immunofluorescent staining, though it is limited to proteoglycans that are retained on the cell membrane. Thus, this study is not entirely representative of the in vivo environment and the range of CSPGs generated because different CNS cells produce different CSPGs found in different niches. For example, brevican can be found attached to astrocyte membranes but neurocan and phosphacan are shed into the surrounding milieu and can bind to underlying substrates [21-23], and neuron-glial antigen 2 (NG2) is predominantly produced by oligodendrocyte progenitors but not astrocytes [24]. Therefore, this study
indicates the presence of membrane-associated core proteins, aggrecan and versican, produced by reactive astrocytes but does not take into account those secreted or produced by other glial cells and neurons. Evidence has shown the active moiety responsible for imparting CSPGs’ inhibitory effect lies in the GAG substitutions, although core proteins can still inhibit axon growth [25-26]. Both specific core proteins and intact CSPGs were detected in this study. The use of the antibody CS-56 is directed toward chondroitin sulfate chains [27] and is thus considered a general marker of CSPGs. It is interesting that CS-56 marker expression levels in this study were lower than both aggrecan and versican, given that it should be directed at all CSPGs. This could be attributed to the type of astrocyte, as one study showed some astrocyte cell lines demonstrate differences in CS-56 immunoreactivity [25]. It is also possible that the conformation of certain CSPG structures was able to block appropriate chondroitin sulfate binding sites.

Another aspect that was not considered and could prove important in future studies was to further evaluate these reactive astrocytes that have been cultured with conditioned media for neurite outgrowth potential using a co-culture system or astrocyte-conditioned medium. It is also possible that, due to the array of CSPGs known to be produced by reactive astrocytes, this study missed any differences from collagen-conditioned macrophage media. Because some CSPGs are shed into the media and surrounding ECM, C-MCM cultured astrocytes could be producing an entirely different profile of proteoglycans. There could also be a temporal effect at play, where ceratin CSPGs were missed because of production at different timepoints than those included in the experimental design.
Controls for this study suggest that the macrophage media alone (RPMI) does not have an effect on reactive astrocyte production of CSPGs or upregulation of GFAP. Still, more controls could aid in the interpretation of this data. Specifically, a control of the macrophage culture media from the M1 and M2 positive controls containing LPS/IFNγ and IL-4, respectively, without cells could serve to represent any direct effects due to treatment factors still present in the media. It is expected that there would be increased CSPG and GFAP immunoreactivity in the presence of LPS/IFNγ because previous studies suggest these molecules are in close association with reactive astrocytes [28-29].

Future studies for this aim should take into account more controls that may help distinguish a clearer role for keratin-derived macrophages in a model of reactive astrogliosis. In addition, the next steps should utilize studies that involve the neuronal response to these reactive astrocytes as mentioned above, as well as continuing this work in vivo by investigating reactive astrocytes present in the injured spinal cord.

In conclusion, this dissertation work has confirmed the potential role for a keratin biomaterial in SCI applications. By showing an ability to modulate macrophage activation that correlated with decreased reactivity in astrocytes, this suggests, in part, a possible mechanism by which keratin is able to promote improved functional recovery following a hemisection SCI. Many questions remain and further development of keratin biomaterials in SCI models and cell-based assays should be addressed in future studies.


APPENDIX I

Figure 1

![Figure 1](image1.png)

**Figure 1.** Triple labeling in injured dorsal column area with a CD206 M2 marker in red and a CD86 M1 marker shown in green. M1 macrophages are dominant in the saline-treated rat, where M2 macrophage are more dominant in the keratin treated rat.

Figure 2

![Figure 2](image2.png)

**Figure 2.** A silver stain cross section just rostral to the epicenter shows greater degeneration in gray/black across the left half of the cord in the saline treated rat, as well as some degeneration contralateral to the lesion (right side) (A). A high power area from the spared gray matter shows a greater number of degenerating axonal and cell body profiles with saline treatment (B) compared to a similar area in a keratin treated rat (C). Cresyl violet staining at the lesion epicenter shows scar formation by 7 days is limited to the lesion side with keratin treatment.
**Figure 3.** Modified image of Figure 5 from Chapter II, indicated rostral and caudal directions and white matter (WM) and grey matter (GM).
1.2 Modified Discussion for Thesis (Not included in original publication)

For hind limb motor function, the TreadScan® treadmill was set to a constant speed of 10cm/s for a duration of 20s periods. This may impact gait analysis as baseline levels (pre-SCI) were taken at an average speed of 15cm/s for 20s periods and differences in treadmill speeds can alter the gait properties (such as stride lengths). Although it is not unusual to use different speeds for gathering baseline data due to injured animals being incapable of achieving a similar walking speed, if the post-injury speed is not an appropriate it can over- or under-interpret hindlimb motor function.

Age-matched controls for cystometry and histology were matched to the age of the animals at the time of sacrifice (not at the time of injury).
APPENDIX II

2.1 Tables and Figures

Table 1.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Vendor</th>
<th>Cat. No.</th>
<th>Host</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD206</td>
<td>R&amp;D</td>
<td>AF2534</td>
<td>Polyclonal goat</td>
</tr>
<tr>
<td>NL493</td>
<td>R&amp;D</td>
<td>NL-003</td>
<td>Donkey anti-goat</td>
</tr>
<tr>
<td>CD86</td>
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<tr>
<td>NL557</td>
<td>R&amp;D</td>
<td>NL-001</td>
<td>Donkey anti-goat</td>
</tr>
</tbody>
</table>

Table 1. Antibody information from those used for the study in Chapter III.

Figure 1.

Figure 1. Unactivated THP-1 cells attach significantly more to a keratin coating compared to a laminin coating and no coating. THP-1 cells do not adhere until activated to a mature macrophage phenotype.
Figure 2. Flow cytometry analysis of PMA-activated THP-1 cells demonstrates a significant increase in CD14$^+$ cells, indicating PMA treatment is effective at producing a mature macrophage phenotype. **$p<0.01$
Figure 3. Modified version of Figure 5 from Chapter III showing different box fills for easier interpretation.
2.2 Modified Figure Legend (Not included in original publication)

**Figure 2.** Percent expression of each macrophage phenotype. M1 and M2 control treatments polarize TDMs (A, B). Non-treated macrophages do not express M1 or M2 cell surface markers (C), with the exception of 24 hours. This is denoted by the horizontal bar showing there are significantly higher levels of unlabeled M0 macrophages at 3d, 7d, and 14d compared to M1, M2, and M1/M2 macrophages. Collagen coatings promote expression of mostly co-staining M1/M2 macrophages, as indicated by the bottom horizontal bar showing there are significantly higher amounts of M1/M2 macrophages at 3d, 7d, and 14d compared to M1, M2, and M1/M2 macrophages (D). There are also significantly higher amounts of M1 macrophages compared to M2 macrophages at 24h, 3d, 7d, and 14d, as marked by the top horizontal bar (D). TDMs cultured on keratin coatings express significantly higher amounts of M2 macrophages compared to M1, M0, and M1/M2 macrophages at 24h, 3d, 7d, and 14d, as shown by the horizontal bar (E). **p<0.01, ***p<0.001.

**Figure 3.** CD86 and CD206 expression over time. Collagen coatings promote an M1 macrophage phenotype significantly more than keratin (A). Top horizontal line represents significance of LPS/IFNγ treatment in producing higher amounts of CD86+ staining cells compared to all other treatments and conditions at all timepoints. Bottom line is showing collagen produces significantly more CD86+ macrophages compared to keratin across all timepoints. Keratin polarizes macrophages to an M2 phenotype better than collagen or no coating (B). Horizontal line is showing keratin produces significantly
more CD206+ macrophages compared to collagen, no coating, and LPS/IFNγ across all timepoints. LPS, lipopolysaccharide. IFNγ, interferon γ. ***p<0.001, **p<0.01.

2.3 Modified Methods for Thesis (Not included in original publication)

Macrophage Quantitative Analysis, Selection of Microscope Fields
Nine pre-determined areas were chosen on each sample in order to avoid overlapping areas. These pre-determined fields were consistent and in the same locations on all sample slides. Regions used for quantification were as follows: 3 fields located within the top third of the slide, 3 fields in the middle third of the slide, and 3 fields on the bottom third of the slide.

2.4 Modified Discussion for Thesis (Not included in original publication)

Figure 4 indicates similar levels of TNF-α with keratin, collagen, and IL-4 treatment at 7 days and 14 days. Individual t-tests indicate there is no statistical difference between these levels and the only group to produce significantly higher amounts of TNF-α is the M1 control treatment, LPS/IFNγ.

Figure 5C shows a trend of higher amounts of IL-10 with keratin coatings compared to no coating at 7 days and 14 days. However, there is no statistical difference between the two. Additionally, the cytokine profile of the no coating group (Fig. 4D) shows no significant changes in IL-10 production at any timepoint.
These results suggest there is still an effect seen with keratin coatings, although it is possible the trends observed are due to the *in vitro* environment that doesn’t take into account other factors that may influence the observed effect.
EDUCATION

Ph.D. Wake Forest University, Molecular Medicine and Translational Sciences, May 2014

B.S. Guilford College, Biology and Health Sciences; Minor: Chemistry, May 2007

PROFESSIONAL INTERESTS

Research Synopsis
My thesis work focused on the immunomodulatory abilities of a keratin biomaterial and its capacity as such to promote repair and regeneration following a spinal cord injury.

Research Skills
Flow cytometry, fluorescent and confocal microscopy, cell and tissue culture, PCR, real-time RT-PCR, gel electrophoresis, ELISA, immunohistochemistry, immunocytochemistry, histology, rat models and husbandry; proficient with statistical software GraphPad Prism and Microsoft Office (Excel, PowerPoint, Word); limited experience with large scale protein purification using dialysis and liquid column chromatography, viral transfection, stem cells, and decellularization for scaffolds.

PROFESSIONAL EXPERIENCE

Research
Pre-Doctoral Fellow/PhD Research, Wake Forest Institute for Regenerative Medicine, Department of Orthopaedic Surgery, Wake Forest University, Winston-Salem, NC, August 2008-present
• Design and conduct experiments to characterize the inflammatory response following treatment with a keratin biomaterial
• Test keratin biomaterial in vivo in a rat spinal cord hemisection injury model
Research Assistant, Department of Pathology, Wake Forest Baptist Health, Winston-Salem, NC, January 2007-November 2007
- Conducted research in the areas of anti-tumor immunity and prostate cancer biology
- Created various reporter constructs for use in non-invasive imaging

Internships
Technology Transfer Intern, Wake Forest University Office of Technology and Asset Management, Winston-Salem, NC, January 2012 - March 2013
- Involved in many aspects of technology transfer including inventions and patent policy, licensing, start-up ventures, and marketing evaluations
- Conduct intellectual property analysis and evaluations on Wake Forest University inventions and technologies
- Compose executive and non-confidential summaries of existing IP available for licensing

Teaching
Laboratory Instructor, Department of Chemistry, Guilford College, Greensboro, NC
August 2005-May 2006
- Taught two laboratory sections for undergraduate introductory Chemistry course
- Prepared laboratory materials and equipment
- Assisted students in experiments and graded laboratory reports

Teaching Assistant, Department of Biology, Guilford College, Greensboro, NC
August 2006-December 2006
- Taught methods such as aseptic/sterile technique, micropipetting, and PCR
- Prepared materials for the laboratory course, including growth media, bacterial cultures, and electrophoretic gels

HONORS/AFFILIATIONS
Society for Biomaterials, 2013-present
Society for Neuroscience, 2010-present
Tissue Engineering Regenerative Medicine International Society, 2009-present
Biomedical Engineering Society, 2010-present
Dean's List, Fall 2005; Spring 2006
President’s Dinner, Honoree, Guilford College, October 2006
Tri Beta National Biological Honors Society, 2006-2007
AWARDS
Society for Biomaterials, Student STAR award honorable mention, April 2013.
Wake Forest University Graduate Student Research Day, 1st place, March 2013.

PUBLICATIONS
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ABSTRACTS
First Author on Accepted Abstracts as Oral Presentations


Co-author on Abstracts Accepted as Oral Presentations


First Author on Accepted Abstracts as Poster Presentations


