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Immunomodulation by Shark Cartilage Extracts

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IMMUNOMODULATION BY SHARK CARTILAGE EXTRACTS

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Liza Merly

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To: Dean Kenneth Furton
College of Arts and Sciences

This dissertation, written by Liza Merly, and entitled Immunomodulation by Shark Cartilage Extracts, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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DEDICATION

I dedicate this dissertation to my husband, Michael Menendez,

and to my children, Mikey and Manny. Without their patience, understanding,

support, and love, the completion of this work would not have been possible.

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ABSTRACT OF THE DISSERTATION

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The immune system is composed of innate and adaptive mechanisms. Innate immune responses are significantly modulated by immunomodulatory factors that act through the induction of specific patterns of cytokine production in responding cells. Human leukocytes have been shown to respond to substance(s) present in acid extracts of commercial shark cartilage (SC). Shark cartilage is a food supplement taken by consumers as a prophylaxis and for the treatment of conditions ranging from arthritis to cancer. No reliable scientific evidence in the literature supports the alleged usefulness of shark cartilage supplements, but their use remains popular. Cartilage extracts exhibit immunomodulatory properties by inducing various inflammatory, Th1-type cytokines and potent chemokines in human peripheral blood leukocytes (HPBL) *in vitro*. The objectives of the study were to (1) to determine the nature of the active component(s), (2) to define the scope of cellular response to SC extract, and (3) to elucidate the molecular mechanisms underlying bioactivity. Results showed that there are at least two cytokine-inducing components which are acid stable. One anionic component has been identified as a small (14-21 kDa) glycoprotein with at least 40% carbohydrate

content. Shark cartilage stimulated HPBL to produce cytokines resembling an inflammatory, Th1 polarized response. Leukocyte-specific responses consist of both initial cytokine responses to SC directly (i.e., TNF-α) and secondary responses such as the IFN-γ response by lymphocytes following initial SC stimulation. Response of RAW cells, a murine macrophage cell line, indicated that TNF-α could be induced in macrophages of another mammalian species in the absence of other cell types. The results suggest that the human monocyte/macrophage is most likely to be the initial responding cell to SC stimulation. Stimulation of cells appears to engage at least one ligand-receptor interaction with TLR 4, although the role of TLR 2 cannot be ruled out. Initial activation is likely followed by the activation of the JNK and p38 MAPK signal transduction pathways resulting in activation, release, and translocation of transcription factor nuclear factor κB (Nf-κB). This dissertation research study represents the first in-depth study into characterizing the bioactive component(s) of commercial shark cartilage responsible for its immunomodulating properties and defining cellular responses at the molecular level.

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LIST OF ABBREVIATIONS AND ACRONYMS

HPBL	Human peripheral blood leukocytes
PBMC	Peripheral blood mononuclear cells
Nf-κB	Nuclear factor kappa B
APC	Antigen presenting cell
PAMP	Pathogen associated molecular pattern
PRR	Pattern recognition receptor
DAMP	Damage associated molecular pattern
TLR	Toll-like receptor
MAPK	Mitogen activated protein kinase
LPS	Lipopolysaccharide
iNOS	Inducible nitric oxide synthase
IL-	Interleukin
TNF	Tumor necrosis factor
IFN-γ	Interferon gamma
MCP-1	Monocyte chemoattractant protein-1
RANTES	Regulated upon Activation, Normal T-cell Expressed, and Secreted
ECM	Extracellular matrix
GAG	Glycosaminoglycan
SCAE	Shark cartilage acetic acid extract
SC-SPE	Shark cartilage solid phase extract

INTRODUCTION

The immune system is composed of innate and adaptive mechanisms that serve to protect the host from infection. Invading organisms are detected by microbial sensors on the surface of innate immune cells. The subsequent intracellular signaling and release of mediators influence downstream events that include both innate and adaptive responses (Iwasaki and Medzhitov, 2010). Innate immune responses are significantly modulated by both exogenous and endogenous factors that either activate or suppress immune function. Often, immunomodulatory factors act through the induction of specific patterns of growth factor, cytokine, and/or chemokine production in responding cells (Clarke and Mullin, 2008). Protein receptors expressed at the cellular surface must first recognize distinct epitopes on stimulating substances and then induce the intracellular signaling events that lead to the production of immune mediators like cytokines. Cytokines are soluble protein messengers that are involved in cell-cell communication. They are the primary mechanism by which communication occurs between leukocytes such as macrophages and lymphocytes, and other immune and non-immune cells (Romagnani, 2007).

Human leukocytes have been shown to respond to substance(s) present in acid extracts of commercial shark cartilage (Merly et al., 2007). Cartilage extracts exhibit immunomodulatory properties by inducing various inflammatory, Th1-type cytokines and potent chemokines in human peripheral blood leukocytes (HPBL) *in vitro*. Given the response to cartilage observed in previous studies, it was important to determine (1) the nature of the stimulating component(s) of SC, (2) through which cell type(s) this

complex cell-mediated cytokine response previously observed in a mixed culture of HPBL is initiated, (3) how immune recognition of shark derived factors is accomplished at the cellular surface by responding cells, and (4) the mechanism of the cellular downstream inflammatory responses mediated by intracellular signal transduction. The principal hypothesis was that shark cartilage contains bioactive component(s) that elicit specific cytokine/chemokine responses in immune cells. The active component(s) is likely a modified protein that is recognized at the cellular surface and induces intracellular signaling that involves more than one biochemical pathway and eventually up-regulates the expression of Nf-κB, a potent transcription factor involved in cytokine production and inflammatory responses. To test these hypotheses, two *in vitro* cell culture models were used in this study, (1) a primary culture of human peripheral blood leukocytes and (2) a single cell type murine macrophage-like cell line.

The conceptual framework was that macrophages, through receptors such as pattern recognition receptors, recognize ligands like the exogenous factors derived from shark cartilage. Typically, the first encounter between an immune-stimulating agent and the immune system is with an antigen presenting cell such as a monocyte/macrophage and/or dendritic cell (Mosser, 2003). The ability of innate immune cells like macrophages to recognize diverse ligands and initiate immune responses is largely dependent on molecular pattern recognition by distinct surface receptors, such as the Toll-like receptor family (Medzhitov, 2008). Receptor collaboration at the cellular surface aids in orchestrating the specificity of these innate immune responses to diverse ligands (Kawai and Akira, 2007). Specificity is derived

from the extensive receptor repertoire and their ability to recognize various ligands in the form of proteins, saccharides, lipids, and nucleic acids. Variations in intracellular signaling molecule expression increase the ability of innate immune cells to generate distinct pro- or anti-inflammatory responses (Taylor et al., 2005).

Distinctive immune responses include Th1-type inflammatory cytokines and chemokines. Cytokines can be classified as Th1 or Th2-type depending on the subset of T helper cells which produce them during an immune response. The differentiation of T-helper cells into Th1 and Th2 cells is tightly regulated by the cytokines present in the local environment and the type of initiating infection and/or immune stimulus (Kidd, 2003). Up-regulation of Th1 subsets typically induces cell-mediated responses to intracellular pathogens while up-regulation of Th2 subsets typically induces increases in antibody production and response to extracellular pathogens.

At a fundamental level, this study was undertaken to investigate the mechanism by which factors derived from an unrelated taxonomic group (i.e., factors present in shark cartilage) can affect immune responses of mammalian cells. Given the recent evidence for the role of endogenous 'danger' signals from proteins such as those found in glycosaminoglycans, it is plausible that constituents of cartilage may stimulate immune responses (Taylor et al., 2004). There is substantial evidence in the literature that the conservation of immune system proteins allows for potential cross-talk between molecules of evolutionarily distant taxa, like the shark and human (Smith et al., 1997).

Shark cartilage is currently sold worldwide as a dietary supplement that is believed to augment human immune function. Initially, cartilage was investigated for its putative anti-cancer properties, but this line of research has not yet yielded any supportive evidence for the use of shark cartilage supplements as treatment for any disease/condition or as prophylaxis (Finkelstein, 2005). One of the problems with use of nutritional supplements is that a comprehensive understanding of their biochemistry, pharmacology, and immunological potential is lacking. Consequently, their claimed efficacy is often questionable and, furthermore, the risk of deleterious effects remains. From a public health point of view, the scientific consensus regarding dietary supplements like shark cartilage has long been that the worst case scenario is that these products are ineffective. Given the results of this study, the worst case scenario may in fact be much worse if these products contain substance(s) with unanticipated and potentially harmful immunological activity.

Results from this study will impact the use of shark cartilage as a 'cure-all'. As a consequence, a reduction in the demand for the commercial product will at least slow the indiscriminate harvesting of sharks worldwide, which if allowed to continue will cause serious ecological imbalance and permanent loss of shark species. The significance of this study lies in its (a) relevance to human health, particularly for individuals taking shark cartilage as a daily oral supplement, and (b) its potential influence on the commercial market for shark cartilage.

The results of this study have yielded information on the type of surface molecules that likely bind to shark cartilage-derived ligands and the signal transduction

molecules activated in this process. Furthermore, partial purification of the active component in shark cartilage has allowed for the development of a model for the mechanism of action in eliciting both initial and downstream cell-mediated responses. Results from this study are pertinent to future study of functional reactivity of diverse immune systems and the potential application of exogenous factors as adjuvants and/or stimuli for modulating the human immune system and thus orchestrating a specific immune response.

BACKGROUND

Immune recognition and macrophage activation

Monocytes/macrophages are specialized cells that play a role in both innate and adaptive immunity. Activated macrophages are potent phagocytes and aid in the clearance of both intracellular and extracellular pathogens. As antigen presenting cells (APC), they are also responsible for immune recognition in the form of initial capture and processing of potential antigens and the activation of downstream specific T and B lymphocyte responses. In addition, they are able to secrete a diverse repertoire of soluble mediators upon activation that induce and regulate both local and systemic immune responses (Mosser, 2003).

When bone marrow-derived circulating monocytes migrate into tissues, they differentiate into tissue macrophages (Mosser, 2003). Tissue macrophages express a wide range of different surface receptors and phenotypes depending on the specialized function within their microenvironment. During the early phase of an immune response, APCs recognize foreign ligands through innate pattern recognition molecules (i.e., receptors) expressed on their cellular surface. It is now known that the receptor repertoire for APC- like macrophages is extensive and recognizes both exogenous and endogenous ligands (Gordon and Taylor, 2005). Receptor diversity is likely increased by combinatorial expression at the cellular surface, alternate splicing to produce multiple isoforms, post-translational glycosylation, lipid modifications, and proteolysis. It is also likely that variations in intracellular signaling molecule expression increase the ability of innate immune cells to generate distinct responses (Taylor et al., 2005).

It is generally accepted that the ability of innate immune cells to recognize diverse ligands and initiate immune responses is largely dependent on pattern recognition by distinct surface receptors. Janeway and Medzhitov (1992) first proposed the concept of pattern recognition which depends on immune recognition of conserved microbial structures referred to as pathogen-associated molecular patterns or PAMPs. Pathogen-associated molecular patterns are recognized by germ-line encoded pattern recognition receptors (PRRs) on the surface of immune cells (Janeway and Medzhitov, 2002). Pattern recognition receptors can recognize a wide array of microbial ligands and are typically categorized into two major classes: those that mediate phagocytic uptake and those that lead to activation of pro-inflammatory pathways. Among those that mediate phagocytosis of microorganisms are the scavenger receptors (Taylor et al., 2005). There is mounting evidence of receptor collaboration at the cellular surface that aids in orchestrating the specificity of these innate immune responses to conserved patterns unique to microbial surfaces (Mukhopadhyay and Gordon, 2004).

Included in the large repertoire of germ-line encoded cell-surface glycoprotein receptors in the PRR family are the Toll-like receptors (TLRs) which act as pathogen sensors by recognizing PAMPs and orchestrating subsequent immune responses. Toll-like receptors were discovered initially as a type I transmembrane receptor in fruit flies (*Drosophila melanogaster*) that plays an important role in embryonic development (St. Johnston, 1992) and later was shown to play a role in anti-fungal responses. Homologues of this type of receptor have been identified in mammals and are referred to as Toll-like receptors (Janeway, 1992). They typically recognize conserved motifs on

the surface of pathogens and induce inflammatory signals in response to receptor-ligand binding at the cell surface.

Toll-like receptors contain an extracellular domain that is rich in leucine repeats that are essential for the recognition of PAMPs. Microbial components such as LPS, peptidoglycan, flagellin, and dsRNA are among the PAMPs recognized by the TLR family of receptors. It is now well accepted that TLR-mediated signaling is the primary mechanism of pathogen detection in innate immunity. Currently, the TLR family consists of 10 and 13 family members in humans and mice, respectively (Medzhitov, 2008).

Most TLRs share a common signaling pathway that often includes the adaptor myeloid differentiation factor 88 or MyD88. TLR-mediated pro-inflammatory cytokine induction in response to pathogen recognition is dependent on MyD88 as well as its downstream mediators IRAK-4 and TRAF-6 that activate transcription factors such as JNK and nuclear factor NF-κB (Mukhopadhyay and Gordon, 2004).

In addition to receptor collaboration on the cellular surface, the use of adapter molecules increases the specificity of TLR-mediated immune responses. A cascade of kinases can be activated following initial receptor-ligand interactions that are organized by multiple adapter molecules into signaling complexes, leading to the activation of downstream transcription factors such as NF-κB. Intracellular signaling molecules from the MAPK family have been linked to TLR signaling as well as to other pathways including PI-3 kinase and G-protein coupled receptors, although the interaction of these

pathways with TLR signaling is less well understood (Sabroe et al., 2008). It is likely that several different TLRs can utilize variations in the typical signaling pathway by using different combinations of adapter molecules (Li, 2005). Additionally, the combinatorial effect of various TLR homodimers and heterodimers expressed on the cellular surface, can in turn collaborate with other receptors and surface molecules, to produce enhanced specificity in the signaling and gene expression that follows activation (Kawai and Akira, 2007).

Innate immune responses

Innate immune mechanisms are an evolutionarily ancient part of host defense against infection. Toll-like receptors and other PRRs allow innate immune cells like the macrophage to recognize and distinguish between noninfectious self molecules and infectious non-self molecules. Toll-like receptors play a major role in initiating inflammatory responses. When microbial ligands bind these receptors, there is subsequent activation of signaling pathways that lead to the induction of antimicrobial genes and inflammatory cytokines (Janeway and Medzhitov, 2002).

In response to TLR signaling, genes that encode proteins important in many different innate immune responses are expressed de novo. When sentinel cells like macrophages and dendritic cells are activated, they produce and release endogenous alarm signals as a first line of defense. In the activated macrophage, the two major subsets of genes expressed are those that code for proteins involved in microbial killing mechanisms such as inducible nitric oxide synthase (iNOS) and those that code for

inflammatory cytokines such as tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), and also IL-12 and IL-18 (Medzhitov, 2008). Pro-inflammatory cytokine IL-1 and IL-18 receptors and TLRs share a cytoplasmic motif, the Toll-IL-1 receptor (TIR) domain (Yamamoto and Akira, 2005). The TIR cytoplasmic domain is necessary for initiating intracellular signaling and downstream effector mechasnisms (i.e., induction of other cytokine/chemokine genes) (Martin and Wesche, 2002).

The inflammatory response that is typically induced as a response to infection or injury is carried out by tissue macrophages that in turn induce the production of many inflammatory mediators. Those mediators are responsible for allowing plasma proteins and leukocytes like neutrophils to leave blood vessels and enter extravascular tissues where they can reach the site of infection or injury. Once there, other killing mechanisms that include reactive oxygen and nitrogen species are initiated. Unfortunately, these factors do not distinguish between pathogen and host effectively and this can lead to damage to host tissues and pathological inflammation (Medzhitov, 2008).

The production and release of cytokines and/or chemokines, which interact with specific receptors in either an autocrine or paracrine manner, contributes significantly to regulating and controlling cellular responses (Belardelli, 1995). Recent studies have shown that the cytokine network can be modulated by treatment with specific cytokines or with cytokine inducers that act as antagonists or as inducers for the production of particular cytokines. Because of their role in immune regulation, cytokines represent a

potential source of new therapeutic approaches to various disease states (Jankovic et al., 2001; McGuirk and Mills, 2002). When TLRs are activated on responding cells, they initiate the expression of co-stimulatory molecules and production of the cytokine microenvironment that drives cellular differentiation into distinct T cell subsets (Re and Strominger, 2001). For example, recognition of microbial and host ligands by TLR 2 and TLR 4 in arthritic joints is involved in the progression of rheumatoid arthritis because they initiate the upstream signals that determine T cell activity and downstream cytokine production (Abdollahi-Roodsaz et al., 2008).

The profile of cytokines produced at any one time during an immune response is largely governed by two subsets of T-helper cells designated Th1 and Th2. The Th1 and Th2 subsets are mainly characterized by the type of cytokines they secrete in response to a specific stimulus, such as an infection. An upregulated Th1 response is usually observed against intracellular pathogens such as viruses, intracellular bacteria, and intracellular parasites. The cell-mediated response involves macrophage activation and occurs via cytotoxic T cell activation (Mosmann and Coffman, 1989) . Activated macrophages will produce Th1 cytokines like IL-12, IL-18 and TNF-α in initiating a Th1 cell expansion. The typical cytokine profile produced by Th1 cells primarily consists of IFN-γ, IL-2, and TNF-α. IFN-γ and IL-2 are also produced by natural killer cells and macrophages. These cytokines induce maturation of Th1 cells (Abbas et al., 1996; Mosmann and Sad, 1996).

The Th2 response, however, is characterized by increased levels of antibody production, including the production of IgE, and the activation of eosinophils (Spencer and Weller, 2010). The Th2 response is effective against extracellular pathogens such as bacteria and parasites, and induces protective humoral immunity. Typically, Th2 cells produce cytokines such as IL-4 and IL-5 that induce immunoglobulin class switching to IgM, IgG1, IgA, and IgE. IL-3, also produced by Th2 cells, induces eosinophilic degranulation which can take part in the destruction of parasitic membranes (Shinkai et al., 2002).

Each subset of T-helper cells can regulate the activities of the other by producing cytokines that either work in an autocrine manner to upregulate the production of cytokines characteristic of that subset or inhibit the maturation of the other subset of T-helper cells consequently inducing downregulation of cytokines representing the other subset. IL-4 and IL-10 produced by Th2 cells inhibit Th1 cell responses. Similarly, IFN-γ production by Th1 cells inhibits Th2 cells. Furthermore, IFN-γ has an inhibitory effect on IL-4-induced B cell activation and interferes with the inhibitory effect of IL-4 on IL-2-induced T and B lymphocyte proliferation (Fitzgerald, 2001).

The differentiation of T-helper cells into Th1 and Th2 cells is tightly controlled by the cytokines present in the local environment and the type of infection and/or immune stimulus. IL-4 has been shown to be a principal factor in whether Th0 cells differentiate into Th1 or Th2 cells. In the presence of IL-4, Th0 cells will preferentially differentiate into Th2 cells. However, in the presence of IL-12, a cytokine produced by APCs like

macrophages, Th0 cells will differentiate into Th1 cells. The balance of Th1 and Th2 cellular responses is crucial to achieving the appropriate response to combat diseases (Mosmann and Coffman, 1989). In addition, there is another class of T cells that are referred to as T regulatory cells (Treg). They act to suppress immune responses inducing immune tolerance and, in some cases, preventing the development of autoimmune disorders. T regulatory cells can have both advantageous and deleterious effects. In the context of the current study, it is interesting to note that they often play a role in stemming the effects of chronic inflammatory responses (Vignali et al., 2008).

If an inappropriate response to a given pathogen is induced by disequilibrium in the cytokines normally present during that infection, the immune response of the host to that infectious agent may be ineffective. In some diseases where pathogenesis is caused by an excess of either Th1 or Th2 activity, treatment of the condition can be achieved by administration of cytokines that regulate or induce a specific T-helper cell subset to regain immunological balance. Polarized Th1 and Th2 responses can contribute to the pathogenesis of immune-mediated diseases (Romagnani, 2000). Immunomodulatory agents that cause shifts in the Th1/Th2 balance can greatly alter immune responses. In cases where innate immunity and cell-mediated immune responses can be promoted using Th1 adjuvants, a natural product or drug capable of stimulating a specific Th1 response would be beneficial to the host. Such products could potentially be used, for example, as topical applications for treating and/or controlling skin lesions caused by parasitic *Leishmaniasis* (Hepburn, 2000).

Beyond PAMPs

The field of innate immune recognition has served to confirm the importance of cellular immunity and the role of APCs in both innate and adaptive immune responses, confirming Metchnikoff's original assertion that innate immune cells contribute to long-term adaptive responses and placing the innate arm of the immune system on an even playing field with that of adaptive immunity (Metchinikoff, 1905; O'Neill, 2004). Innate immune recognition has largely been associated with APCs expressing germ-line encoded receptors like TLRs that recognize microbial surfaces and initiate inflammatory responses in much the same way as Janeway first proposed (Janeway, 1989; Medzhitov, 2009). However, an alternative view dubbed the 'danger' hypothesis, proposed by Polly Matzinger (Matzinger, 1994), although less well developed, has remained viable in light of the mounting evidence that inflammation and tissue damage can occur under sterile conditions. Matzinger's hypothesis states that APCs are equipped to respond to endogenous signals released from damaged tissues and necrotic cells and sense 'danger' in much the same way as they respond to infection. The 'danger' signals she first described are now typically referred to as damage-associated molecular patterns or DAMPs (Foell et al., 2007; Matzinger, 1998).

In the cases of autoimmunity and response to tumors, it has long been recognized that the immune system can respond without the presence of exogenous stimuli. In many cases, pathological inflammation is an underlying cause of disease and it is thought to be initiated in some cases by endogenous danger signals released as a response to cell stress or necrosis. It is likely that this kind of inflammation occurs as a

consequence to initial cell injury and/or infection so that there can be an increase in the inflammatory response that is related to endogenous signals up-regulating or maintaining inflammatory states even when the primary stimuli (i.e., bacterial endotoxin) has been cleared (El Mezayen et al., 2007). However, some endogenous signals or DAMPs can initiate and maintain inflammatory responses in the absence of microbial stimuli (i.e., sterile inflammation). Among these are high-mobility group box 1 proteins (El Mezayen et al., 2007), heat shock proteins Hsp 60 and Hsp 72 (Johnson and Fleshner, 2006), uric acid (Shi, 2010), and glycosaminoglycan fragments (Johnson et al., 2002). Many of these factors have now been associated with TLR signaling and, in some cases, specifically identified as TLR 4 agonists. For the scope of the present study those DAMPs that are derived from proteoglycans, the extracellular matrix, and other components of cartilage are of particular interest.

Cartilage proteins and immunomodulation

Cartilage is a connective tissue that composes the skeleton of vertebrate embryos, but is replaced almost entirely by bone during ossification. In elasmobranchs, ossification does not take place and the skeleton remains cartilaginous throughout ontogeny. Cartilage is primarily composed of a single cell type, the chondrocyte, and an extracellular matrix (ECM) (Hay, 1981). Chondrocytes are derived from fibroblasts. In the formation of new cartilage, fibroblasts can differentiate into chondroblasts, form new cartilage, and then mature into chondrocytes. The ECM is made up mostly of water (75% of it by weight), proteoglycans, and collagen fibers. The fibroblasts and

chondrocytes regulate the composition of the ECM by producing collagen, proteoglycans, and enzymes necessary for cartilage metabolism.

Proteoglycans are complex macromolecules that contain a core protein to which at least one glycosaminoglycan (GAG) side chain is attached. These complex molecules are synthesized following a highly coordinated series of enzymatic steps (post-translational processing). The structure of proteoglycans can differ significantly depending upon the nature of core proteins, the different classes of glycosaminoglycan side chains, and the variable number and length of glycosaminoglycan chains. Glycosaminoglycan chains commonly found in cartilage tissue include chondrotoin sulfate, keratin sulfate, heparin sulfate, and O-/N-linked oligosaccharides. Cartilage proteoglycans must also be organized into the ECM through a specific aggregation process involving additional macromolecules, such as hyaluronic acid (HA) and link proteins that contain additional oligosaccharides and bind to the HA-binding site on the core protein, forming a stable structure. In addition, these proteoglycans are associated with collagen fibers, organized into highly ordered triple helical fibrils that together form a network structure typical of cartilage (Heinegard, 1974; Lindahl, 1978).

Cartilage structures are highly conserved across widely distributed taxa. Tissues that are similar to vertebrate cartilage are described for most taxa within the metazoa. There are specific structural criteria for a tissue to be considered cartilage, including the cellular components and the composition of the extracellular matrix. Chondroid cartilage-like connective tissue is widely distributed within the invertebrate lineages (Cole and Hall, 2004). Among vertebrates, cartilage proteoglycans appear to exhibit

high degrees of homology (Knudson and Knudson, 2001). There is evidence in the literature that antibodies to vertebrate cartilage and bone have some immunoreactivity with invertebrate cartilage, suggesting that there may be extensive homologous structures even between distantly related taxa (Cole and Hall, 2004)

Several endogenous agonists for TLR 4 and stimulators of the innate immune response are derived from cartilage. Hyaluronan is an important structural component of the ECM and it is often released following tissue injury. The presence of hyaluronan can exacerbate an inflammatory state by inducing the production of chemokines in macrophages through TLR 2 and TLR 4, potentially leading to persistent inflammation (Jiang et al., 2005). Hyaluronan fragments have also been shown to stimulate endothelial cells and induce the production of a potent chemokine, IL-8. Activation of endothelial cells is TLR 4-dependent and appears to be a mechanism for detecting injury in the early stages of wound healing. Inflammation is a critical component of wound healing and allows for tissue repair and remodeling as well as defense against opportunistic pathogens. Components of the ECM such as GAGs appear to act as innate signals of injury or distress in tissues like the skin and elsewhere (Taylor et al., 2004).

Trauma in other tissues like the bones and joints can release GAG fragments from the ECM which induce inflammatory responses via TLR 4 as well (Taylor et al., 2007). Among these are fibronectin fragments, tenascin-C an ECM glycoprotein, and soluble heparin sulfate (Goh et al., 2010; Johnson et al., 2002; Okamura et al., 2001). Studies have shown that heparin sulfate GAGs can activate macrophages and alter downstream T cell responses (Wrenshall et al., 1999). Specifically, heparin sulfate

triggered the up-regulation of adhesion molecules and the release of IL-1, IL-6, TNF-α, and IL-12. It also triggered the production of nitric oxide. The authors suggested that the glycosaminoglycan composition of a given tissue might aid in regulating the behavior of antigen-presenting cells in that microenvironment, particularly during inflammatory processes.

It seems likely that most of these ECM/cartilage components that initiate innate responses do so following damage or stress and are involved in maintaining the integrity and health of tissue through surveillance. Many of these agonists for TLR 4 are believed to be the source of some of the unabated inflammation that persists with conditions such as arthritis and are, therefore, being targeted therapeutically. To investigate the role of TLR 4 agonists in inflammation one must distinguish between responses induced by the putative agonist itself and that which might result from contamination by bacterial molecules like LPS. This is a challenge because the interaction between TLR agonists and their receptors and the initiation of subsequent intracellular signaling is poorly understood (Sabroe et al., 2008).

In the context of the present study, one must consider the possibility that cartilage-derived factors might represent a group of molecules that can activate APCs like tissue macrophages and have localized and/or systemic effects on downstream innate immune responses. Given the evolutionary conservation of effectors and receptors in innate immunity, it is likely that ligands derived from an unrelated taxonomic group like sharks can interact with cell surface recognition molecules on

mammalian immune cells (Magor and Magor, 2001). This is particularly true for inflammatory responses and the receptors and intracellular signaling molecules related to these responses, since they represent such an ancient form of host defense. Cross-reactivity and interspecies functional compatibility between factors derived from shark cartilage extracts and mammalian immune cells may lead to a better understanding of the evolutionary conservation among these immune recognition systems in the vertebrate lineage.

Mammalian cells may recognize the active component(s) in shark cartilage either because the putative ligand is recognized as a foreign, non-self molecular structure or because the ligand is similar enough in composition and structure to endogenous ligands that activate immune cells as 'danger' signals.

Shark cartilage's troubled history

Research on cartilage began following the observation by Brem and Folkman that bovine cartilage exhibited anti-angiogenic properties (Brem and Folkman, 1975). The apparent anti-angiogenic properties of cartilage suggested its potential use in preventing the formation and controlling the proliferation of cancerous tumors (Brem and Folkman, 1975; Prudden and Balassa, 1974). Because only a relatively small yield of cartilage can be recovered from mammalian (nonhuman) tissues, investigators were prompted to examine shark cartilage for similar properties, because a higher yield of cartilage per unit weight could be obtained given that the entire shark skeleton is made of cartilage. Results from these studies and subsequent studies have confirmed that certain derivatives of shark cartilage have anti-angiogenic properties against tumors in

experimental animal studies (Gonzalez et al., 2001a). The use of shark cartilage, however, has not been limited to the treatment of cancer as it has been marketed as a therapeutic agent for a number of different diseases (Gonzalez et al., 2001b). Unfortunately, reliable scientific data in support of the therapeutic use of cartilage for a variety of medical conditions remains to be obtained.

While several studies have shown that cartilage extracts have analgesic, anti-inflammatory, anti-oxidant, and anti-angiogenic capabilities under certain experimental conditions, none have clearly established their role in modulating or enhancing immune function (Chen et al., 2000; Dupont et al., 1998; Felzenszwalb et al., 1998; Fontenele et al., 1997; Fontenele et al., 1996; Gingras et al., 2000; Lee and Langer, 1983; McGuire et al., 1996; Miller et al., 1998; Oikawa et al., 1990; Rabbani-Chadegani, 2008; Sheu et al., 1998). Many of these studies focused on the effect of cartilage-derived compounds on inhibition of angiogenesis and tumor progression in cellular and animal models and the compounds were derived from shark cartilage prepared by investigators themselves (i.e., relatively "pure" preparations), not from the commercial shark cartilage products sold as dietary supplements which in addition to cartilage may contain any number of contaminants and other tissue derivatives.

Oral administration of powdered, commercial shark cartilage has been tested in an *in vivo* rabbit cornea assay and shown to inhibit neovascularization and basic fibroblast growth factor-induced angiogenesis (Gonzalez et al., 2001a). Several studies where commercially available powdered shark cartilage was administered orally in murine and rat models found that oral ingestion inhibited angiogenesis and, although it

did not abolish tumor progression, it did delay its development significantly (Barber et al., 2001; Davis et al., 1997). These results suggest that, under these experimental conditions, enough of the "active" anti-angiogenic ingredient in shark cartilage is being absorbed to have an effect following oral ingestion.

Relatively few studies have examined the effects of shark cartilage extracts or products on humans either *in vitro* or *in vivo* (Bukowski, 2003; Gingras et al., 2000; Hillman et al., 2001; McGuire et al., 1996; Miller et al., 1998). Liquid cartilage extract (LCE) was administered orally along with a placebo to male volunteers and wound angiogenesis was measured indirectly by endothelial cell density. The results indicated that LCE contains an antiangiogenic component that is bioavailable to humans following oral administration (Berbari et al., 1999). There have been anecdotal reports of several clinical trials investigating the effect of shark cartilage ingestion on cancer patients' health, some of which elicited wide public attention although their experimental methods did not undergo the scrutiny of peer review. Moreover, the results of these studies have not been replicated successfully.

More recently, scientists chose to examine the efficacy of one of the most well-known commercial shark cartilage products, Benefin, in a placebo-controlled clinical trial. Benefin was developed as shark cartilage treatment for cancer following Dr. William Lane's publication 'Sharks don't get cancer' in 1992 and remains on the market today despite having had an FDA injunction placed against it and its manufacturer, Lane Labs, in 1999 for promoting Benefin as a cancer treatment. In the placebo-controlled trial, no benefit was found with the intake of shark cartilage with respect to cancer

progression (Loprinzi et al., 2005). Shark cartilage continues to be promoted as an alternative therapy for anti-cancer on websites such as the 'shark cartilage information exchange, although it is done quite cautiously given the reaction of the scientific community to Lane Labs in recent years. Furthermore, shark cartilage is also promoted as a potential treatment for other diseases such as arthritis and as a prophylaxis to prevent disease.

Commercial shark cartilage and immunomodulation

Some scientists have begun to investigate the potential immunomodulatory properties of shark cartilage. One study focused on the effect of shark cartilage on the infiltration of lymphocytes in a murine tumor model. Intraperitoneal injection of shark cartilage protein fractions into tumor-bearing mice increased T cell infiltration into the tumor and significantly increased the CD4/CD8 ratio in tumor infiltrating lymphocytes. The study also showed that a 15 kDa fraction of shark cartilage enhanced immune response by augmenting delayed hypersensitivity against sRBC in mice (Feyzi et al., 2003). Another study investigated the immunostimulating potential of various preparations of shark cartilage extracts using an *in vitro* murine model. The extracts were potent stimulators of B cells and macrophages isolated from BALB/c mice spleen. The active components were shown to be thermally stable proteoglycans with molecular masses exceeding 100 kDa (Kralovec et al., 2003).

To date, few studies have investigated the effect of commercial shark cartilage products on human immune cellular responses. The first such study (Simjee, 1996) was

conducted in our laboratory which investigated the cytokine (TNF-α) response of cultured human leukocytes to various extracts of several brands of commercially available shark cartilage. The level of detectable TNF-α in culture supernatants of leukocytes stimulated with shark cartilage extract was significantly higher than that of LPS-stimulated control cultures and levels were highest after 24 hours of exposure. This study showed that different commercial brands of shark cartilage differed in the level of biological activity (i.e., cytokine induction) and protein content. In addition, when three different types of extracts (a salt-soluble, a phosphate-buffered, and an acid extract) were assayed for activity, the acid extract induced higher levels of cytokine production than the others. Furthermore, when the studies compared shark cartilage extracts to bovine cartilage extracts, the former induced a significantly higher level of cytokine. The study also showed that extracts lost 80% of their biological activity (cytokine induction) following treatment with trypsin and chymotrypsin (i.e., protein digesting enzymes), indicating that the active component(s) in shark cartilage may be proteinaceous. It should be noted that a low level of activity (20%) remained following enzymatic digestion, suggesting that oral ingestion and digestion will not necessarily eliminate all activity and some level of activity can be expected to be retained which may be sufficient to be absorbed and be immunostimulatory. In addition, shark cartilage extracts were subjected to heating conditions up to 95^{o}C and heating the extract to high temperatures abolished its cytokine-inducing activity, serving as further evidence that the bioactivity in SC extracts is protein in nature (Simjee, 1996).

A follow-up study analyzing the overall cytokine production in response to *in vitro* stimulation with shark cartilage showed that cartilage significantly induced the production of Th1-type, inflammatory cytokines, namely, IL-1β, IL-2, TNF-α, and IFN-γ as well as a potent chemokine, IL-8 (Merly et al., 2007). Many of the diseases (i.e., rheumatoid arthritis) for which shark cartilage products are recommended by commercial manufacturers are caused by and are the result of undesirable inflammatory reactions. The use of shark cartilage which induces up-regulation of inflammatory cytokines is, therefore, counter indicated. However, if active components in shark cartilage that stimulate and/or modulate innate immune response are isolated, they may serve as immunostimulatory agents. A better understanding of the active component(s) of shark cartilage, the scope of their bioactivity, and the mechanisms by which they modulate the immune response of cells can lead to their potential application in clinical medicine. One such application would be topical immunomodulation where an agent that induces monocytes/macrophages to produce Th1 cytokines and promote cell-mediated immunity, especially as a TLR agonist, may be clinically useful in treating viral infections and/or improving wound healing (Hengge and Ruzicka, 2004). The goal of the present study was to define the scope of bioactivity, to characterize the nature of the active component(s), and to elucidate the molecular mechanisms underlying their biological activity.

MATERIALS AND METHODS

Reagents, stock solutions, and buffers

All buffers and reagents are described in detail in Appendix 1. Manufacturer's information and catalog numbers have been provided in the text where appropriate. Additionally, all electrophoresis and chromatography reagents are listed in Appendix 1.

Methods

The objectives of the study were (1) to define the scope of the cellular response to SC extract, (2) to further characterize the nature of the active component(s) of SC extract, and (3) to elucidate the molecular mechanisms underlying bioactivity. A three-pronged experimental approach was taken and experiments were designed and carried out as described below. Figure 1 is a diagrammatic overview of the experimental plan.

1. Cartilage Extracts

Extracts were prepared from commercially available shark cartilage (SC) capsules (Solgar Vitamin and Herb, Leonia, NJ 07605, USA) and for the duration of this study, cartilage capsules from a single lot (Lot #47580) were used. To compare efficacy of extracts, multiple extraction protocols varying in extraction temperature and grams of starting material were evaluated for both acetic acid and hydrochloric acid extractions. Five different amounts of shark cartilage powder starting material were used: 0.2 g, 0.4 g, 0.8 g, 1.0 g, and 1.5 g. In each case, the dry powder was placed in a 25 ml sterile flask and dissolved in 10 ml of either 0.5M acetic acid with 0.1% TFA, pH 4.2 or in 1 N HCl, pH 2. Each extract was prepared overnight either at room temperature or at 4^{o}C. All extracts were prepared in duplicate. In addition, an acid extract using a larger amount

of starting material, equivalent to the recommended daily dose stated on the SC label (8 capsules per day; i.e. 6.41 g), was prepared in 50 ml acetic acid and its cytokine inducing activity was compared to that of the standard SC extract prepared in the laboratory from lesser capsule amounts.

The standard extraction protocol for the present study consisted of adding 10g cartilage powder from capsules to 250 ml 0.5M acetic acid with 0.03% toluene pH 4.2, incubating the mixture overnight at 4^{o}C with stirring. Insoluble material was removed from the soluble extract by centrifugation for 45 minutes at 250 x g, followed by vacuum filtration (0.45 μm filter). Acid extracts (SCAE) were then dialyzed against phosphate-buffered saline pH 7.4 (0.15M NaCl, 0.003M KPO_4, 0.001M $NaPO_4$) at 4^{o}C, and stored at -20^{o}C until tested for endotoxin (see below) and used in bioassays.

1.1 Protein Estimation

Protein estimation was performed using the Bicinchonic acid (BCA)-protein assay employing microtitration (Pierce, Cat #23225) and using bovine serum albumin (BSA) as the standard. Bicinchonic acid is a highly sensitive reagent for the spectrophotometric measurement of protein concentration (Smith *et al.,* 1985). Briefly, the assay is based on the principle of the Biuret reaction where, in the presence of protein, Cu^{2+} in an alkaline medium is reduced to produce Cu^{1+}. Bicinchonic acid is highly specific for the cuprous ion Cu^{1+} and will react to form a colored product that is water soluble and exhibits a strong absorbance at 562 nm, allowing the spectrophotometric quantitation

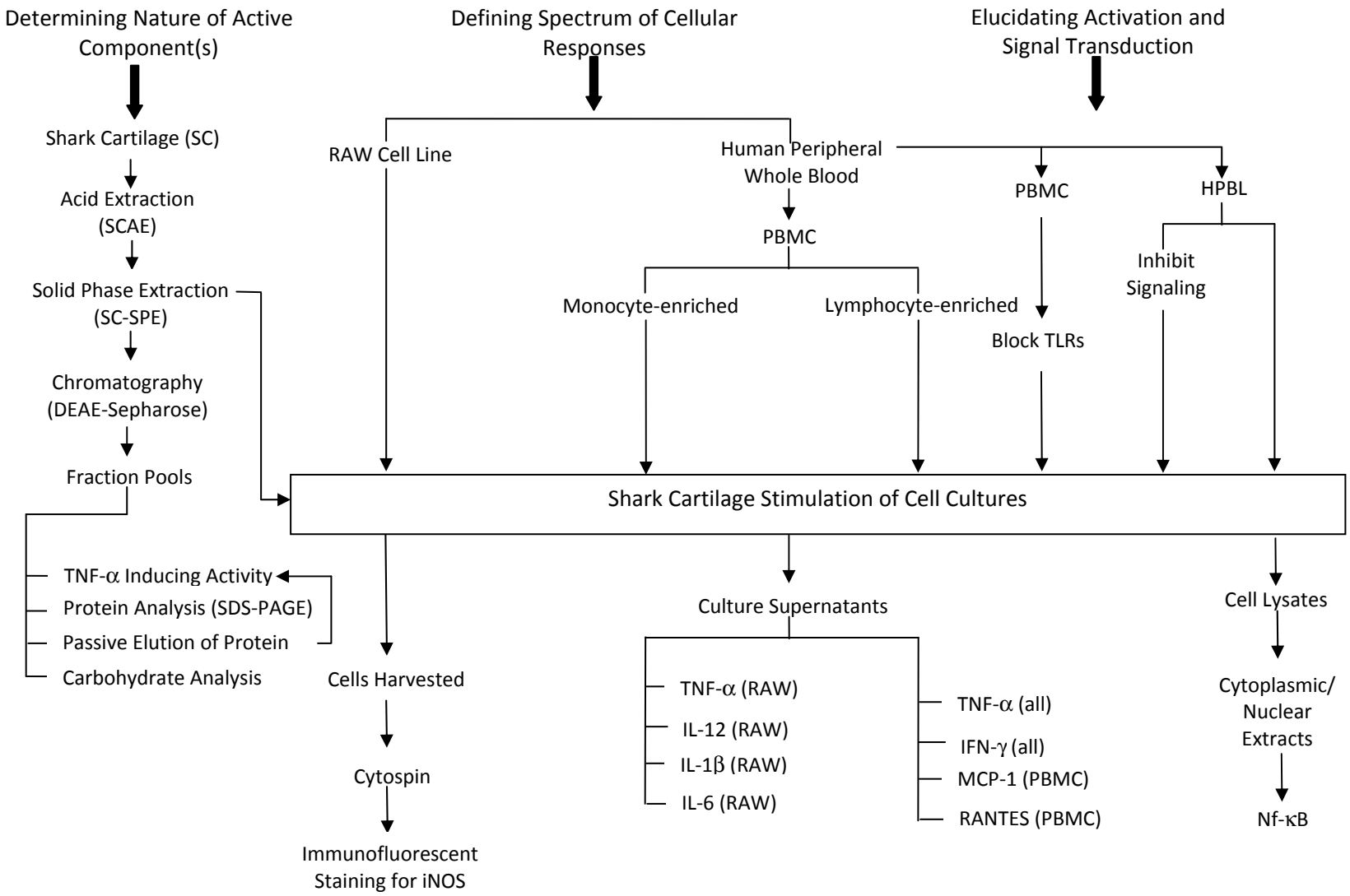


Figure 1: A diagrammatic overview of the experimental approach.

of protein in aqueous solutions. The assay was carried out in microtiter plates by mixing 0.1 ml of each cartilage extract with 0.25 ml of the working reagent prepared by mixing 50 parts of reagent A (which contains sodium carbonate, bicarbonate, and sodium tartarate in 0.2 N NaOH) and 1 part reagent B (4% copper sulfate solution). The mixture was incubated for 30 minutes at 37^{o}C. Following incubation and color development, samples were allowed to cool to room temperature for approximately 5 minutes before the absorption (Abs_{562nm}) of each sample and control was measured in duplicate. A standard curve was generated by plotting the optical density (absorbance) of BSA standards against protein concentrations.

1.2 Fractionation of crude extracts

1.2.1 Solid phase extraction (SPE)

Solid phase extraction was employed to concentrate crude extracts and to remove inorganic salts, by applying the extract to a pre-packed SPE C18 column (Waters, Sep-Pak 6g 1cc) that was equilibrated with approximately 10 ml of 100% Methanol (Sigma, HPLC grade) before being rinsed with 10 ml of distilled water. The acid extract (approximately 250 ml) was then added drop-wise and allowed to run through the column under slight vacuum pressure (flow rate approximately 2 ml/min). The filtrate was collected and stored for activity and protein analysis. The column was then rinsed briefly with approximately 10 ml distilled water. The bound material was eluted from the column with 10 ml of 50% Acetonitrile with 0.1% TFA. The resulting yellow fractions were collected in microcentrifuge tubes and vacufuged to remove all Acetonitrile. Remaining solids were re-suspended in chromatography buffers (1 ml) and/or culture

medium (1 ml) and stored at -20^{o}C until used in bioassays or for further fractionation. The acid extract that was separated and recovered from solid phase extraction was referred to as SC-SPE for the remaining analysis. The column was given a final wash in 80% Acetonitrile with 0.1% TFA to remove any remaining material from the column before storing column at 4^{o}C for future use.

1.2.2 Ion exchange chromatography

Acid extracts separated by solid phase extraction (SC-SPE) were further fractionated by ion exchange chromatography. DEAE Sepharose was packed into a Fast-Protein Liquid Chromatography (FPLC) column (8 cm x 2 cm) and washed with 20% ethanol, followed by distilled water. The column bed was equilibrated first with high concentration stock buffer, 0.5M Tris-HCl pH 7.5 followed by the equilibration buffer, 0.02M Tris-HCl buffer pH 7.5. When the pH and conductivity of the effluent was similar to that of the equilibration buffer, the sample was loaded. The column was run on the ACTA prime LC system (Amersham). Bound material was eluted from the column by applying a linear gradient using an elution buffer consisting of 0.02M Tris-HCl with 1M NaCl pH 7.5.

Vacufuged samples (SC-SPE) were re-suspended in the column equilibration buffer and syringe-filtered (0.2 µm filter) prior to injection into the loading chamber. Extracts were run using the following volume-based method designed for this protocol: 0-10ml (100% equilibration buffer); 10.1-20ml (Injection of 2 ml sample with 100% equilibration buffer); 20.1-40ml (100% equilibration buffer wash); 40.1-70ml (0-100%

gradient of elution buffer); 70.1-80ml (final wash with 100% elution buffer); and 80.1-110ml (100% equilibration buffer wash). All fractions (each 1 ml) were collected and pooled, according to protein content (i.e. peak fractions) and tested for activity. Pools were screened for TNF-α inducing activity following confirmation that pool was endotoxin-free. Protein/peptide composition of active pools was determined by SDS-PAGE analysis.

1.2.3 Analysis of solid phase extracts by High Pressure Liquid Chromatography (HPLC)

Acid extract (SCAE) and solid phase extract (SC-SPE) were subjected to HPLC analysis. Each extract was rapidly thawed to room temperature before undergoing short, rapid centrifugation (250 x g for 3-4 minutes) to remove any particulate matter from the sample. An YMC C18 column, 250 x 4.6mm (Waters), was used for HPLC. The column was cleaned with 20% methanol and equilibrated with water/acetonitrile before and between each extract sample applied. Samples were filtered (0.2 μM) and approximately 100 μl of each sample was applied for each run. Material was eluted with acetonitrile with 0.1% TFA in water using the following protocol: 0-5 min (100% water; 0% ACN); 5-40 min (0-70% ACN in water); and 40-45 min (100% ACN; 0% water). The flow rate was maintained at 0.300 ml/min and all protein peaks were resolved within 30 minutes of elution using this method. Fractions (approximately 250 μl each) were collected in a sterile, 96-well plate. Following chromatographic separation, extract fractions were evaporated under vacuum pressure (Ependorf Vacufuge) at room temperature until all fluid evaporated (approximately 2 hours). Each plate was sealed

using parafilm and stored at -20^{o}C until use in bioassays. Fractions were thawed briefly before complete RPMI culture medium (50 µl) was added to each microtiter well to re-suspend protein. Re-suspended fractions were pooled according to HPLC retention time (i.e., each pool represents 2 minutes retention time) and used as stimulants in HPBL cultures. Culture supernatants were screened for TNF-α inducing activity by ELISA.

1.3 Concentration of Extracts and/or Pooled Fractions

1.3.1 Concentration by ultrafiltration

Pooled fractions of extracts were concentrated by ultrafiltration using a centrifugal filter device. Briefly, samples were loaded onto the YM3 filter device (Amicon, MWCO 3,000) and centrifuged at 3,000 x g for 4 hours. Following centrifugation, a retentate vial was placed over the sample reservoir and the assembly was inverted before undergoing centrifugation at 2,000 x g for 3-4 minutes to transfer concentrated sample into the retentate vial which was stored at -20^{o}C until thawed for chromatographic fractionation.

1.3.2 Concentration by lyophilization

Fractions and/or fraction pools from ion exchange columns were concentrated by lyophilization. Briefly, samples were dialyzed to remove salts and approximately 5-10 ml transferred to a 50 ml falcon tube and frozen at -70^{o}C overnight at a 45^{o} angle. Once frozen, tubes were placed in a 500 ml glass lyophilization vial and connected to the lyophilizer. Samples were lyophilized overnight for approximately 16-18 hours before

being removed and lyophilized samples were re-suspended in appropriate buffers and/or deionized distilled water (ddH_2O).

1.4 Test for endotoxin: E-Toxate assay

To confirm that all samples, extracts, and fractions were free of endotoxin, each was assayed for endotoxin using the E-toxate assay (*Limulus polyhemus* amoebocyte lysate assay, Sigma Catalog #210-50) which detects and semi-quantitates endotoxin in test samples. The assay is able to detect endotoxin levels as low as 0.015 EU/ml, considered to be insufficient for significant biological activity. Only endotoxin-free samples were used in bioassays. Briefly, 0.1 ml of each test sample was added to a tube containing 0.1 ml of E-toxate working solution (lyophilized and reconstituted with endotoxin-free water according to the manufacturer's directions). The reaction tubes were incubated for 1 hour at 37^{o}C in a water bath. Following incubation, tubes were gently inverted 180 degrees and examined for the degree of gelation of contents. The formation of a hard gel was interpreted as a positive reaction for endotoxin. All other reaction results, including soft gels, viscous fluid, and turbidity were interpreted as a negative reaction for endotoxin. Endotoxin standards of LPS from *E. coli* (Sigma) in concentrations of 0.5 EU/ml, 0.25 EU/ml, 0.125 EU/ml, 0.06 EU/ml, 0.03 EU/ml, and 0.015 EU/ml were used as positive controls. Endotoxin-free water provided by the manufacturer was used as a negative control by adding 0.1 ml of endotoxin-free water to 2 ml of E-toxate working solution. Samples were also tested for the presence of any inhibitors for the E-toxate test by incubating 0.1 ml of each sample with 0.1 ml of E-

toxate inhibitor (provided by the manufacturer) with 2 ml of E-toxate working solution and assayed as described above.

1.5 Protein analysis of samples

1.5.1 SDS-PAGE

Acid extracts subjected to solid phase extraction (SC-SPE) were analyzed for protein composition by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). Samples were mixed 1:5 in 5x sample buffer, Tris-HCl with or without mercaptoethanol as the reducing agent (Appendix 1), centrifuged to remove any particulates, and heated at 95^{o}C for five minutes. Samples were then loaded onto a 10% SDS-PAGE protein gel made using the Oxford formula (Appendix 1) or onto a Ready-made 10% or 12% Tris-HCl protein gel (BioRad Criterion Ready-made gels). Gels were run at room temperature with SDS running buffer at 150 volts for 1 hour. Gels were removed from cassettes and stained for either protein or glycoprotein using the methods described below. All pooled fractions from DEAE Sepharose were also analyzed further by SDS-PAGE.

1.5.2 Staining for Protein

Protein gels were stained with Coomassie Brilliant Blue (Appendix 1) for 1 hour with gentle agitation and then placed in fix/de-stain solution (Appendix 1) overnight. Alternatively, gels were rinsed three times with ultrapure water and then stained with Simply Blue Safestain (Invitrogen) for 1 hour. Gels were then rinsed in two washes of ultrapure water to visualize bands.

1.5.3 Staining for Glycoprotein

Protein gels were stained for glycoprotein using the GelCode glycoprotein staining kit (Pierce #24562). For this staining procedure, negative and positive glycoprotein controls were run in separate lanes on the gel. Soybean trypsin inhibitor and horseradish peroxidase served as the negative and positive controls (10µl/lane), respectively. Protein gels were removed from cassettes and fixed in 100 ml 50% methanol solution for 30 minutes. The gel was then washed twice in 100 ml 3% acetic acid with gentle agitation for 10 minutes. The washed gel was transferred to 25 ml oxidizing solution and agitated gently for 15 minutes. Following oxidation, it was washed three times in 3% acetic acid for 5 minutes per wash and transferred to 25 ml GelCode Glycoprotein Staining Reagent and agitated gently for 15 minutes. The gel was transferred to 25 ml of reducing solution and agitated gently for an additional 5 minutes. The gel was washed extensively with 3% acetic acid and finally rinsed several times in ultrapure water. Glycoproteins appeared as magenta bands on the gel.

1.5.4 Passive elution of protein bands

Protein gels were stained with a reversible E-Zinc stain (Pierce Catalog #24582) for the purposes of visualizing protein bands for passive elution. Gels were immersed in 50 ml of E-Zinc stain and agitated gently for 10 minutes. The stain solution was decanted and 50 ml of E-Zinc developer solution was added. The stain was allowed to develop for 1-2 minutes and checked against a dark background (gel becomes opaque and protein bands are visualized as clear, unstained areas). Bands of interest were then

excised from the gel. The same sample was run in multiple lanes to obtain several identical bands for excision. Additionally, two areas of the gel without bands were excised as negative protein controls for the passive elution procedure. Excised bands were de-stained in 50 ml of Tris-glycine buffer pH 8 with gentle agitation for 10 minutes and then rinsed several times with ultrapure water. Excised gel pieces of each sample were placed in a sterile microcentrifuge tube and 500 µl of elution buffer (0.05M Tris-HCl, 0.15M NaCl, and 0.001M EDTA pH 7.5) was added so that gel pieces were completely immersed. The gel pieces were homogenized manually with a sterile pestle and incubated in a rotary shaker at 30^{o}C overnight. Following incubation, tubes were centrifuged at 10,000 x g for 10 minutes and the supernatant carefully transferred to fresh tubes and stored at -20^{o}C until used in bioassays.

1.6 Glycoprotein/Carbohydrate Estimation

Pooled fractions that showed positive magenta bands upon glycoprotein staining were tested for carbohydrate content using the glycoprotein carbohydrate estimation kit (Pierce Catalog #23260). All samples were standardized to 0.25 and 2.5 mg/ml protein in glycoprotein assay buffer using the BCA protein estimation kit described above. Lysozyme (2.5 mg) and bovine serum albumin (2.5 mg) served as the negative control standards. Ovalbumin (2.5 mg), human apotransferrin (2.5 mg), fetuin (0.25 mg), and α_1-acid glycoprotein were the positive control standards. All samples and standards were tested in triplicate in microtiter wells at 50 µl/well. Blank wells were loaded with the glycoprotein assay buffer. The samples were oxidized by the addition of

25 µl of sodium meta-periodate solution per well and mixed on a microplate shaker for 30 seconds. Plates were then covered with plate sealers and incubated at room temperature for 10 minutes. Following incubation, 150 µl of glycoprotein detection reagent (50 mg detection reagent dissolved in 10 ml of 1N NaOH) was added to each well. Plates were mixed for 30 seconds on a microplate shaker and then incubated at room temperature for 1 hour. Absorbances of the reaction mixture in each well were measured at 550 nm in a microplate reader and a standard curve was constructed based on the absorbance readings for each standard. Carbohydrate content of glycoprotein samples was estimated based on a line of best fit for the standard curve.

1.7 Identification of the bioactive glycoprotein

Protein bands of interest were transferred to PVDF membranes using a wet electroblotting technique and stained using Coomassie blue before being sent for N-terminal amino acid sequencing to identify proteins of interest. Alternatively, protein pools of interest were lyophilized and reconstituted in sterile, distilled deionized water before being sent for LC-MS/MS analysis at the Interdisciplinary Center for Biotechnology Research at the University of Florida, Gainesville, Florida.

2. Primary cell cultures

2.1 Isolation of Human Peripheral Blood Leukocytes (HPBL)

The protocol for the collection and isolation of leukocytes from peripheral blood of healthy human donors was approved by FIU's Institutional Review Board (IRB). Leukocytes were separated by mixing heparinized whole blood 1:1 with sterile 3% dextran solution (Sigma) in a 15 ml tube by gently inverting tube several times and

allowing mixture to sit upright for 30 minutes. Erythrocytes sedimented to the bottom and leukocytes suspended in the supernatant were transferred to fresh tubes and centrifuged at 250 x g for 10 minutes to pellet the cells. Supernatant fluid was discarded and the leukocyte pellet re-suspended with gentle tapping in approximately 5 ml of sterile 0.2% NaCl solution for 15-20 seconds to lyse contaminating erythrocytes (hypotonic lysis). To return the solution to the proper isotonicity, 5 ml (similar to the volume of hypotonic solution used) of sterile 1.6% NaCl solution with 0.2% dextrose (hypertonic solution) was immediately added to the tube and contents gently mixed to achieve physiological isotonicity. Tubes were centrifuged at 250 x g for 10 minutes to sediment leukocytes. The pellet (white) was gently re-suspended by tapping in a small quantity of fluid remaining before transfer to a fresh, sterile tube and then washed several times in phosphate-buffered saline. Final cell suspensions were prepared in serum-free RPMI-1640 medium (supplemented with 0.3 mg/ml glutamine and 25mM HEPES, 100 μg/ml of streptomycin and 100 U/ml of penicillin to inhibit bacterial contamination). Cell viability was checked using the Trypan blue exclusion test. Cell counts were performed manually using a hemacytometer and leukocyte suspensions were standardized to 2.5 x 10^5 cells/ml for setting up leukocyte cultures.

2.2 Isolation of Peripheral Blood Mononuclear Cells (PBMC)

To obtain mononuclear enriched cell suspensions, freshly drawn heparinized peripheral blood was diluted 1:1 with sterile, physiological saline and layered onto MonoPoly Ficoll-Paque Resolving medium (Sigma, Cat#17-1440-02). Briefly, 7 ml of

diluted blood was layered carefully atop 3 ml of the separation medium in a 15 ml centrifuge tube. Tubes containing Ficoll-Paque blood mixtures were centrifuged immediately at 250 x g for 30 minutes at room temperature with the brake off. The buffy layer containing the mononuclear enriched cell fraction was carefully aspirated and transferred to a fresh, sterile tube and washed several times in phosphate-buffered saline. Cells were finally suspended in serum-free RPMI-1640 medium (supplemented with 0.3 mg/ml glutamine and 25mM HEPES, 100 µg/ml of streptomycin and 100 U/ml of penicillin to inhibit bacterial contamination) and checked for viability as described above. A cell viability of 95% or higher was required before proceeding with cell culture set-up. Cell suspensions were standardized to desired concentrations.

2.3 In vitro stimulation of HPBL or PBMC

Leukocyte cultures were set up in triplicate in 24-well flat bottom tissue culture plates (Falcon, Becton Dickinson). Each well contained 50 µl of the test stimulant or control stimulant, 100 µl of culture medium (RPMI-1640 supplemented with 10% fetal bovine serum or FBS), and 200 µl of cell suspension (2.0-2.5 x 10^5 cells/ml). Test stimulant consisted of shark cartilage extract alone or fractionated samples of the cartilage extract. Lipopolysaccharide, i.e. LPS, (*E. coli,* Difco laboratories) served as the stimulant for positive control cultures. Unstimulated or negative control cultures contained culture medium in lieu of stimulant. Cultures were incubated for periods ranging from 24 hours up to 96 hours in a humid chamber at 37^{o}C in 5% CO_2. For cytokine and growth factor assays, culture supernatants were harvested at 2-8 hour

intervals by aspiration, centrifuged, and cell-free supernatants stored at –20^{o}C until further use. Harvested supernatants representing 0 hour are estimated to be samples collected within five minutes immediately following culture set-up.

2.4 Leukocyte-specific responses

Peripheral blood mononuclear cell (PBMC) suspensions obtained by density gradient centrifugation using Ficoll-Paque were standardized to 1 x 10^{6}cells/ml. Cell cultures (1 ml of cell suspension per well) were set-up in sterile 24-well plates and incubated for 2 hours at 37^{o}C with 5% CO_2 and high humidity in complete RPMI-1640 growth medium in the absence of any stimulants. Monocytes/macrophages were allowed to adhere to the plate bottom while non-adherent lymphocytes suspended in the medium were removed and, using a hemacytometer, were standardized to 2.5 x 10^{5}cells/ml. Lymphocytes (200 µl of standardized suspension) were re-plated on fresh 24-well plates and stimulated over 96 hours. Test and control culture supernatants were collected every 24 hours. Simultaneously, fresh culture medium was added to the remaining adherent monocyte/macrophage population and cultures were stimulated as described above for 96 hours, with culture supernatants harvested every 24 hours. Using dextran sedimentation (described above) with the same sample of whole blood, HPBLs were isolated and concurrent cultures were set up for comparison. Culture supernatants from these three sets of leukocyte subpopulations were assayed for the production of TNF-α and IFN-γ. Each leukocyte-specific suspension was also used to

prepare cytospin monolayers that were stained with Wright's stain and used to confirm the leukocyte types represented in each cell population.

3. RAW264.7 cell culture

The RAW 264.7 cell line was obtained commercially from the American Type Culture Collection (ATCC). It is a murine macrophage-like cell line originally isolated from an Abelson leukemia virus transformed cell (Raschke *et al*., 1978). A vial containing RAW cells was thawed rapidly upon arrival in a 37^{o}C water bath, cleaned with 70% ethanol rinse, and cells were transferred to a sterile, 15 ml centrifuge tube containing pre-warmed culture medium as per ATCC instructions. The cell suspension was centrifuged at 250 x g for 5 minutes to remove DMSO (preservative) and re-suspended gently in approximately 10 ml of supplemented culture medium (RPMI-1640 with 5% fetal bovine serum, 100 μg/ml of streptomycin and 100 U/ml of penicillin to inhibit bacterial contamination) in sterile, T25 culture flasks and incubated at 37^{o}C with 5% CO_2 and high humidity. Cell growth and confluence were monitored by inverted light microscopy. Cells (approximately 80% confluent) were sub-cultured every 2-3 days. Cells were released and removed from the flask using a cell scraper and transferred to new sterile T25 culture flasks containing culture medium (1:4). At every other passage, cell subcultures were frozen in 10% DMSO as follows. Released, cultured cells were transferred to a 15 ml centrifuge tube after two washes in RPMI-1640 with no added supplements. Cells were standardized to 5-10 x 10^{4}cells/ml and 1 ml of cell

suspension transferred to 1 ml cryovials which were frozen overnight in an ethanol container before transfer to long-term liquid nitrogen storage.

3.1 Stimulation of RAW cells

Cultured RAW cells were harvested from flasks using a cell scraper and transferred to a 15 ml centrifuge and standardized as described above. Cells (200 µl cell suspension per well) were plated onto sterile, 24-well tissue culture plates (BD Falcon) in complete RPMI-1640 growth medium (supplemented with 10% fetal bovine serum and 100 µg/ml of streptomycin and 100 U/ml of penicillin). Cultures were stimulated with cartilage extracts or extract fractions for varying periods of time. Simultaneously, appropriate control cultures were treated with either medium alone or LPS (5 µg/ml in RPMI-1640). Culture supernatants were harvested at 2-4 hour intervals and assayed by ELISA for cytokines/chemokines of interest.

3.2 Macrophage activation assay for iNOS (inducible nitric oxide synthase)

RAW cultures at about 80% confluence were harvested and standardized to 2.5 x 10^5cells/ml in supplemented RPMI and plated in 12-well tissue culture plates. Each well contained a 800 µl cell suspension along with either 200 µl of culture medium or 200 µl of stimulant. For this assay, positive control cultures were stimulated with either 200 µl of LPS (5 µg/ml) alone or a combination of 100 µl of LPS (5 µg/ml) and 100 µl of IFN-γ (2.5 ng/ml). The solid phase extract of SC (SC-SPE) was used as the test stimulant. Plates were incubated at 37^{o}C with 5% CO_2 in a humid chamber for 24 hours.

Cultures were assayed for induction of iNOS using a modified version of the Cellomics iNOS activation kit (Thermo Scientific, Catalog #2001.0). The assay was modified so that cells could be fixed to microscope slides rather than the plate bottom and the presence of iNOS in the cytoplasm of responding cells detected by antibody using fluorescent microscopy. Following incubation, cells were harvested by gentle scraping from the plate bottom and 500 µl of each cell suspension was transferred directly into a cytospin chamber (tan). Clean, lysine-coated microscope slides were placed with each cytospin chamber containing the RAW macrophage suspension in a cytocentrifuge (Westcor). Chambers were spun at 300 x g for 5 minutes to allow cells to settle as a monolayer on the slide. Slides were removed and placed on a flat surface. Monolayers were fixed to the slide by pipetting 100 µl of fixative solution (5% formaldehyde solution provided with kit) directly onto the monolayer and incubating the slides at room temperature for 15 minutes. Following fixation, the fixative solution was aspirated completely and the surface of slides was washed twice in 1x wash buffer (100 µl per wash). Wash buffer was aspirated and cells permeabilized by addition of 100 µl of permeabilization buffer and incubated for 15 minutes. Monolayers were washed twice again and 100 µl of primary antibody blocking buffer was added and incubated for 15 minutes. Blocking buffer was removed and cells were covered with 50 µl of primary antibody solution (anti-mouse iNOS) and incubated for 1 hour at room temperature. Monolayers were subjected to a series of washes (3x). A second blocking buffer (100 µl) supplemented with 2% FBS was added to the monolayers and the slides were incubated for an additional 15 minutes. Blocking buffer was removed and 50 µl of staining solution

with secondary antibody (goat anti-mouse Ig) was added and slides incubated for 45 minutes. The staining solution contained a nuclear stain (Hoechst dye) and a conjugated secondary antibody (DyLight 549) for visualizing cytoplasmic iNOS. The staining solution was removed and slides washed (4x) in wash buffer. Monolayers were air dried briefly before mounting with luminol and covered with a cover slip. Cells were visualized under fluorescence microscopy for both nuclear and cytoplasmic staining and photographed.

4. Cytokine/Chemokine assays

Cell culture supernatants harvested from all three experimental cell culture models described above (i.e., HPBL, PBMC, RAW cells) were tested for cytokines/chemokines by ELISA following the manufacturer's instructions. Specifically, RAW 264.7 cell culture supernatants were assayed for the production of mediators such as TNF-α, IL-6, IL-12, and IL-1β (eBioscience murine cytokine ELISA). Human peripheral blood mononuclear cells (PBMC) supernatants were assayed for the production of MCP-1 and RANTES (Pierce). Human peripheral blood leukocyte (HPBL) culture supernatants were tested for the presence of TNF-α and IFN-γ (Pierce) in multiple experiments as a screen for biological activity.

5. Cellular activation

To determine the mechanism of interaction by which shark cartilage stimulates monocyte/macrophages through surface molecules, assays involving blocking antibodies were employed. Antibodies to immune surface receptors TLR2 and TLR 4 were used to block these receptors on target cells prior to stimulation with shark cartilage extract.

Cell culture supernatants from such treated cultures were assayed for TNF-α by ELISA and results compared to TNF-α levels in supernatants harvested from cell cultures to which no blocking antibody was added. Comparative values were used to determine whether TLR2 and/or TLR4 receptors are involved in cellular activation of leukocytes by cartilage-derived extracts.

5.1 Blocking TLR 2 and TLR 4

A suspension of peripheral blood mononuclear cells (PBMC) was prepared (2 x 10^6cells/ml) in complete growth medium and plated onto 24-well tissue culture plates as previously described. Cultures were set up in triplicate for each treatment. Leukocytes were pre-incubated for 30 minutes at room temperature with either medium alone, anti-TLR 2 antibody (20 µg/well; eBioscience Catalog #16-9024), or isotype control antibody (10µg/well; eBioscience Catalog #16-4724) to ensure that antigen/antibody reaction alone does not stimulate cells. Following pre-incubation/blocking, stimulants or medium in lieu of stimulant were added to each well. For these experiments, SC-SPE was used as the test stimulant and LPS (at 0.1ng/ml) was used as the positive control stimulant. Cells were incubated for 14 hours at 37^{o}C with 5% CO_2 and high humidity. Cell culture supernatants were harvested and tested for TNF-α production by ELISA.

A suspension of PBMC were set up in a similar manner to that described above for TLR 2 except that the blocking antibody used for pre-incubation was an anti-TLR 4 antibody (20 µg/well; eBioscience Catalog #16-9917). Cultures were pre-incubated for

60 minutes at 37°C. Cells were stimulated and incubated for 24 hours at 37°C with 5% CO_2 and high humidity as described above. Cell culture supernatants were harvested and tested for TNF-α production by ELISA.

6. Examination of intracellular signaling by kinase inhibition

The role of intracellular kinases in leukocyte stimulation by cartilage extract was investigated using a series of inhibitors for specific kinases and/or signal transduction pathways. For each pathway studied, two concentrations of each inhibitor were used in the pre-treatment of leukocytes (HPBL) prior to stimulation with shark cartilage extract (SC-SPE), LPS (5 μg/ml), or medium alone. Dextran sedimentation was employed to obtain HPBL suspensions (described above) that were standardized to 2.5 x 10^5cells/ml in supplemented RPMI. Cultures were established by adding 200 μl of suspension in each well of a 24-well tissue culture plate (done in triplicate). An additional 100 μl of supplemented RPMI medium was added to each well. Cell cultures were pre-incubated with 50 μl of either a specific inhibitor or a vehicle control for 60 minutes at 37°C with 5% CO_2 in a humid incubator. The two negative control cultures consisted of: pre-incubation with medium alone and pre-incubation with DMSO/PBS vehicle with no inhibitor. Following pre-treatment, leukocytes were stimulated with 50 μl of SC-SPE, LPS (5 μg/ml), or medium alone and incubated for 24 hours at 37°C with 5% CO_2 in a humid incubator. Culture supernatants were harvested after 24 hours and assayed for TNF-α to determine the effect of kinase inhibition on TNF-α production. Experimental details for inhibition of each test kinase are described below.

6.1Phosphatidylinositol 3 kinase inhibition

Phosphatidylinositol 3 kinase or PI-3 kinase is a central intracellular mediator of several biochemical pathways related to g-protein coupled receptors that recognize growth factors and survival factors. When PI-3 kinase is blocked, these pathways are shut off. Wortmannin is a potent inhibitor of PI-3 kinase activity in HPBL and is used at both high and low concentrations (Nakanishi et al., 1992; Arcaro and Wymann, 1993). In this experiment, HPBL were pre-treated with Wortmannin at low (0.2 μM) and high (2 μM) concentrations before stimulation.

6.2 Jun-kinase inhibition

Jun kinase (c-Jun amino terminal kinase) or JNK is a stress activated protein kinase which is a member of the MAPK family and is involved in several pathways which lead to cytokine gene expression and is typically part of intracellular signaling activated after ligand binding to g-protein coupled receptors and/or toll-like receptors. BI 78D3 is one of several small molecules that mimics a scaffolding protein, JIP1, and is able to act as a substrate competitive inhibitor of JNK (Stebbins et al., 2008). HPBL were pre-treated with BI 78D3 at low (0.2 μM) and high (2 μM) concentrations before stimulation.

6.3 Protein kinase C inhibition

Protein kinase C or PKC is involved in the production of cytokines in human monocytes and its activation and cytoplasmic translocation appear to be early events in signaling cascades associated with the up-regulation of Nf-κB and inflammatory responses. Rottlerin is an isoenzyme selective inhibitor of PKC in human leukocytes

(Kontny et al., 2000). HPBL were pre-treated with Rottlerin at low (20 μM) and high (50 μM) concentrations before stimulation.

6.4 p38 MAP kinase inhibition

The p38 (mitogen-activated protein) kinase or p38 MAPK intracellular pathway is involved in multiple cellular functions including cytokine expression, cell proliferation and apoptosis. It is specifically inhibited by the cell permeable inhibitor SB202190 (Laan et al., 2001). HPBL were pre-treated with SB202190 at low (5 μM) and high (50 μM) concentrations before stimulation.

6.5 Raf/MEK/ERK2 MAPK kinase inhibition

In addition to the JNK and p38 MAPK kinase families, a third MAPK kinase cascade has been identified as the extracellular signal-regulated kinase or ERK. Specifically, the Raf/MEK/ERK signaling cascade is involved in the transduction of signals originating from activation of g-protein coupled receptors which recognize growth factors or mitogens and initiate cell growth, differentiation, and development. GW5074 is a specific inhibitor for the Raf/MEK/ERK pathway (Edwards et al., 2006). HPBL were pre-treated with GW5074 at low (1 μM) and high (10 μM) concentrations before stimulation.

7. Nf-κB analysis

The production of Nf-κB and its translocation from the nucleus to the cytoplasm of responding cells was investigated using nuclear and cytoplasmic protein extracts of leukocytes following stimulation with shark cartilage extract (SC-SPE), LPS (5 μg/ml), or medium alone. Since Nf-κB induction and translocation is likely to be an early event

preceding cytokine production (i.e. gene expression), HPBL cultures were stimulated and assayed at short time intervals; 30, 60, 90, 120 minutes, and 4 hours.

7.1 Cytoplasmic and nuclear protein extracts from leukocyte lysates

Leukocytes for extraction were prepared as follows. Leukocyte (HPBL) suspensions were standardized to 3 x 10^6cells/ml and 600 µl aliquots were plated into each well of a 24-well plate. Supplemented RPMI (300 µl) and 150 µl of stimulant (SC-SPE, LPS, or medium) were added to each well. Cultures were incubated at 37^{o}C with 5% CO_2 with high humidity and cells harvested at 30 minute intervals. Harvested leukocytes were centrifuged and washed twice in cold, sterile PBS and the supernatant discarded. The cell pellet was transferred to a pre-chilled microcentrifuge tube on ice and gently re-suspended in 500 µl 1x hypotonic buffer (Appendix 1) by pipetting. Leukocytes were incubated on ice for 15 minutes.

A cytoplasmic extract was prepared from the leukocyte lysate by adding 25 µl of detergent (Igepal 40, Sigma) to the chilled leukocyte pellet and vortexing the mixture for 10 seconds at the highest setting. The homogenate was then centrifuged at 400 x g at 4^{o}C for 10 minutes. The supernatant containing the cytoplasmic fraction was transferred to a new tube and stored at -80^{o}C until used for bioassay. The remaining cell pellet constituted the nuclear fraction. The nuclear pellet was re-suspended in 50 µl cell extraction buffer (Appendix 1) on ice for 30 minutes with vortexing at 10 minute intervals. The pellet was centrifuged at high speed at 4^{o}C for 30 minutes and the supernatant containing the extracted nuclear fraction was transferred to a clean microcentrifuge tube and stored at -80^{o}C until used for bioassay. Both cytoplasmic and

nuclear extracts prepared from HPBL were tested for Nf-κB by ELISA using the human total Nf-κBp65 immunoassay kit (Invitrogen Catalog #KHO0371).

8. Statistical analysis

Cell cultures were set up in duplicate and cytokine/chemokine assays were set up in triplicate unless otherwise stated. Experiments for each treatment were repeated at least twice. Statistical analyses consisted of the student t test and ANOVA used to identify significant differences between untreated and treated cell cultures. A p value of less than 0.05 was considered statistically significant.

RESULTS

To achieve the objectives of the study, a three-pronged experimental approach was taken to (a) determine the nature of the active component(s) of cartilage, (b) define the spectrum of cytokine/chemokine response of cells stimulated with SC, and (c) elucidate the mechanism of signal transduction of SC-stimulated cells. Results are as follows.

Determination of the nature of active component(s) in shark cartilage

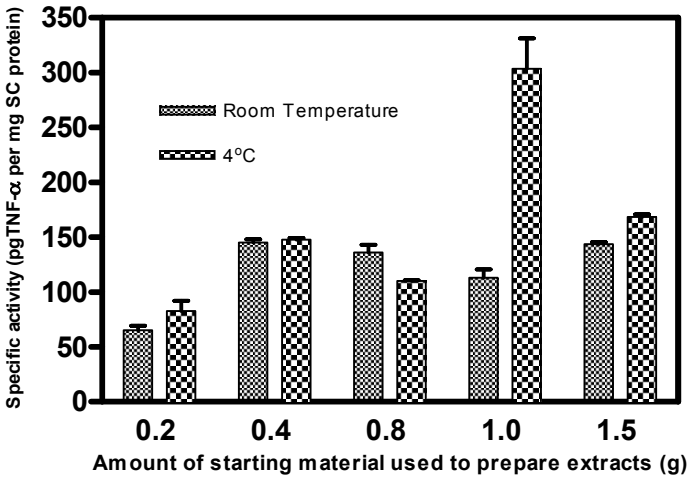
Preparation of shark cartilage (SC) extract

Various extraction protocols were employed to determine the optimal extraction conditions for obtaining active extracts (i.e., with TNF-α inducing ability) from commercial shark cartilage. Extracts were prepared using both a weak (acetic) acid and a strong (hydrochloric) acid added to varying amounts of starting material and the mixtures were incubated either at room temperature or at 4^{o}C. All extracts were prepared using the same volume of acid, incubated for the same length of time (i.e., overnight), and further processed in a similar way. Following extraction, the protein concentration for all extracts was estimated (Table 1). Protein content varied significantly depending on the extraction conditions. Hydrochloric acid extracts contained significantly higher levels of protein, almost two-fold higher, when compared to those prepared using acetic acid using corresponding starting material amounts (0.2, 0.4, 0.8, 1.0, and 1.5 g commercial shark cartilage). This was particularly true for extracts made at room temperature. Furthermore, although the amount of protein extracted increased with increasing amounts of starting material, protein

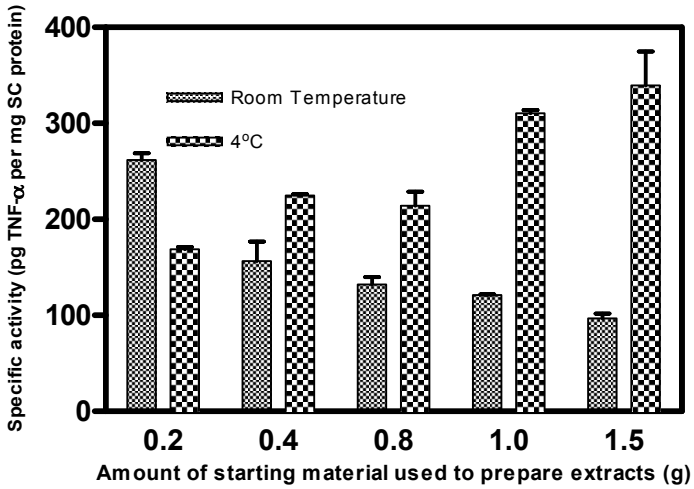
concentrations increased modestly with each two-fold increase in grams of starting material (i.e., a five-fold increase in grams of starting material yielded only a two-fold increase in protein concentration). Temperature also played an important role in the amount of protein extracted. Protein extracted at 4^{o}C was less than when extraction was done at room temperature in most cases (Table 1). The effect of temperature on the amount of protein extracted was more pronounced in hydrochloric acid extracts when compared to its effect on acetic acid extracts. However, when acid extracts were screened for human TNF-α inducing ability in HPBL, the proteins extracted at 4^{o}C for both acetic and hydrochloric acid had equal to or higher specific activity (i.e., pg TNF-α produced per mg SC protein) than the same extracts prepared at room temperature, particularly for extracts made with increased amounts of starting material (Figure 2). The highest specific activity was associated with acetic acid extract prepared with 5x starting material (i.e. 1.0 g SC) at 4^{o}C. Hydrochloric acid extracts prepared with 5x and 7.5x amounts of starting material at 4^{o}C had a similar level of specific activity. Preparing extracts from increasing amounts of starting material did not yield increased amounts of specific activity. Furthermore, there appeared no significant difference (except in the case of the five-fold (5x) preparation) between extracts prepared at room temperature or 4^{o}C (Figure 2a). At room temperature, specific activity recovered decreased in extracts prepared from increasing amounts of starting material. Conversely, at 4^{o}C, specific activity extracted increased with increasing amounts of starting material (Figure 2b).

Table 1: Protein concentrations (µg/ml) of acid extracts of shark cartilage. Extracts were prepared by dissolving varying amounts of SC starting material in 10 ml of either acetic acid or hydrochloric acid. Extraction was performed either at room temperature (RT) or at 4°C overnight. Protein concentrations were estimated based on protein assay using bovine serum albumin as a standard.

Amount of SC starting material (g)	Extraction Conditions			
	Acetic Acid (µg/ml)		Hydrochloric Acid (µg/ml)	
	RT	4°C	RT	4°C
0.2	458	495	973	765
0.4	698	548	1505	943
0.8	883	718	1548	1258
1.0	1053	955	1673	1393
1.5	1018	1110	1855	1363



(a)



(b)

Figure 2: Comparison of extraction protocols using acetic acid and hydrochloric acid with varying amounts of SC starting material and extraction temperatures. Extracts were prepared from 0.2, 0.4, 0.8, 1.0, or 1.5 g of shark cartilage powder dissolved in 10 ml of either (a) acetic acid or (b) hydrochloric acid overnight either at room temperature or 4°C. The highest specific activity was associated with acetic acid extract prepared with 5x starting material (i.e. 1.0 g SC) at 4°C ($p < 0.01$).

Shark cartilage acetic acid extracts (SCAE) prepared using 2 g of starting material routinely used in previous studies were compared to extracts prepared using 6.41 g of shark cartilage powder starting material (the amount found in daily recommended dose, i.e., high dose). The high dose extract had significantly higher protein content, approximately twice as much as that observed for the low dose extract (Table 2). Protein estimations were performed before and after concentrating extracts using centrifugal devices, employing membranes with cut-offs of 3,500 kDa molecular size, and before and after dialysis against PBS and Tris-HCl buffers and RPMI medium. The amount of protein in low dose versus high dose extracts was estimated for samples pre- and post-dialysis to determine the effect on protein retention following centrifugal concentration and dialysis (MWCO 3, 500 kDa). After dialysis, in all cases, there was a significant loss of protein. Centrifugal concentration devices with a MWCO of 3,500 kDa (500 µl samples reduced to 50 µl) used to concentrate 10-fold did concentrate the samples, but the estimation of protein concentration indicated that only three-fold rather than ten-fold protein concentration was achieved, indicating that low molecular weight molecules had been lost. Also, concentrating the extract prior to dialysis appeared to minimize protein losses associated with dialysis for both low dose and high dose extract samples (Table 2). This is to be expected since the low molecular weight proteins were presumably already lost through concentration.

To further test this dose-dependent response, the extract was compared to a high dose extract that was prepared from a larger amount of starting material equivalent to the daily dose recommended on the shark cartilage capsule label. The

amount of TNF-α produced in response to high dose extract was not significantly different (only 20% increase in TNF-α response despite three-fold increase in the amount of starting material) than that observed in response to the SC extract used as the standard in this study (Figure 3). When the specific activity of the low dose and high dose extract were compared, the low dose extract actually has a higher specific activity.

Table 2: Protein concentration (μg/ml) of acid extracts of shark cartilage before and after concentration and dialysis. Protein concentrations of acid extracts prepared from varying amounts of starting material (i.e. low dose, LD vs. high dose, HD) were estimated before and after concentration and following dialysis against PBS, Tris-HCl buffer, and RPMI cell culture medium. Protein concentration was estimated by protein assay using bovine serum albumin as a standard.

	Protein Concentration (μg/ml)			
Source of SCAE sample	**Prior to dialysis**	**Dialysis against PBS buffer**	**Dialysis against Tris-HCl buffer**	**Dialysis against RPMI medium**
Low Dose (0.2g) cartilage powder	594	289	309	191
High Dose (6.41g) cartilage powder	1070	509	450	329
Extract of LD: concentrated (3x)	1458	1341	629	1252
Extract of HD: concentrated (3x)	2622	2089	846	2154

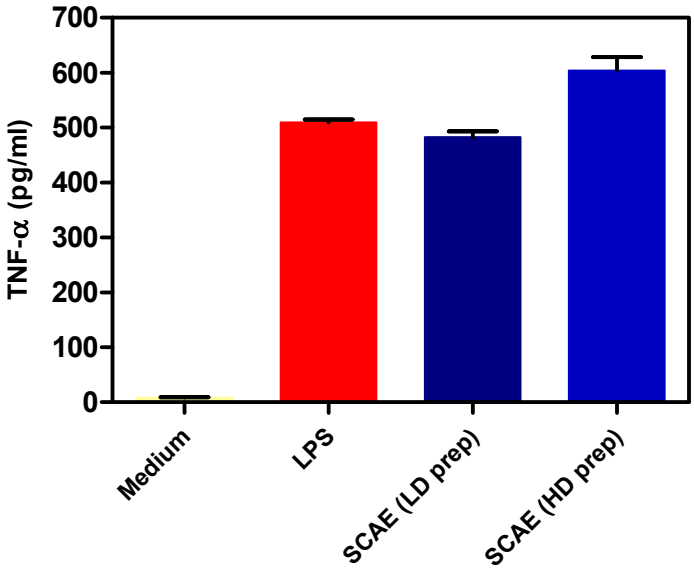


Figure 3: TNF-α production by HPBL following stimulation with low and high dose shark cartilage extracts. Acid extracts were prepared from 2 g (low dose or LD) or 6.41 g (high dose or HD, equivalent to recommended daily dose of SC) of SC dissolved in 50 ml of acetic acid overnight at 4°C (standard extraction protocol). Extract prepared from a three-fold higher dose of starting material induces only a slightly higher level of cytokine production in responding cells ($p < 0.05$).

Fractionation of SC extract

Shark cartilage acid extract (SCAE) was applied to a solid phase extraction (SPE) C18 column. A yellow fraction bound to the column was eluted with 50% Acetonitrile with 0.1% TFA (ACN). The yellow fraction (SC-SPE) when assayed for protein concentration was found to have a much higher protein concentration (3x) at 1.5 mg/ml when compared to crude SCAE at 0.575 mg/ml, although total protein in the fraction represented only 10% of total protein applied to the column. Thus, the solid phase extraction process served to concentrate the active protein contained in the crude sample. The SC-SPE sample obtained was tested for its TNF-α inducing ability using HPBL cultures and compared with crude SCAE, SCAE filtrate (unbound fraction from the

column), SC-SPE (80%) fraction, an acetonitrile control, LPS, and culture medium alone (Figure 4). The filtrate from this column did not contain any cytokine inducing ability. Results show that the SC-SPE sample induced a significant amount of TNF-α production by HPBL, when compared to the SCAE starting material, likely a result of the increased protein concentration. There was no significant difference between the TNF-α activity induced by SC-SPE 80% fraction, SCAE filtrate, and ACN treated controls and cultures treated with medium alone ($p < 0.05$).

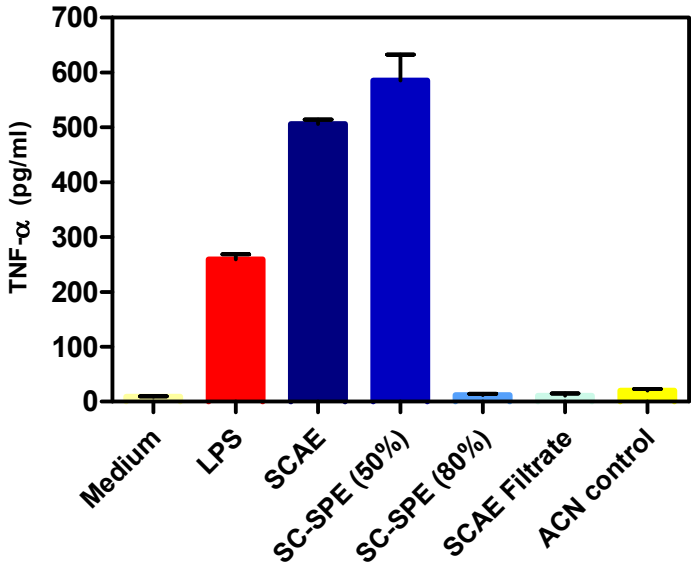


Figure 4: TNF-α production following stimulation with various fractions from solid phase extraction (SC-SPE). HPBL (2.5 x 10^5 cells/ml) were stimulated with either medium alone, LPS (5 µg/ml), SCAE, SC-SPE (50%), SC-SPE (80%), SCAE filtrate, and ACN control for 24 hours at 37°C with 5% CO_2 and high humidity. Error bars represent standard error from mean of three replicate ($p < 0.05$).

SCAE was also fractionated and analyzed using HPLC in an attempt to obtain a chromatographic profile of the crude extract (vertical bars, Figure 5). Individual fractions representing 1 minute time intervals from the HPLC run of SCAE were re-suspended in culture medium and tested for their TNF-α inducing ability (Figure 5). Activity was detected in fractions eluted after 6-8 minutes retention time. Separation of activity in distinct individual peaks, however, was not achieved from this procedure.

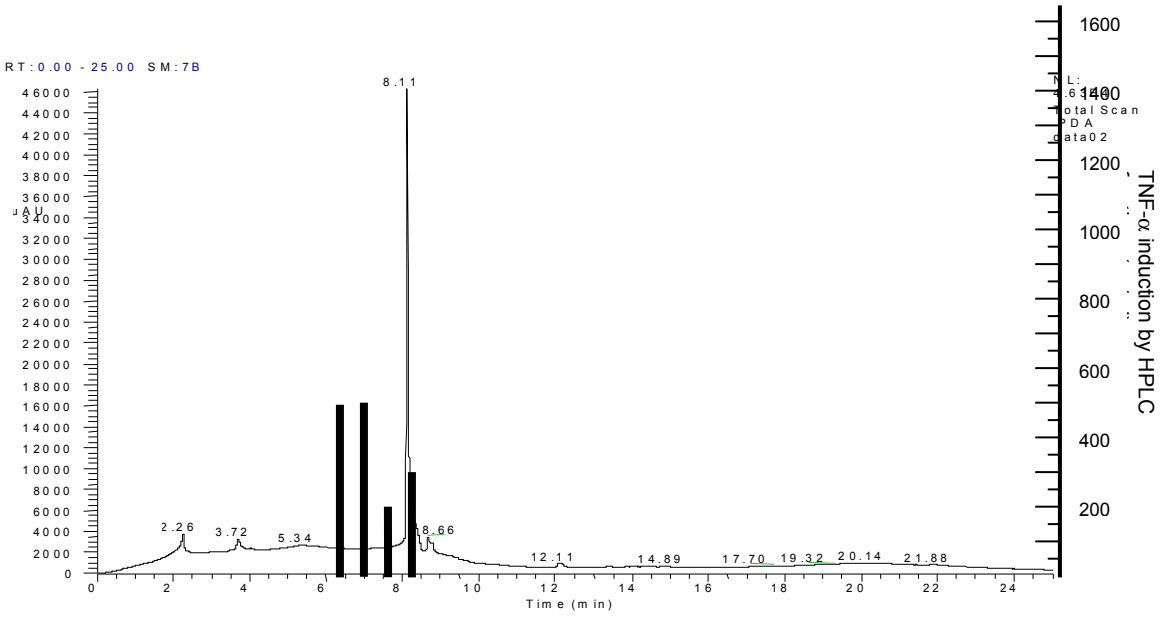
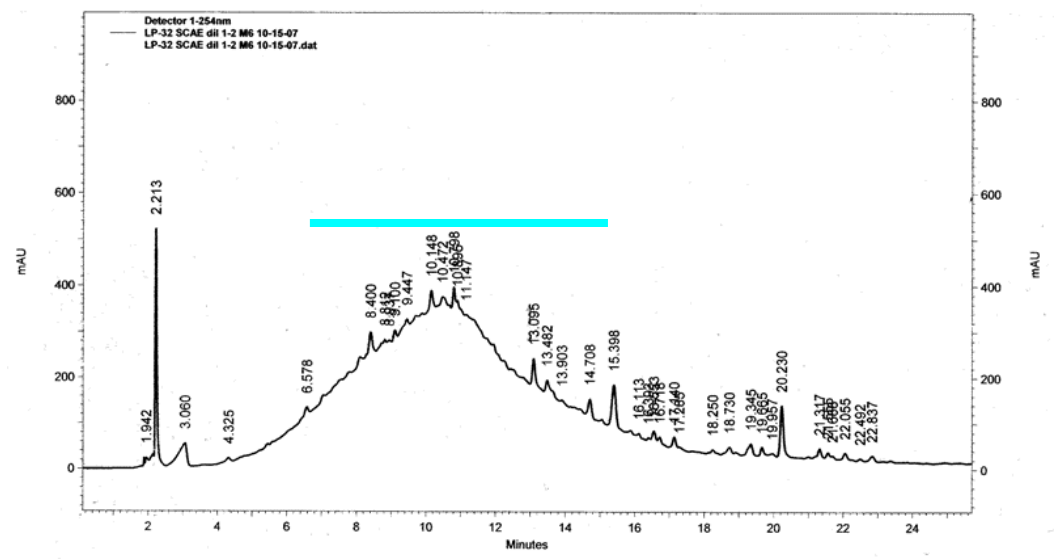


Figure 5: TNF-α response induced by RP-HPLC fractions of acetic acid extract of shark cartilage (SCAE). SCAE was prepared in 0.5 M acetic acid with 0.03% Toluene, pH 4.2, protein estimated at 477 µg/ml. The column (YMC C18, 250 x 4.6 mm) was eluted with an acetonitrile gradient (50-80% during 25 min) in water. Flow rate 0.4 ml/min. Individual fractions were tested for activity. Black bars represent fractions exhibiting TNF-α-inducing activity.

Next, the SC-SPE sample was analyzed employing HPLC and the resulting profile compared to the HPLC profile obtained for SCAE. HPLC fractionation and analysis of SC-SPE was carried out under similar chromatographic conditions. When SC-SPE was applied to a C18 column with an acetonitrile gradient, one broad peak was eluted off the column (Figure 6a). Fractions were pooled as twelve pools representing approximately 2 minutes of retention time on the chromatogram (P1-P12, corresponding to 0-24 minutes). The TNF-α activity profile for the fractions tested showed that pooled fractions P4-P7 corresponded tightly with the elution peaks on the chromatogram. The highest level of activity was seen with pool 6, which corresponds to the highest point on the elution peak at around 10-11 minutes retention time (Figure 6b).



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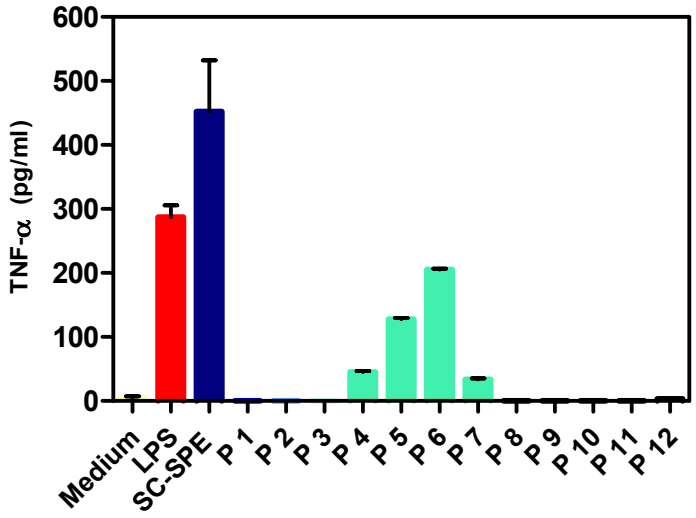
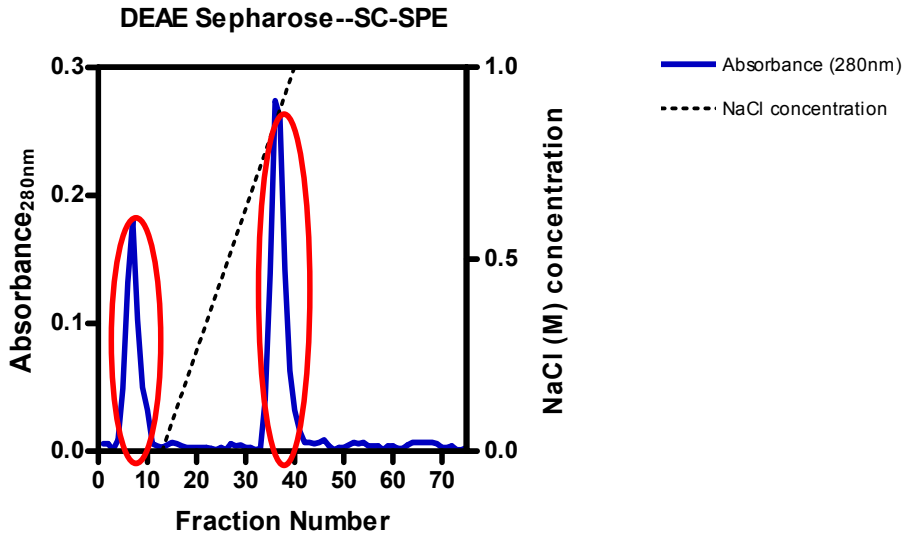


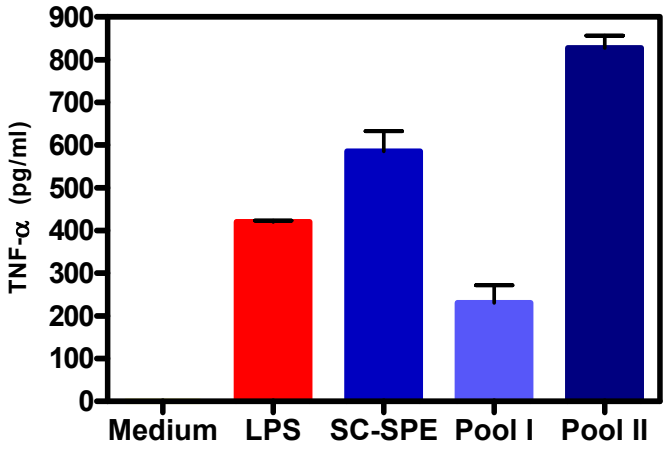
(b)

Figure 6: Fractionation of SC-SPE by HPLC and identification of TNF-α inducing activity of fractions in HPBL. (a) SC-SPE was subjected to RP-HPLC on a C18 column (YMC C18, 250 x 4.6 mm) and was eluted with an acetonitrile gradient (up to 50% during 25 min). Flow rate was 1 ml/min. Fractions were collected in a 96-well plate and pooled for every two minutes retention time in 12 fraction pools (P1-P12). Horizontal bars indicate pools with TNF-α activity. (b) TNF-α activity of pooled fractions P1-P12. Positive control cultures were treated with LPS and SC-SPE starting material and negative control cultures with culture medium alone. P4-P7 exhibited significant cytokine induction when compared to cultures stimulated with medium alone ($p < 0.05$).

In addition to fractionation by HPLC, SC-SPE was also fractionated by applying to a DEAE Sepharose column to separate the TNF-α inducing activity from other components. Two major protein peaks were obtained, a pre-elution peak and a post-elution peak eluting with a linear salt gradient over 30 ml of a 1 M NaCl solution (Figure 7a). The post-elution peak was eluted off the column between 75% and 90% 1 M NaCl concentration, suggesting that components of this peak tightly bound to the anionic exchanger. Fractions within each peak were pooled and were designated pool I and pool II. Pool I and Pool II were estimated to contain approximately 570 μg/ml and 552 μg/ml protein, respectively. Pool II was dialyzed against ddH_2O overnight at 4^oC to remove high NaCl concentration and concentrated by centrifugation (Centriplus 3,500 MWCO) prior to use as a stimulant of HPBL. Pool I was also dialyzed against ddH_2O and similarly concentrated prior to stimulating cell cultures. Using the standard assay, HPBL were stimulated with pool I, pool II, SC-SPE, LPS, and culture medium alone. Culture supernatants were harvested after 24 hours and assayed for TNF-α released by stimulated cells. Pool II induced significant levels of cytokine when compared to cultures treated with medium alone (Figure 7b). Pool I also induced TNF-α, but at a level significantly less than that induced by either SC-SPE (starting material) or Pool II. It appears that chromatography on DEAE Sepharose served to separate two potentially different bioactive components, a cationic and an anionic component(s). The level of TNF-α induced by Pool II was three fold higher than that induced by Pool I ($p < 0.02$). For this reason, Pool II was selected for further isolation and characterization.



(a)



(b)

Figure 7: Fractionation of SC-SPE on DEAE Sepharose and TNF-α inducing activity of pooled fractions. (a) SC-SPE (2 ml) was applied to a DEAE Sepharose column (6 x 2 cm) equilibrated with 0.02M Tris-HCl buffer, pH 7.5 at a flow rate of 1 ml/min using the ACTA prime FPLC system. Proteins were eluted with a 0-100% 1M NaCl gradient. Protein in each fraction was estimated by measurement of absorbance at 280 nm. Peak fractions were pooled (circled) into Pool I and Pool II. (b) Each pool was assayed for ability to induce production of TNF-α by HPBL. HPBL (2.5×10^5 cells/ml) were stimulated with SC-SPE (starting material), pool I, pool II, LPS (positive control), or medium alone (negative control) and culture supernatants assayed for TNF-α. Pool II induced significantly higher level of cytokine production when compared to pool I ($p < 0.02$).

The TNF-α inducing activity in Pool II was also higher than that of the starting material (SC-SPE) indicating that ion exchange chromatography not only separated component(s) based on differences in charge and most likely molecular size, it also concentrated the specific activity in Pool II. However, from the activity profile recovered following ion exchange chromatography, one must also consider that the increased activity of Pool II may also be the result of separation and removal of inhibitors of activity that might be present in the starting material. Total specific activity and yield obtained at different steps of the isolation procedure are summarized in Table 3. Activity was purified four-fold with a 1.45 increase in specific activity from starting material, although only 4% was recovered.

Table 3: Specific activity and yield in the isolation and purification of TNF-α inducing component(s). Different preparations of SC were compared by calculating specific activity using the total activity (cytokine induction) and the total protein content at each stage in purification. The final yield was 4% and the purification obtained in pool II was 4-fold.

Sample	Volume (ml)	Total TNF-α inducing activity (pg)	% Yield	Total Protein Content (μg)	Specific Activity	Purification
SCAE	250	125,000	100	143,750	0.87	
SC-SPE	25	14,638	12	37,500	0.39	0.46
DEAE—Pool I	7	1,400	10	3,990*	0.35	0.88
DEAE—Pool II	7	5,600	4	3,860*	1.45	4.14

Protein analysis of SC samples

SC-SPE was analyzed under reducing and non-reducing conditions by SDS-PAGE initially using a 10-20% Tris-Tricine gradient gel (BioRad ready-made gel) where multiple bands were observed, but the resolution of bands was poor (Figure 8). To obtain better resolution, following fractionation of SC-SPE on DEAE Sepharose, Pool I and Pool II were analyzed on a 16% Tris-HCl gel (BioRad ready-made gel). Pool I contained multiple protein bands ranging in molecular size from 14 to 103 kDa. Pool II appeared to contain significantly fewer bands, possibly only one major protein band, although it was difficult to determine due to streaking in the lanes (Figure 9).

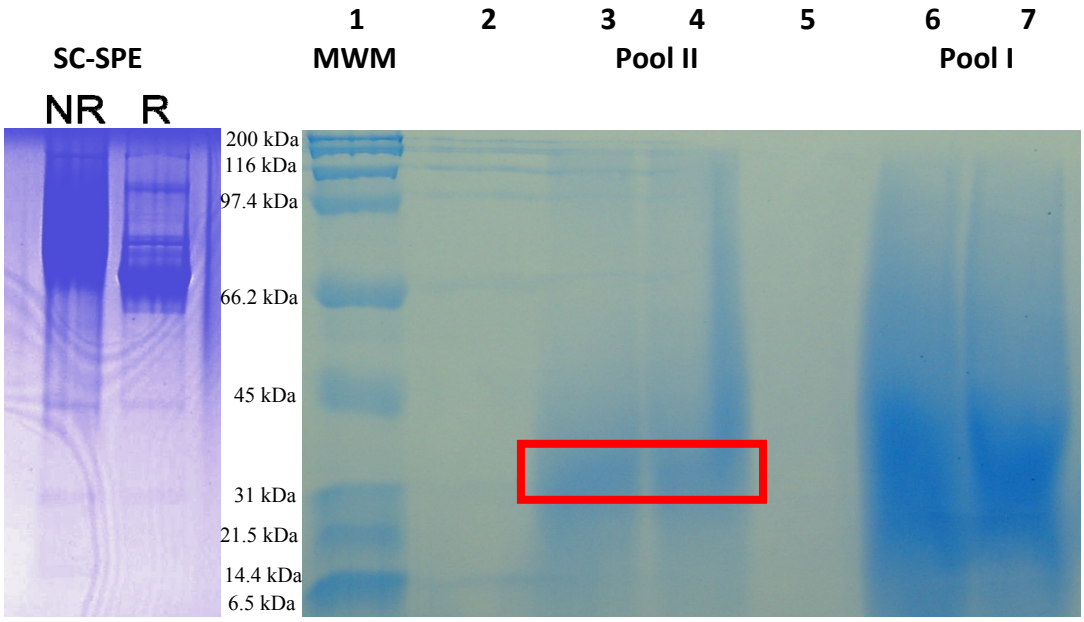


Figure 8 (left panel): SDS-PAGE analysis of SC-SPE under reducing and non-reducing conditions. SC-SPE run on 10-20% gradient Tris-Tricine gel (BioRad) under both non-reducing (NR) and reducing (R) conditions and stained with Coomassie blue to visualize protein bands.

Figure 9 (right panel): SDS-PAGE analysis of pooled material recovered from SC-SPE fractionated on DEAE Sepharose. Pool I and Pool II run on 16% Tris-HCl ready-made gel and stained with Coomassie blue. Molecular weight marker was run in lane 1. Pool II was run in lanes 3 and 4. Pool I was run in lanes 6 and 7. Lanes 2 and 5 contained only sample buffer. The putative protein band(s) in Pool II are highlighted in red.

Since pool II of DEAE Sepharose-fractionated SC-SPE exhibited a high level of TNF-α inducing activity, resolving the major protein band further was undertaken. Pool II was subjected to SDS-PAGE analysis on a 10% gel. A relatively broad yet distinct protein band (~14-21 kDa) was visible when the sample was run under non-reducing conditions (Figure 10).

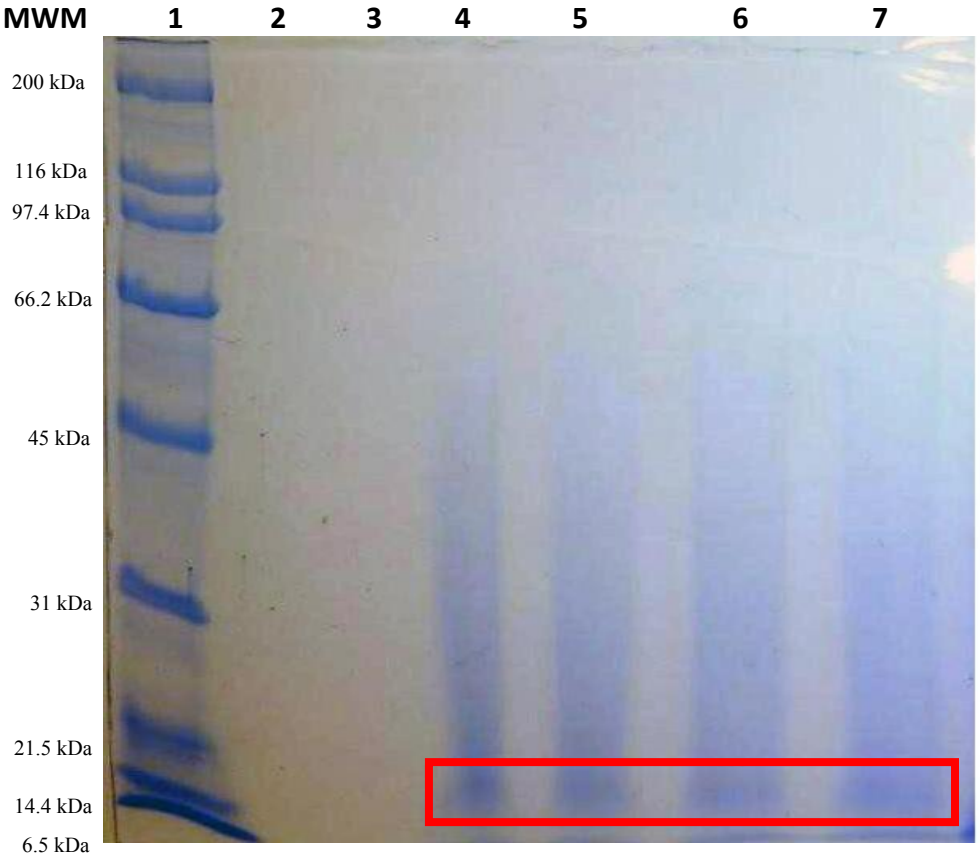


Figure 10: SDS-PAGE analysis of DEAE Sepharose Pool II. Pool II was subjected to SDS-PAGE under non-reducing conditions on a 10% Tris-HCl gel and stained with Coomassie blue. Molecular weight standards were run in lane 1. Duplicate samples of Pool II were run in lanes 4-7. The protein band in Pool II is highlighted in red.

To determine whether the prominent protein band represented the bioactive protein in pool II that was responsible for TNF-α induction, the sample was re-run on a 12% gel that was reverse stained using E-Zinc to visualize bands for excision and bioactivity was assayed. Pool II was applied to four lanes on the gel to maximize the amount of recoverable protein from the gel. Protein band(s) at approximately 14-21 kDa were excised and protein was passively eluted from the gel. In addition, similar bands from the region of the gel that did not stain for protein were also eluted as controls. Eluted protein material recovered from excised bands was used to stimulate HPBL. Control cultures were stimulated with material recovered from gel regions that did not stain for protein. Culture supernatants from stimulated HPBL were assayed for TNF-α (Figure 11).

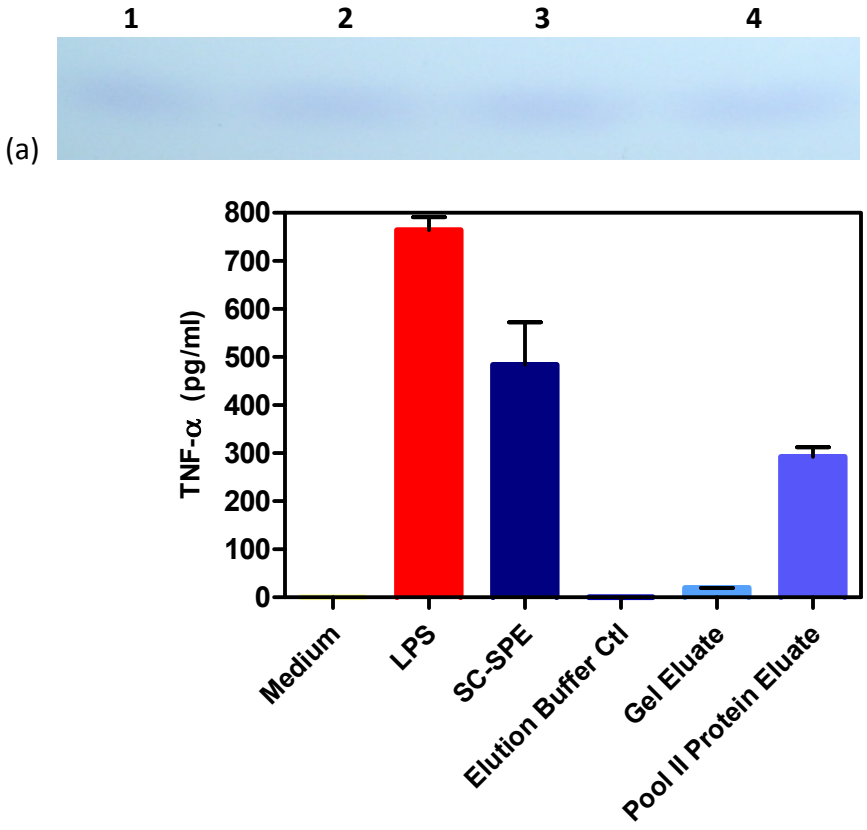


Figure 11: TNF-α produced by HPBL stimulated with material eluted from protein band(s) separated by SDS-PAGE. (a) Pool II was run on a 12% Tris-HCl gel under non-reducing conditions in four lanes (1, 2, 3, and 4). Gel was reverse stained to visualize bands (clear bands against opaque, white gel). Protein bands were excised and passively eluted. Material eluted from additional bands excised from area of the gel that showed no protein served as controls. (b) Following passive elution, HPBL were stimulated over 24 hours with culture medium, LPS, SC-SPE, elution buffer, and eluates from pool II protein band and excised gel. Culture supernatants were assayed for TNF-α. Eluate from pool II protein band induced significant level of TNF-α compared to cultures stimulated with medium alone. There was no significant TNF-α induction in cultures stimulated with elution buffer alone or with gel eluate controls ($p < 0.01$).

Carbohydrate analysis of SC fraction pools

The prominent protein band seen in SDS-PAGE gels of pool II recovered from DEAE Sepharose was further analyzed for carbohydrate content using a glycoprotein staining kit. Pool II was run on a 10% SDS-PAGE gel under non-reducing conditions and

gels were fixed and stained. Horseradish peroxidase was simultaneously run and served as a positive control for carbohydrate staining. Soybean trypsin inhibitor served as a negative control for carbohydrate and showed no protein bands when stained. Carbohydrate staining protein bands were seen in all lanes containing Pool II, under both non-reducing and reducing conditions (Figure 12). A putative glycoprotein band was observed at 14-21 kDa upon glycoprotein staining, corresponding to the bioactive protein band identified in coomassie-stained gels and from which the eluate stimulated leukocyte cultures to produce TNF-α.

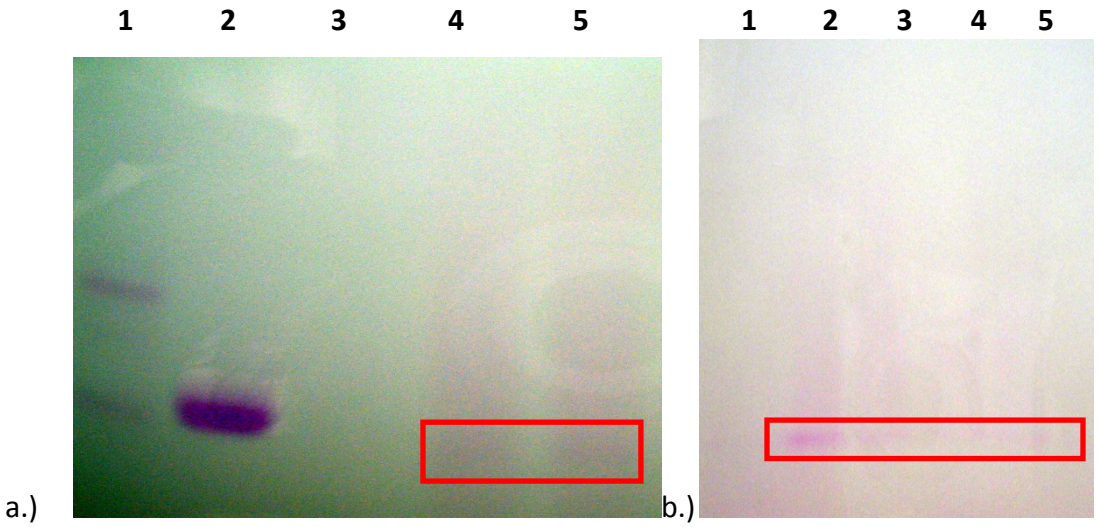


Figure 12: SDS-PAGE analysis of pool II stained with GelCode glycoprotein stain. (a) Pool II was run in lanes 4 and 5 on a 10% Tris-HCl gel under non-reducing (NR) conditions. Soybean trypsin inhibitor was run in lane 3 as a negative control. Horseradish peroxidase was run in lane 2 as a positive control. A ladder of carbohydrate standards was run in lane 1. (b) Initially, Pool II sample was run without a positive control on a 10% gel under both non-reducing (lanes 2-3) and reducing (lanes 4-5) conditions. Staining was observed more clearly in non-reduced samples. Putative bands are highlighted in red.

Pool II was analyzed for total carbohydrate content using a colorimetric estimation kit. The standard curve was constructed using lysozyme and bovine serum albumin (negative for CHO content) and a series of positive controls: ovalbumin, human apotransferrin, fetuin, and α_1-acid glycoprotein. The standard curve underwent linear regression to determine the CHO content of unknown samples (Table 4). The SC-SPE starting material was estimated to contain between 5 and 15 % carbohydrate based on the standard curve. Colorimetric results also indicated that the putative glycoprotein in Pool II contained at least 40% CHO content, although the exact CHO content (in percentage) could not be determined since the measurement fell outside the range of the standard curve.

Table 4: Estimated carbohydrate content of shark cartilage extracts. Shark cartilage extract sample, SC-SPE, was tested for carbohydrate content along with pool II from DEAE Sepharose column. Carbohydrate content was determined using a standard curve constructed from the following standards: lysozyme (2.5 mg/ml), bovine serum albumin (2.5 mg/ml), ovalbumin (2.5 mg/ml), apo-tranferrin (2.5 mg/ml), fetuin (0.25 mg/ml), and alpha acid glycoprotein (0.25 mg/ml). Standards run at 0.25 mg/ml were multiplied by ten. Linear regression yielded an R^2 value of 0.9907. Pool II exhibited CHO content outside the standard curve. Based on the curve, it is estimated that Pool II contains at least 40% CHO content.

Standard or Sample	**Mean Absorbance**	**CHO Content (%)**
Lysozyme	0.109	0
Bovine Serum Albumin	0.136	Trace amounts
Ovalbumin	0.254	3.2
Apo-Transferin	0.846	5.8
Fetuin	0.313 (10x)	22.9
Alpha Acid Glycoprotein	0.355 (10x)	41.4
SC-SPE (0.25 mg/ml and 2.5 mg/ml)	0.791/2.143	**5-15**
DEAE Sepharose Pool II	Over limit 3.5	**> 40**

Spectrum of cellular responses to shark cartilage

Leukocyte-specific responses

Leukocyte populations enriched for specific leukocytes were stimulated with shark cartilage to determine which populations of leukocytes are primary responders to SC directly and to show whether there are secondary leukocyte responses to mediators released in culture after initial stimulation of cultures. Peripheral blood mononuclear cell (PBMC) fraction of whole blood was further separated into monocyte/macrophage and lymphocyte populations (Figure 13). Each leukocyte population (exhibiting at least 95% viability) was stimulated with either SC-SPE, LPS (5 μg/ml), or medium alone over 96 hours. HPBL cultures representing a mixed total leukocyte population were set up in tandem and similarly stimulated for comparison.

HPBL cultures of heterogenous leukocyte populations produced significant levels of TNF-α in response to both LPS and SC-SPE (Figure 14a), similar to the results obtained in previous studies. Cultures of the adherent monocyte/macrophage-enriched population also produced significant levels of TNF-α in response to SC-SPE. The level of TNF-α induced was higher than that observed in HPBL cultures (Figure 14b). Cultures of lymphocyte-enriched populations, however, did not produce significant amounts of TNF-α in response to SC-SPE when compared to medium alone, although low levels of TNF-α was produced in response to LPS (Figure 14c). These results suggest the primary responder in a heterogenous population of leukocytes is most likely the monocyte, with the response noted for the lymphocyte-enriched population being a secondary response to mediators released by stimulated monocytes/macrophages.

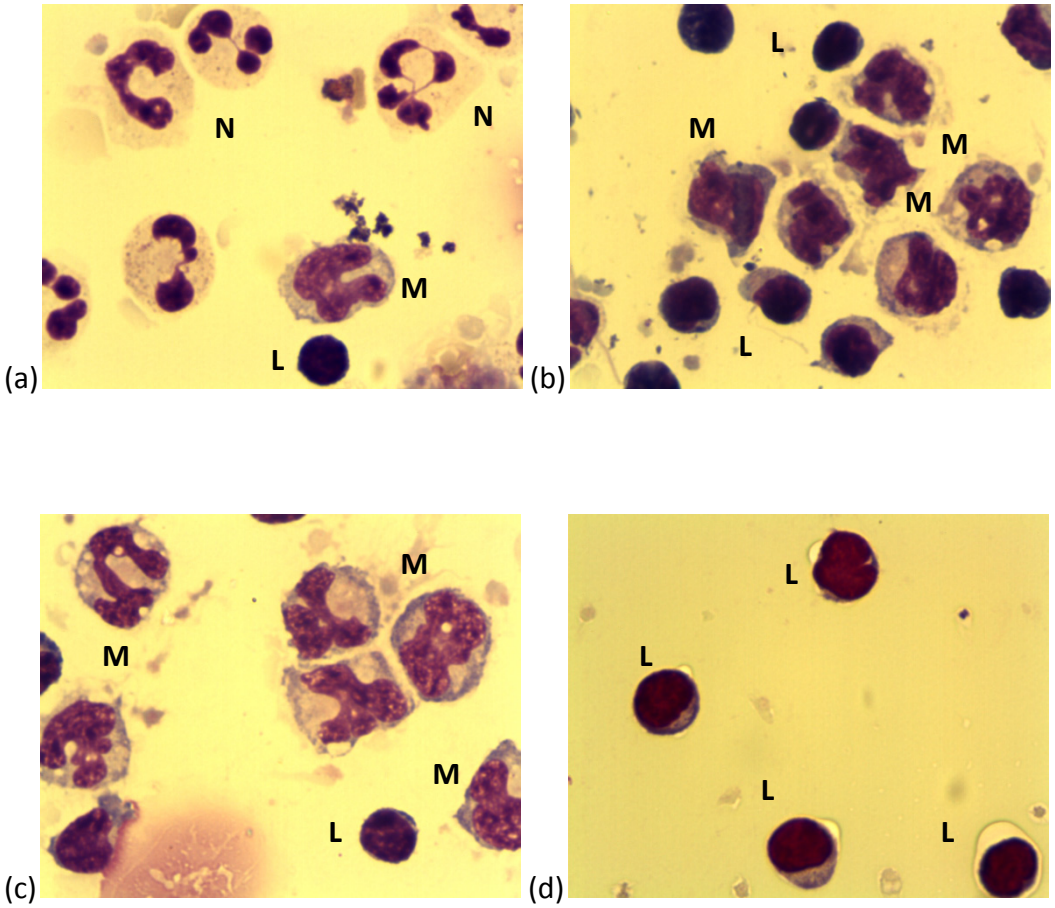
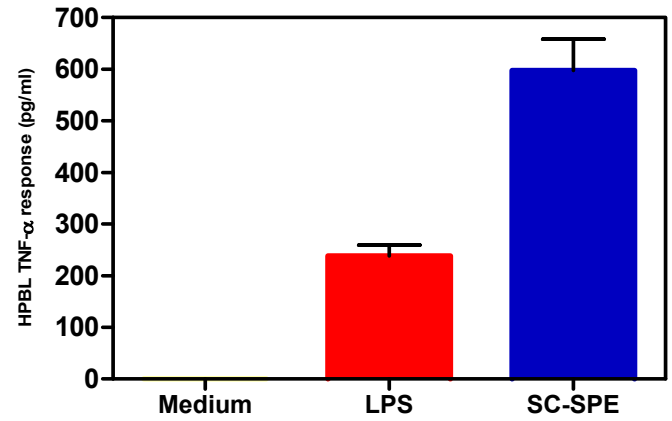
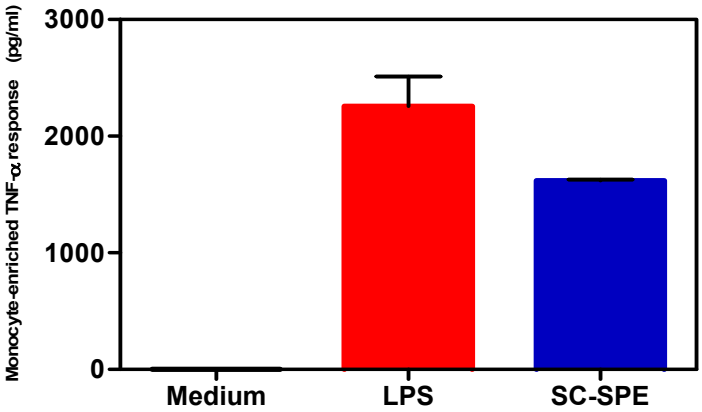


Figure 13: Cytospin monolayers of human leukocyte populations. (a) Heterogenous mixture of several types of leukocytes (HPBL), (b) peripheral blood mononuclear cells (PBMC) prior to separation, (c) monocyte/macrophage-enriched leukocyte population, and (d) lymphocyte-enriched leukocyte population. (**M** = monocyte/macrophage; **L** = lymphocyte; **N** = neutrophil)



(a)



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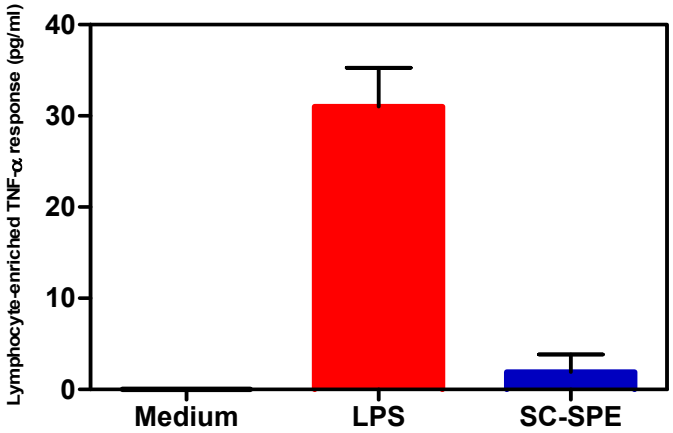


(c)

Figure 14: TNF-α produced by leukocyte populations stimulated with SC-SPE. Leukocyte cultures were stimulated with SC-SPE, LPS, or medium alone over 24 hours. Culture supernatants were assayed for TNF-α. (a) TNF-α response of HPBL cultures, (b) TNF-α response of monocyte-enriched cultures, and (c) TNF-α response of lymphocyte-enriched cultures.

When supernatants from SC-SPE-stimulated monocyte-enriched cultures were assayed for IFN-γ production at 24 and 72 hours no significant level of IFN-γ was detected (results not shown). Similarly, lymphocyte-enriched cultures also did not produce significant levels of IFN-γ in response to SC-SPE stimulation when compared to the responses of positive control cultures, stimulated with PHA and LPS or medium alone (Figure 15). IFN-γ was not produced in lymphocyte-enriched populations in the absence of a primary responders, indicating that the IFN-γ response is likely in response to a mediator produced by a primary responding cell, most likely the monocyte/macrophage.

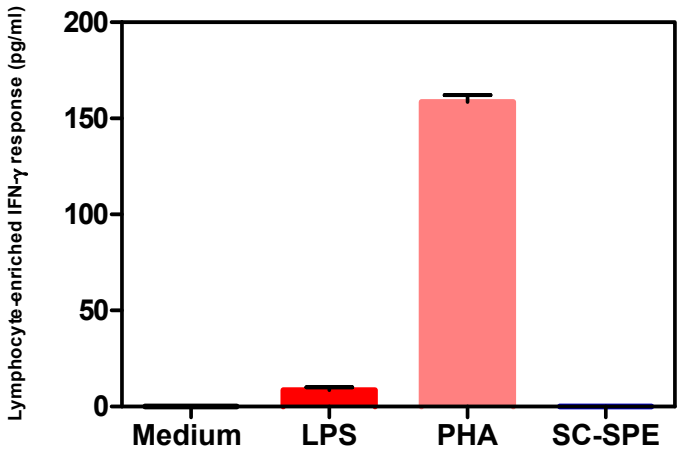


Figure 15: Interferon-γ produced by lymphocyte-enriched cell population stimulated with SC-SPE. IFN-γ production in lymphocyte-enriched population was measured in response to SC-SPE, LPS, PHA, and medium alone. Significant levels of IFN-γ were produced by lymphocytes stimulated by PHA (positive control). IFN-γ was not detected in supernatants of cultures stimulated with SC-SPE at 72 hours.

Chemokine production by PBMC in response to cartilage

To further define the chemokine response to cartilage of mononuclear leukocytes, PBMC were stimulated with crude SCAE over 96 hours and culture supernatants assayed for monocyte-chemoattractant protein-1 and RANTES by ELISA. PBMC cultures produced significant amounts of MCP-1 through 96 hours, at levels significantly higher than that produced in response to either medium alone or LPS (Figure 16). Significant level of MCP-1 was produced within 4 hours and the level continued to rise through 96 hours of stimulation unlike the TNF-α response to LPS which decreased significantly after 24 hours ($p=0.0004$). In contrast, the level of RANTES produced by PBMC for the first 12 hours in both unstimulated (medium alone) and stimulated (SCAE, LPS) cultures was not significantly different and was relatively constant (Figure 17). However, after 24 hours in unstimulated cultures, RANTES levels began to decrease steadily through 96 hours, while levels of RANTES produced by LPS- and SC-stimulated cultures remained elevated ($p = 0.003$).

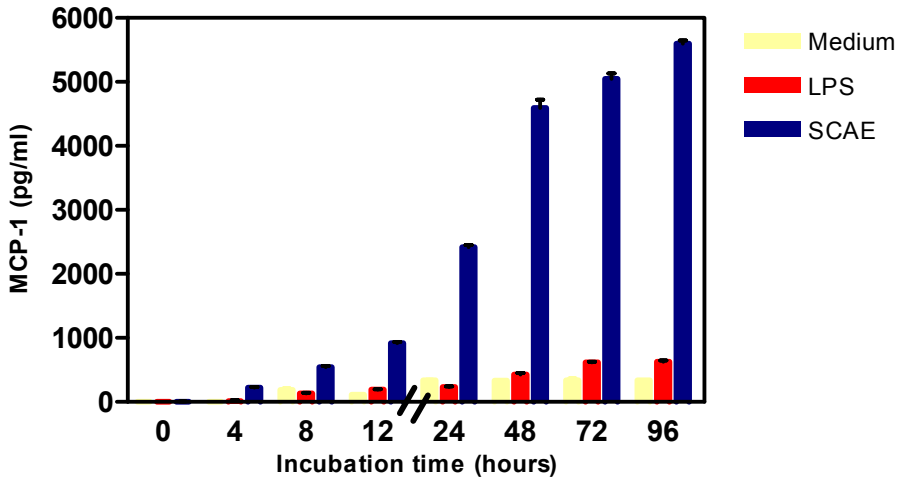


Figure 16: MCP-1 produced by PBMC stimulated with shark cartilage. PBMC (2.5 x 10^5 cells/ml) cultures were stimulated with either SCAE, LPS, or medium alone for 96 hours. Culture supernatants were harvested at 0, 4, 8, 12, 24, 48, 72, and 96 hours following stimulation and assayed for MCP-1 by ELISA. SCAE induced significant levels of MCP-1 production in PBMC at all time points when compared to cultures stimulated with medium alone or LPS ($p < 0.005$).

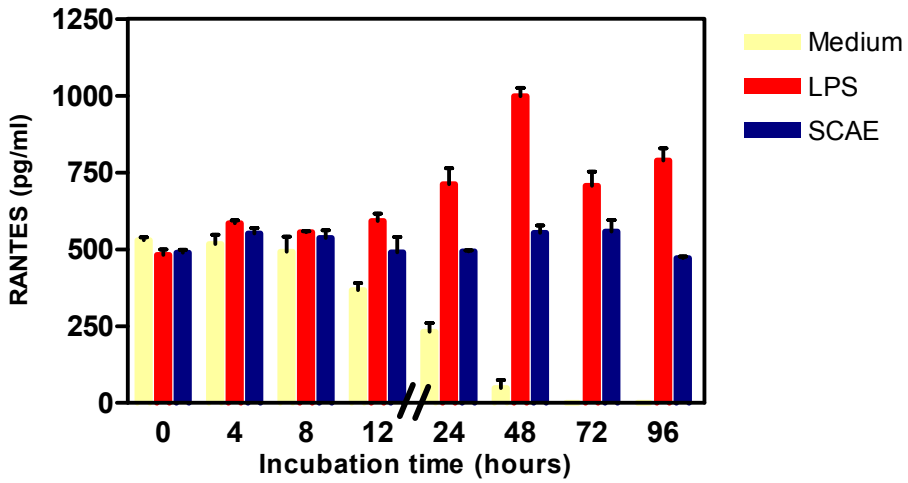


Figure 17: RANTES produced by PBMC stimulated with shark cartilage. PBMC (2.5 x 10^5 cells/ml) cultures were stimulated with either SCAE, LPS, or medium alone for 96 hours. Culture supernatants were harvested at 0, 4, 8, 12, 24, 48, 72, and 96 hours following stimulation and assayed for RANTES by ELISA. There was no significant difference in RANTES production in PBMC through12 hours. However, after 12 hours, the level of RANTES in cultures treated with medium alone (unstimulated) significantly decreased while the response to LPS rose significantly, reaching peak levels at 48 hours. The RANTES response to SC showed very little variation in level over 96 hours and although initially it was not significantly different from treatment with medium alone, it did not decrease over time of experiment ($p < 0.004$).

Cytokine response of stimulated murine macrophages

Murine macrophages (RAW 264.7 cell line) were cultured and used as single cell type targets to investigate cytokine induction following stimulation with SC-SPE. Macrophages were stimulated with either SC-SPE, LPS (5 μg/ml), or culture medium alone for up to 96 hours in culture. To determine whether the macrophage represents a primary responding cell to SC, both early and late responses were investigated. Culture supernatants were harvested at the following time intervals for an early response: 0, 30, 60, 90, 120 minutes, and 4 hours. Supernatants were assayed for the production of TNF-α, IL-1β, IL-12, and IL-6 by ELISA. There was no significant cytokine induction in response to SC-SPE within 4 hours in culture, however, culture supernatants harvested at later time intervals, that is, 0, 6, 12, 18, 24, 36, 48, 60, 72, 84, and 96 hours showed specific cytokine responses. The level of TNF-α produced by macrophages when stimulated with medium alone was significant particularly when compared to the baseline level of the cytokine produced by human heterogenous leukocyte cultures as shown in earlier experiments (Figure 4). TNF-α was detected in medium-stimulated supernatants as early as 6 hours and continued through 96 hours, reaching peak level at 24 hours. In SC-SPE-stimulated cultures, a significant level of TNF-α was induced by 12 hours with level peaking at 24 hours ($p < 0.02$) and the level of TNF-α gradually decreased over 96 hours to a level similar to that induced by medium alone (Figure 18). By contrast, macrophage cultures stimulated with LPS produced TNF-α as early as 2 hours with levels increasing up to 24 hours after which TNF-α levels were maintained through 96 hours ($p < 0.001$).

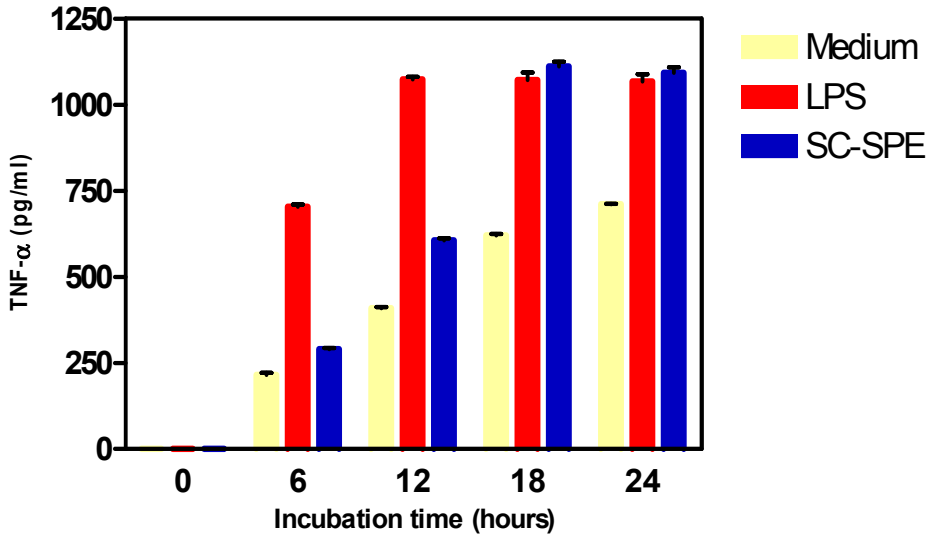
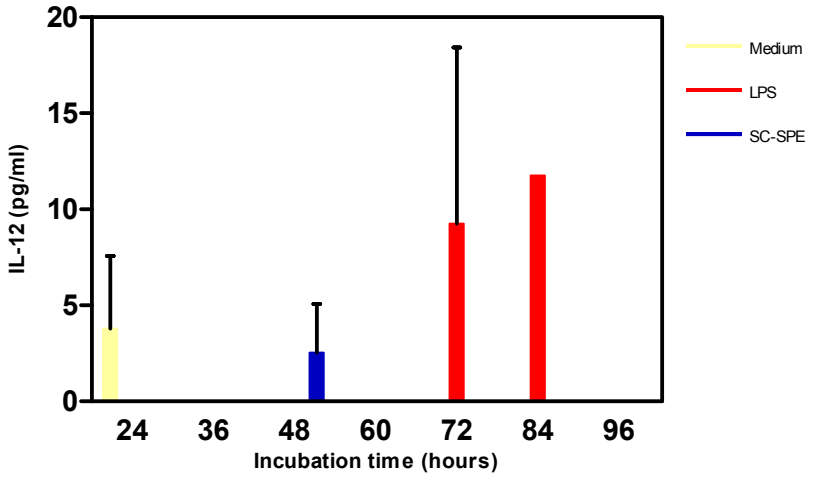
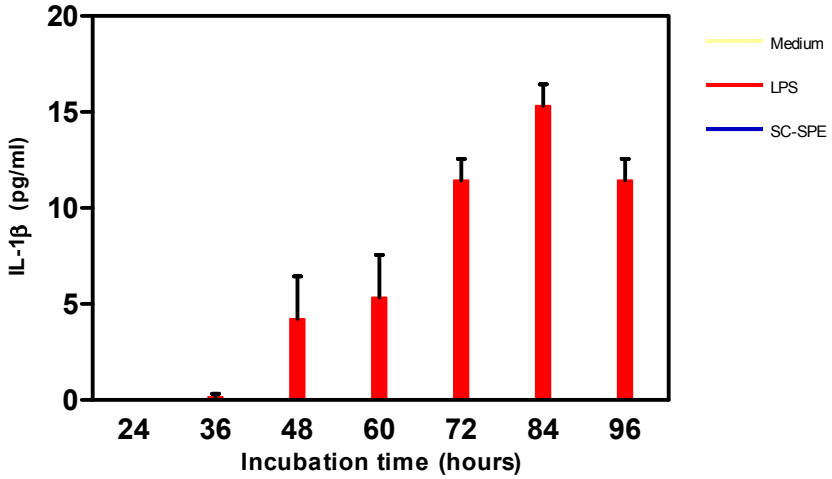


Figure 18: TNF-α produced by RAW 264.7 cells stimulated with SC-SPE. RAW 264.7 murine macrophages (2.5 x 10^5 cells/ml) were cultured and stimulated with either SC-SPE, LPS or medium alone and culture supernatants harvested at 0, 6, 12, 18, 24, 36, 48, 60, 72, 84, and 96 hours. Supernatants were assayed for murine TNF-α by ELISA. SC-SPE induced significant levels of TNF-α production when compared to cells stimulated with medium alone after 12 hours ($p < 0.02$). Macrophages produced peak levels of TNF-α in response to SC-SPE at 24 hours, with levels decreasing gradually between 24 and 96 hours ($p < 0.001$).

Macrophages produced low levels of other cytokines assayed for under the present experimental conditions (Figures 19 and 20). Macrophage cultures did not produce significant levels of IL-12 in response to SC-SPE or LPS, a result which is not unexpected as RAW cells are known to produce low levels of this cytokine (Figure 19a). Furthermore, macrophages stimulated with SC-SPE did not produce significant levels of IL-1β, with only some responses to LPS noted. LPS induced significant levels of IL-1β between 48 and 96 hours when compared to cultures stimulated with SC-SPE or medium alone, although at relatively low levels (< 20 pg/ml) (Figure 19b). LPS also induced significant levels of IL-6 production between 24 and 72 hours (for all time intervals assayed) consistently (Figure 20a). If the level of IL-6 production for SC-SPE and medium alone (without the high levels produced in response to LPS included in the figure) is analyzed, a low level of induction was observed for SC-SPE-stimulated cultures at 24 and 36 hours. The level of IL-6 detected, however, was significantly lower than that produced in response to LPS. Between 48 and 96 hours, the level of IL-6 produced in response to medium alone was actually higher than that produced in response to SC-SPE, although there were significant standard deviations between samples (Figure 20b). SC-SPE did not induce significant production of IL-12, IL-1β, or IL-6 in macrophage cultures.

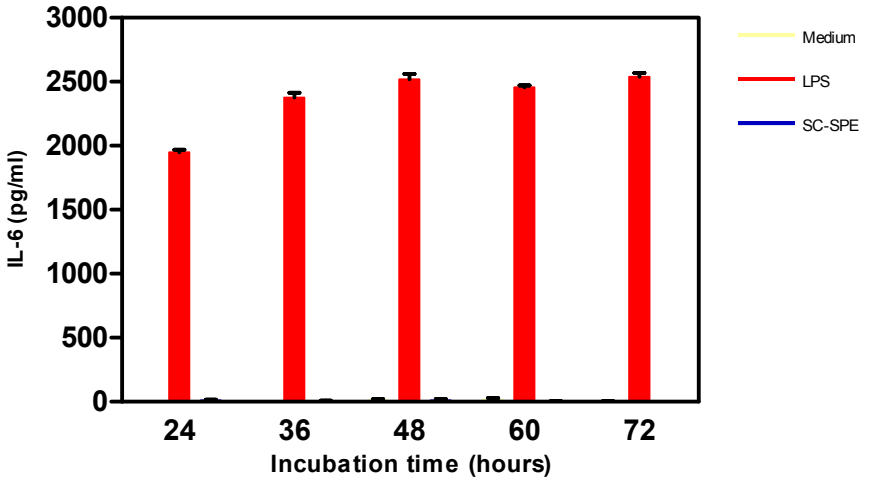


(a)

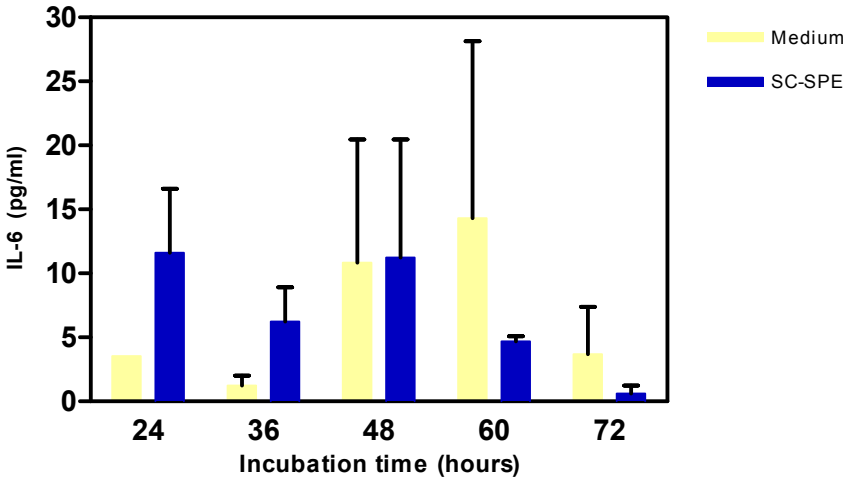


(b)

Figure 19: IL-12 and IL-1β produced by RAW 264.7 cells stimulated with SC-SPE. RAW 264.7 murine macrophages (2.5 x 10^5 cells/ml) were cultured and stimulated with either SC-SPE, LPS, or medium alone over 96 hours and culture supernatants harvested at 24, 36, 48, 60, 72, 84, and 96 hours. Supernatants were assayed for murine IL-12 and IL-1β by ELISA. (a) There was no significant induction of IL-12 production in RAW cells under these experimental conditions. (b) LPS induced significant levels of IL-1β production in RAW cells when compared to cells stimulated with medium alone between 48 and 96 hours in culture, SC-SPE did not induce the production of either IL-12 or IL-1β.



(a)



(b)

Figure 20: IL-6 produced by RAW 264.7 cells stimulated with SC-SPE. RAW 264.7 murine macrophages (2.5×10^5 cells/ml) were cultured and stimulated with either SC-SPE, LPS, or medium alone over 72 hours and culture supernatants harvested at 24, 36, 48, 60, and 72 hours. Supernatants were assayed for murine IL-6 by ELISA. (a) LPS induced significant levels of IL-6 production in RAW cells when compared to cells stimulated with medium alone between 24 and 72 hours in culture. (b) SC-SPE induced the production of IL-6 at 24 and 36 hours but not at significant levels.

Murine macrophage nitric oxide production

Murine macrophages (RAW 264.7 cell line) were cultured and stimulated with either SC-SPE, LPS, or medium alone for 24 hours. Macrophages were harvested and fixed to microscope slides, stained, and viewed for inducible nitric oxide synthase (iNOS) production by fluorescence microscopy. RAW cells when stimulated with SC-SPE induced significant levels of nitric oxide when compared to cells stimulated with medium alone (Figure 21a). LPS failed to stimulate cells to produce NO (results not shown). However, a combination of LPS and IFN-γ as a stimulant induced a significant amount of nitric oxide as shown by the increased cytoplasmic staining when compared to cells stimulated with SC-SPE or medium alone (Figure 21b). Nitric oxide was not induced by medium alone (Figure 21c).

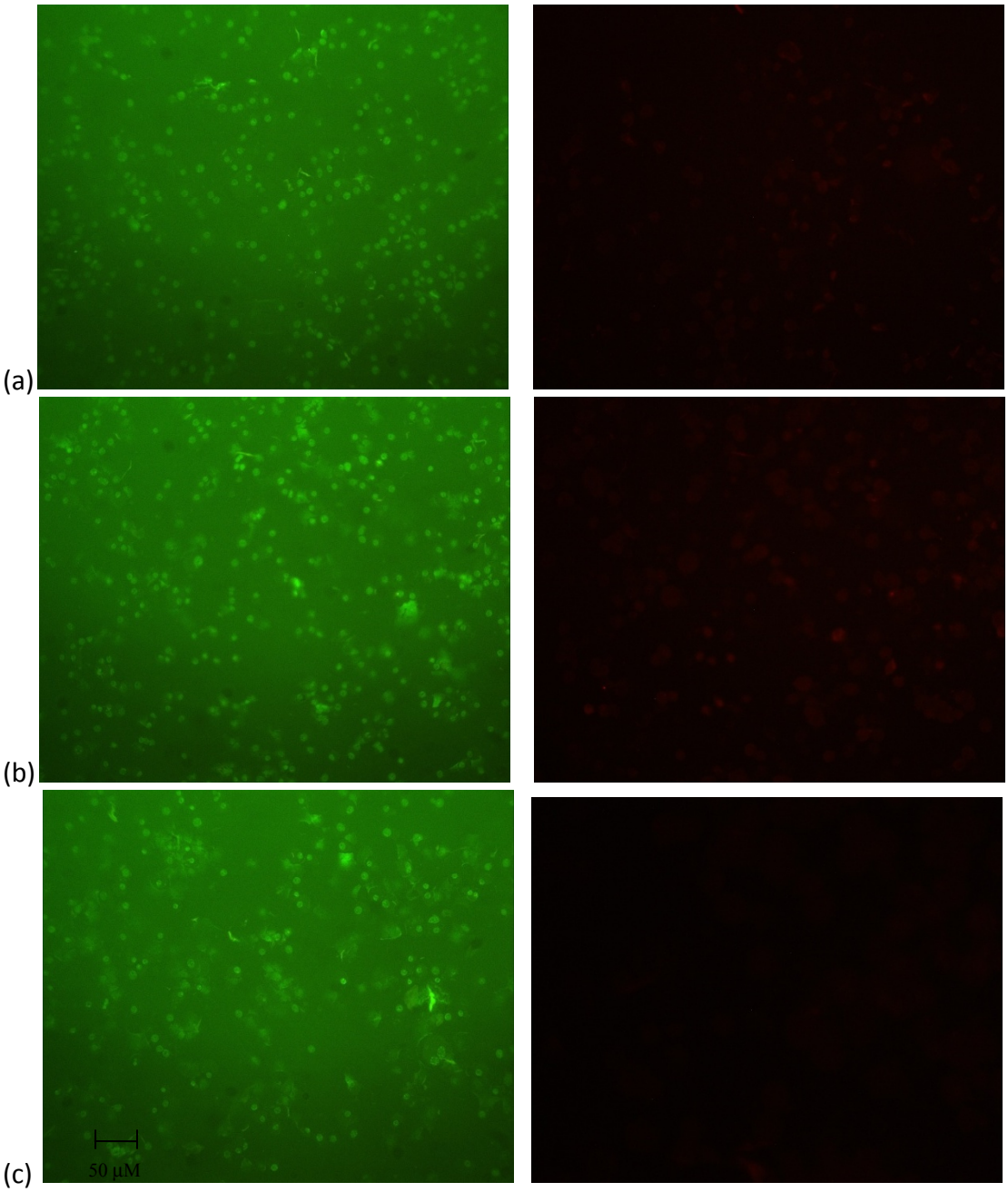


Figure 21: Nitric oxide (NO) produced in RAW 264.7 cells stimulated with SC-SPE. RAW cells were stimulated with either SC-SPE, LPS/IFN-γ, or medium alone and then stained for cytoplasmic iNOS. Left panels are corresponding images of nuclear stains (Hoescht dye) for each treatment and right panels are representative images of NO cytoplasmic stains for each treatment. (a) SC-SPE induced NO production in RAW cells comparable to that observed for cells stimulated with LPS/IFN-γ, but not those stimulated with LPS alone. (b) RAW cells stimulated with a combination of LPS (5 µg/ml) and IFN-γ (2.5 ng/ml) as a positive control exhibited significant NO production. (c) There was no induction of NO production in RAW cells stimulated with medium alone.

Mechanism of cellular activation and signal transduction

Role of TLR receptors in cartilage stimulation

The role of Toll-like receptors 2 and 4 in the stimulation of leukocytes by shark cartilage was investigated by treating cultured HPBL with blocking antibodies prior to stimulation. Control cultures consisted of un-stimulated (pre-treated and stimulated with medium alone) cells and cells pre-treated with isotype control antibodies. The response of leukocytes pre-treated with isotype control antibodies was not significantly different than that of leukocytes pre-treated with medium alone. When TLR-2 receptor was blocked by pre-treatment with specific anti-TLR 2 antibody, leukocytes stimulated with either LPS or SC-SPE produced a TNF-α response not significantly different from that observed in cells pretreated with medium in lieu of antibody ($p < 0.05$). Thus, blocking TLR-2 ligand binding activity did not inhibit the production of TNF-α in response to SC-SPE significantly, although there was a slight reduction in the level of TNF-α produced (Figure 22). LPS did not serve as a good positive control for blocking TLR 2, since the major ligand for TLR 2 is peptidoglycan, not LPS. The results for the TLR 2 experiment, therefore, remain difficult to interpret given the lack of an appropriate positive control.

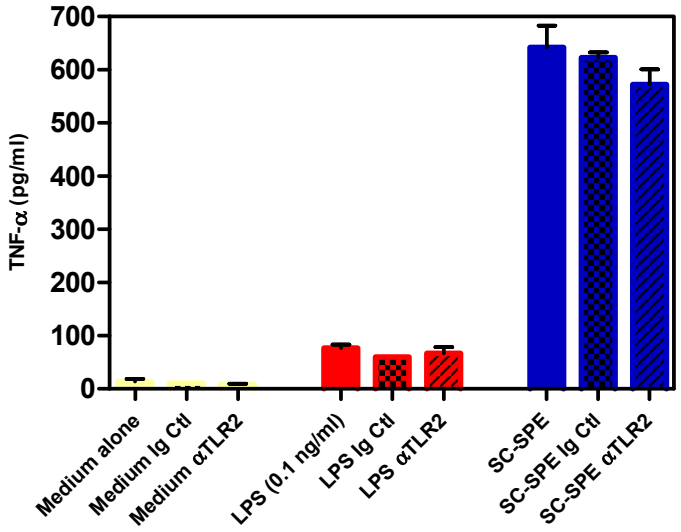


Figure 22: TNF-α produced by HPBL stimulated with SC-SPE following pretreatment with blocking antibody against TLR-2. HPBL (2.5 x 10^5 cells/ml) cultures were pre-treated with either Ig isotype controls or anti-TLR-2 blocking antibodies prior to stimulation over 24 hours with SC-SPE, LPS (0.1 ng/ml), or medium alone. No significant difference in TNF-α production was observed in cultures where the TLR2 receptor was blocked with antibody and those pre-treated with the Ig isotype control antibody or medium alone ($p < 0.05$).

In HPBL cultures, when TLR-4 receptor was blocked with specific antibody, a significant reduction in the TNF-α response was seen when cultures were stimulated with either LPS (0.1 ng/ml) or SC-SPE. The TNF-α response to LPS stimulation was inhibited almost entirely when the TLR-4 receptor was blocked ($p = 0.019$). In SC-SPE-stimulated leukocytes, the level of TNF-α was reduced by over 50% when the TLR 4 receptor was blocked, indicating that TLR4 may be involved in surface recognition of an active component(s) in SC-SPE as a ligand ($p = 0.013$; Figure 23).

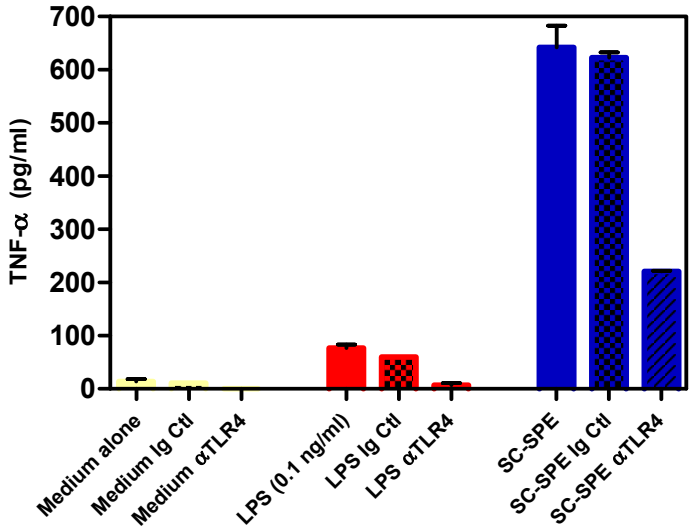


Figure 23: TNF-α produced by HPBL stimulated with SC-SPE following pretreatment with blocking antibody against TLR-4. HPBL (2.5×10^5 cells/ml) cultures were pre-treated with either Ig isotype controls or anti-TLR-4 blocking antibodies prior to stimulation over 24 hours with SC-SPE, LPS (0.1 ng/ml), or medium alone. Blocking of TLR-4 receptor with antibody diminished TNF-α production in LPS- and SC-SPE-stimulated by more than 50%, suggesting that active components in SC-SPE may act via TLR-4 ($p < 0.02$).

Effect of cartilage stimulation on intracellular signal transduction

The involvement of intracellular kinase pathways in the induction of TNF-α by cartilage stimulated cells was investigated employing a variety of specific inhibitor molecules that interfere with intracellular signaling by blocking specific activation pathways. The Jun Kinase (JNK) pathway was blocked using BI 78D3. At low concentrations, no significant difference in the cytokine response of HPBL was seen between cultures pre-treated with inhibitor or those pre-treated with medium alone or a DMSO/PBS vehicle control. At high concentrations of BI 78D3, however, TNF-α production was completely inhibited in both LPS- and SC-SPE-stimulated leukocytes, suggesting that JNK may be an important intracellular signal transduction mediator for TNF-α induction by SC-SPE ($p < 0.02$; Figure 24).

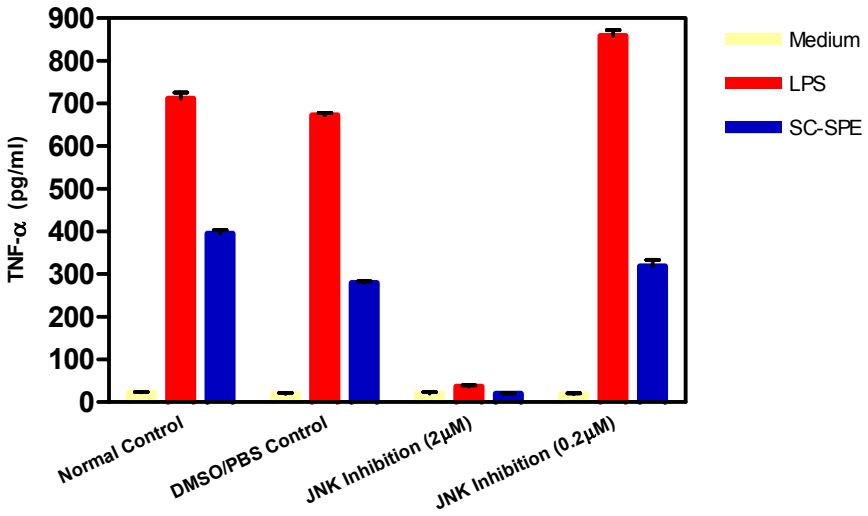


Figure 24: TNF-α produced by HPBL following inhibition of the JNK intracellular pathway. HPBL (2.5×10^5 cells/ml) were pre-treated with BI 78D3, medium, or DMSO/PBS control for 1 hour prior to stimulation with either SC-SPE, LPS, or medium alone for 24 hours. Culture supernatants were assayed for TNF-α by ELISA. At high concentrations (2 μM), there was complete inhibition of TNF-α production in HPBL ($p < 0.02$).

The Phosphoinostidol-3 kinase (PI-3) pathway was blocked by pre-treating cultures with Wortmannin before stimulation. Culture supernatants were assayed for TNF-α production. When PI-3 kinase was blocked with either low or high concentrations of Wortmannin, TNF-α production was not inhibited in SC-SPE- and LPS-stimulated cultures. In LPS-stimulated cultures, when PI-3 kinase was blocked, the level of TNF-α induced was similar to control cultures pre-treated with DMSO vehicle control. However, blocking the PI-3 kinase pathway in SC-SPE-stimulated cultures appears to augment the cytokine response since a significantly higher level of TNF-α was produced in such treated cultures when compared to pre-treated control cultures ($p < 0.002$; Figure 25).

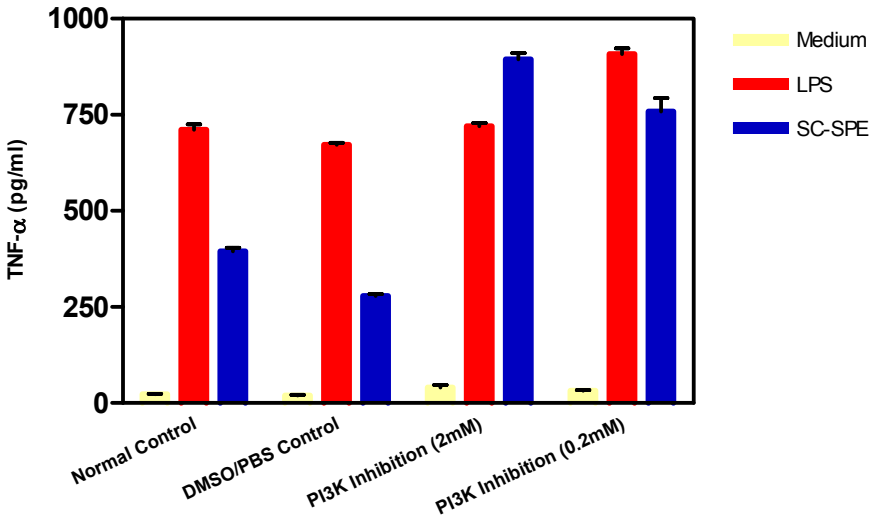


Figure 25: TNF-α produced by HPBL following inhibition of the PI-3 Kinase intracellular pathway. HPBL (2.5×10^5 cells/ml) were pre-treated with Wortmannin, medium, or DMSO/PBS control for 1 hour prior to stimulation with either SC-SPE, LPS, or medium alone for 24 hours. Culture supernatants were assayed for TNF-α by ELISA. At both high and low concentrations (2 μM), there was no inhibition of TNF-α production by blocking PI-3 kinase in HPBL. There was higher level of cytokine production in cells where PI-3 kinase was inhibited, particularly for cultures stimulated with SC-SPE ($p < 0.002$).

Rottlerin was used to block the Protein Kinase C (PKC) pathway. Supernatants from treated cultures were assayed for TNF-α production. At both low and high concentrations, a significant reduction in the amount of TNF-α produced by LPS-stimulated leukocytes was observed when compared to pre-treated leukocyte controls ($p = 0.019$). In SC-SPE-stimulated leukocytes, there was no significant difference in the TNF-α response when PKC was blocked. At high concentrations, there was a slight reduction when compared to pre-treated leukocyte controls, but the results indicate that PKC is likely to be involved as an important mediator of the response to LPS, but not as critical to the cytokine response to SC-SPE (Figure 26).

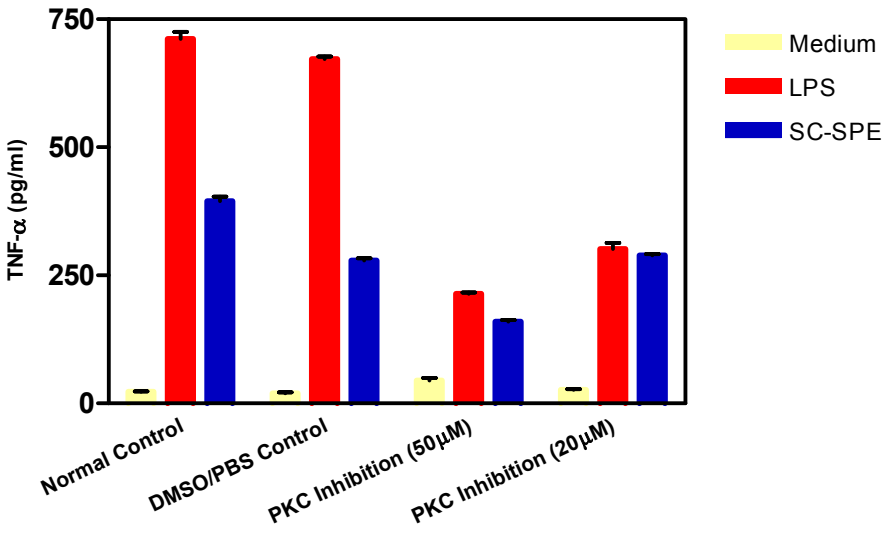


Figure 26: TNF-α produced by HPBL following inhibition of the Protein Kinase C intracellular pathway. HPBL (2.5×10^5 cells/ml) were pre-treated with Rottlerin, medium, or DMSO/PBS control for 1 hour prior to stimulation with either SC-SPE, LPS, or medium alone for 24 hours. Culture supernatants were assayed for TNF-α by ELISA. At both high and low concentrations, TNF-α production was significantly inhibited in response to LPS when PKC was blocked ($p < 0.02$). There was no significant inhibition of TNF-α production in response to SC-SPE by blocking PKC in HPBL.

The p38 MAPK kinase pathway was blocked using SB202190 prior to stimulating HPBL cultures. Culture supernatants were assayed for TNF-α production following treatment. The TNF-α response in both LPS- and SC-SPE-stimulated leukocytes was significantly reduced ($p < 0.05$). The lowest concentration of inhibitor was enough to almost entirely block the TNF-α response to LPS and SC-SPE, suggesting that p38 MAPK is a critical mediator in TNF-α response to cartilage (Figure 27). However, when another member of the MAPK family of kinases, the Raf/MEK/ERK2 pathway, was blocked with GW 5074 no significant difference in the TNF-α inducing activity of either LPS or SC-SPE was noted. The results suggest that inhibition of the Raf/MEK/ERK2 pathway does not affect the ability of leukocytes to produce TNF-α in response to cartilage stimulation (Figure 28).

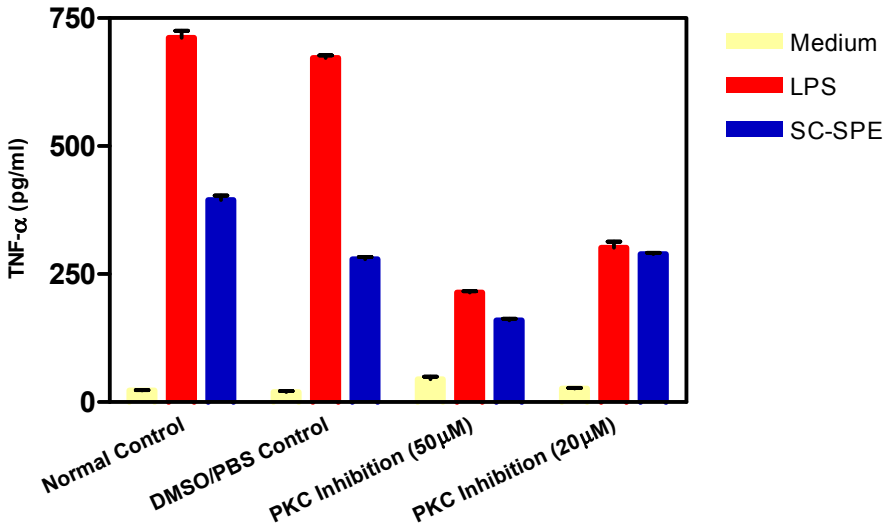


Figure 27: TNF-α produced by HPBL following inhibition of the p38 MAPK kinase intracellular pathway. HPBL (2.5×10^5 cells/ml) were pre-treated with SB202190, medium, or DMSO/PBS control for 1 hour prior to stimulation with either SC-SPE, LPS, or medium alone for 24 hours. Culture supernatants were assayed for TNF-α by ELISA. TNF-α production was significantly inhibited in response to both LPS and SC-SPE ($p < 0.05$).

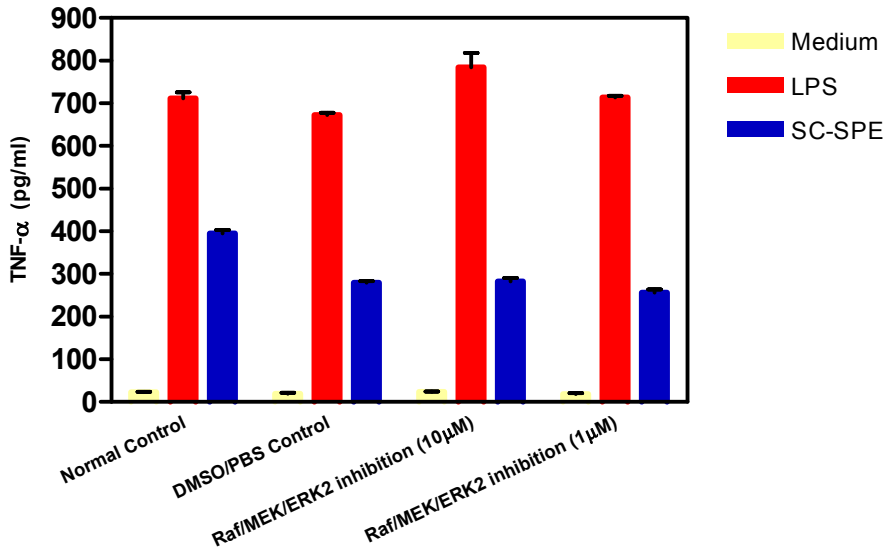
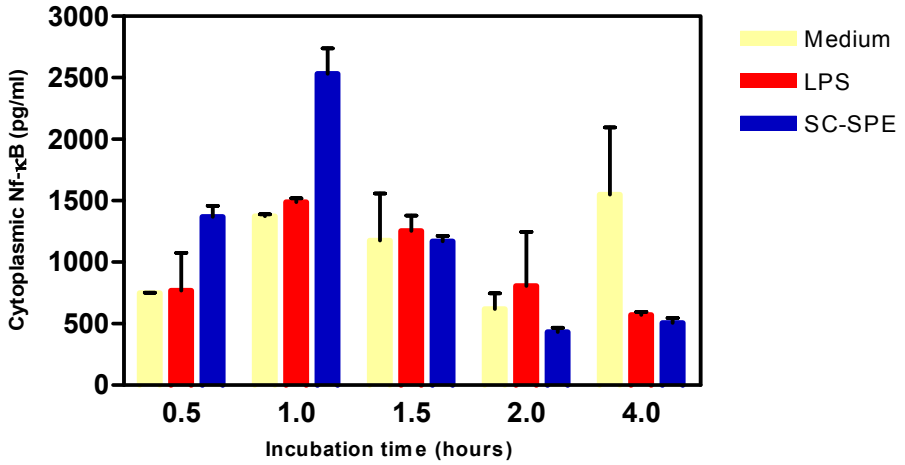


Figure 28: TNF-α produced by HPBL following inhibition of the Raf/MEK/ERK2 MAPK kinase intracellular pathway. HPBL (2.5 x 10^5 cells/ml) were pre-treated with GW 5074, medium, or DMSO/PBS control for 1 hour prior to stimulation with either SC-SPE, LPS, or medium alone for 24 hours. Culture supernatants were assayed for TNF-α by ELISA. At both high and low concentrations, TNF-α production was not inhibited in response to either LPS or SC-SPE when the Raf/MEK/ERK2 MAPK kinase pathway was blocked.

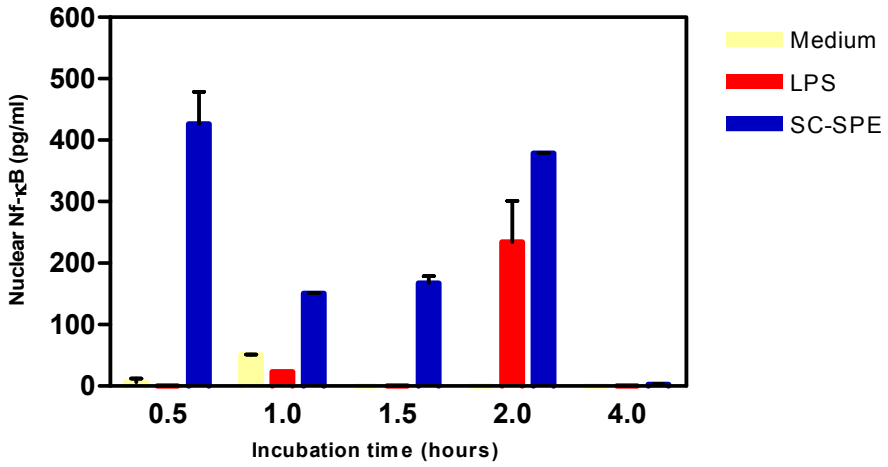
Effect of cartilage stimulation on Nf-κB

The level of Nf-κB in leukocytes stimulated with either LPS or SC-SPE was up-regulated in the cytoplasm compared to levels in leukocytes stimulated with medium alone up to one hour in culture. After one hour, there is a significant decrease in the amount of Nf-κB in the cytoplasm of responding cells (Figure 29a). However, the level of Nf-κB detected in the nucleus of responding cells is significantly elevated in response to SC-SPE when compared to cultures stimulated with medium alone. There was little or no translocation of Nf-κB from the cytoplasm to the nucleus in leukocytes stimulated with LPS or medium alone. In response to SC-SPE, however, there is strong up-regulation of Nf-κB in the nucleus very early in culture after only 30 minutes which

continues through 2 hours. The level of Nf-κB present in the nucleus after 1-2 hours is likely due to translocation from the cytoplasm which explains the decrease in Nf-κB levels observed in cytoplasmic extracts at that time (Figure 29b).



(a)



(b)

Figure 29: Detection of Nf-κB in cytoplasmic and nuclear extracts of HPBL stimulated with SC-SPE. HPBL (1×10^6 cells/ml) were stimulated with either SC-SPE, LPS, or medium alone and leukocytes harvested at 0.5, 1.0, 1.5, 2.0, and 4.0 hours. (a) Total cellular protein was extracted from cellular pellets to obtain the cytoplasmic fraction and extracts were assayed for Nf-κB by ELISA. At 30 minutes and 1 hour time intervals, there was a significant increase in NF-κB in leukocytes stimulated with SC-SPE compared to those stimulated with LPS or medium alone ($p < 0.05$). (b) A nuclear fraction was extracted from total cellular protein and assayed for Nf-κB. Between 0.5 and 2.0 hours, there was significant up-regulation of NF-κB in leukocytes stimulated with SC-SPE compared to those stimulated with LPS or medium alone ($p < 0.02$). Results suggest that there is translocation of Nf-κB into the nucleus in response to SC-SPE.

DISCUSSION

Commercial shark cartilage is a food supplement taken by consumers as a prophylaxis and for the treatment of conditions ranging from arthritis to cancer. No reliable scientific evidence in the literature supports the alleged usefulness of shark cartilage supplements, but their use remains popular. The oral use of shark cartilage and its development as an alternative medicine has largely been based on unreliable data, anecdotal reports, and assertions by manufacturers like Dr. William Lane, an early proponent of shark cartilage use and the author of 'Sharks don't get cancer', who stand to profit considerably from the sale of such products (Ostrander *et al*., 2004). It remains to be determined whether any putative efficacious active components in commercial preparations of shark cartilage can reach appropriate target sites in the body at effective concentrations after oral ingestion, digestive processes, and absorption. Studies in our laboratory have tested various natural shark cartilage products for their immune-stimulating ability and found that cartilage extracts induce human leukocytes to produce potent inflammatory cytokines *in vitro* (Merly *et al*., 2007). The purpose of the present study was (a) to characterize the nature of the active component responsible for the immunological activity observed, (b) to define the scope of cellular responses to shark cartilage, and (c) to elucidate the mechanism by which shark cartilage extracts stimulate human immune cells.

Since shark cartilage is an oral food supplement, acid extraction was chosen to prepare the shark cartilage extract from commercial preparations. Shark cartilage acid

extracts prepared using various methods were shown to have TNF-α inducing activity when used to stimulated human leukocyte cultures. Previous studies had shown that extracts prepared from several different commercial brands all had TNF-α inducing activity. In this study, the bioactivity extracted using acetic acid (i.e., weak acid) and hydrochloric acid (i.e. strong acid) was compared. Hydrochloric acid was used since it resembles more closely conditions found in the human stomach following oral ingestion. Starting with similar amounts of commercial shark cartilage, extraction with a stronger acid (HCl) yielded more extracted protein that contained high levels of cytokine inducing activity. Hydrochloric and acetic acid extractions yielded more total protein when the extraction procedures were carried out at room temperature. However, when the specific activity of extracts was calculated, extractions performed at $4^{o}C$ yielded extracts with specific activity equal to or higher than extractions done at room temperature. Given that hydrochloric acid extracts yielded increased specific activity from increasing amounts of starting material at $4^{o}C$ suggests that the lack of such a result with acetic acid could mean that larger volumes of acid were needed to achieve a more effective extraction when a weaker acid was used. At room temperature, specific activity recovered decreased in extracts prepared from increasing amounts of starting material. Conversely, at $4^{o}C$, specific activity extracted increased with increasing amounts of starting material. With higher doses at room temperature, there appears to be a loss of activity, but at low temperatures with concentrated extracts, there is more effective extraction of specific activity from shark cartilage. It may be that there

are proteases present in the crude extract that chew up active component(s) at room temperature (that are inactive or less active at lower temperatures) given that previous studies showed that there is loss of SC activity following treatment with serine proteases (i.e., trypsin and chymotrypsin). Finally, a threshold effect was observed when a high dose acid extract prepared from the same amount of shark cartilage powder found in the daily recommended dose of shark cartilage (8 capsules) was tested for activity. The results indicate that a three-fold increase in starting material only increased the induction of TNF-α production in responding cells by 20%. There may be a saturation point and conditions for extraction were not ideal under these conditions (i.e., needed longer extraction time or larger volume of acid).

There were significant differences in total extracted protein content using different extraction conditions. Increases in the amounts of starting material did not directly correspond to increased protein concentrations, indicating that there may have been insufficient amounts of acid used for the higher amounts of starting material. In addition, higher protein yields did not always correlate with corresponding higher cytokine inducing activity of the extract. Dialysis of extracts against a variety of buffers and culture medium resulted in loss of protein when dialysis membranes with low molecular weight cutoffs were employed, suggesting that the loss is most likely restricted to small protein molecules. Proportionately, the amount of protein loss following dialysis was less with concentrated extracts, indicating that the small protein molecules are lost during the prior concentration step and overall protein apparent

losses minimized during dialysis. The results suggest that lyophilization may provide a more effective method for concentrating extracts when the goal is to retain as much of the extracted proteins as possible.

Earlier studies had shown that active component(s) in shark cartilage extract were likely proteinaceous in nature since upon treatment with proteases such as chymotrypsin and trypsin, approximately 80% of bioactivity was lost. Residual activity remained, suggesting the presence of another active moiety (Simjee, 1996). An objective of this study was to characterize the nature of the active component(s) in cartilage extracts responsible for cytokine-inducing activity. To do so, a concentrated preparation of the acid extract was obtained by solid phase extraction (SPE). All of the active material in crude extract was recovered in a yellow pigmented fraction. Identification of the yellow pigment was beyond the scope of this study. The SC-SPE sample induced significant levels of TNF-α, but there was a loss in specific activity and a significant reduction in yield. The decreased specific activity following solid phase extraction was likely due to inactivation of certain active components, given the organic solvents used in this process. However, the application of SPE removed the bulk of non-essential material from the crude extract and yielded an active fraction that could be analyzed by SDS-PAGE for protein composition whereby distinct protein bands were observed. Additionally, SC-SPE could be vacufuged and/or lyophilized and the resulting active, protein solids re-suspended without loss of activity.

For this reason, SC-SPE was used as the starting material in further fractionation of the extract and as the test stimulant in bioassays.

Anionic exchange chromatography of SC-SPE identified two distinct protein peaks, representing cationic component(s) and anionic component(s). Much higher levels of TNF-α were induced in HPBL by anionic components than by either the un-fractionated SC-SPE or components of the cationic fractions. Since specific cytokine inducing activity was noted in both peaks, albeit present at different levels, this suggests that there are possibly two or more active components present in shark cartilage extracts differing in chemical composition as indicated by their behavior on the column. The specific activity was higher in pool II and associated with negatively charged component(s).

Protein analysis of pool II employing SDS-PAGE yielded a distinct protein band(s), approximately 14-21 kDa in size. Since most of the cytokine-inducing activity was recovered in pool II, it was reasonable to assume that one or more of these bands represented specific activity. Protein(s) from these bands was passively eluted from SDS-PAGE gel and assayed for activity. TNF-α inducing activity was recovered in the eluate and thus the TNF-α induction in HPBL was correlated directly to a protein contained in pool II. The correlation of bioactivity with specific protein band makes it feasible to obtain N-terminal sequence data on the unknown protein and thus identify an active component in shark cartilage extract.

Given the nature of cartilage-derived proteins, several are glycosylated, it was reasonable to consider that the protein was modified by glycosylation. Glysolated

proteins in general are common in biological processes and are particularly prominent as regulatory proteins involved in almost every aspect of innate and adaptive immune responses (Rudd *et al*., 2001). To obtain further information on the molecular nature of this active component, that is to determine if it was a glycoprotein, a series of stains and assays for glycoprotein were carried out. As stated above, earlier studies had suggested that the active component was a modified protein. Therefore, a glycoprotein stain was used to determine whether the active protein band identified in pool II was glycosylated. Staining results showed a distinct single band of approximately 14-21 kDa stained for glycoprotein. The glycosylated nature of the protein was investigated further by estimating carbohydrate content of pool II. The level of glycosylation was determined specifically for this glycoprotein as no other glycosylated protein band was identified in pool II. Results indicated the protein to be heavily glycosylated and that it likely contained at least 40% carbohydrate given the colorimetric results obtained. When the original SC-SPE sample was analyzed for carbohydrate, it was estimated to contain between 5-15% carbohydrate content. The DEAE Sepharose column, therefore, served to separate this carbohydrate containing anionic, active protein from the remainder of proteins present in the SC-SPE sample. Previous results (Merly et al., 2007) had shown that alkaline treatment of shark cartilage resulted in reducing the cytokine-inducing activity by more than 50%, in some cases abrogating activity of extracts almost entirely. Taken together, these results suggest that the sugar moieties present on the active protein in pool II may be contributing significantly to its cytokine-

inducing ability and may be equally important to the protein residues in terms of the overall reactivity of the molecule.

The present study undertook to determine the scope of cellular response to shark cartilage extracts. One of the limitations in previous studies has been that the cellular responses (i.e., cytokine induction) was that of a heterogenous leukocyte culture, and thus it was difficult to determine which specific cell types are primarily involved in distinct aspects of the cytokine responses observed. The hypothesis was that cytokine responses observed in heterogenous leukocyte cultures were the cumulative results of both initial responses to shark cartilage itself followed by additional responses to mediators released by the initial responding cells early in culture. To test this hypothesis, leukocyte cultures were enriched for a specific leukocyte type and when stimulated, their culture supernatants tested for cytokine production, specifically TNF-α and IFN-γ.

Leukocyte-specific responses indicated that there are both initial, direct responses to shark cartilage and also secondary or downstream responses to mediators up-regulated in cartilage-stimulated cultures. Results showed that TNF-α production was restricted to monocytes/macrophages although there was likely some contribution from neutrophils when HPBL were tested. Lymphocytes did not produce TNF-α in response to cartilage while monocyte-enriched cultures did, which was expected. However, an unexpected finding was that lymphocytes also fail to produce IFN-γ in response to cartilage alone. It is only when lymphocytes are cultured alongside other

leukocytes actively responding to SC that they produce IFN-γ. Monocyte-enriched cultures and HPBL do not produce significant levels of IFN-γ. Therefore, the conclusion that can be drawn is that initial, direct responses to SC are most likely restricted to monocyte/macrophage cells and neutrophils, while secondary/downstream responses are likely mediated by lymphocytes, where Th1 cells are up-regulated in response to the release of mediators following initial stimulation and, consequently, drive a secondary response in T lymphocytes that includes the production of IFN-γ.

To study further the cellular responses to SC, stimulated cultures were examined for induction of two potent mediators, monocyte chemoattractant protein-1 (MCP-1) and RANTES. Previous studies had shown that SC induced the production of a major chemokine, IL-8. The results showed that SC up-regulated MCP-1 production in a mononuclear cell-enriched population of leukocytes (PBMC). The finding corroborates the role of the monocyte/macrophage as the central responding cell to cartilage as MCP-1 is likely acting in an autocrine fashion to maintain macrophage responses to initial stimulation by SC. The MCP-1 response by SC-stimulated cells increases with time and is significantly different from the cellular response to LPS in which MCP-1 production did not increase significantly over 96 hours and the level of cytokine induced did not exceed 1000 pg/ml. The level of MCP-1 induced by SC increased to over 1000 pg/ml within 24 hours.

The production of RANTES by PBMC upon stimulation with SC extract showed that the cytokine level was consistently maintained over 96 hours compared to

unstimulated control cultures in which the level of RANTES decreased steadily after 12 hours until it was undetectable at 96 hours. It is unclear whether the production of RANTES represents a direct response to SC or a secondary response in T lymphocytes to mediators produced early in culture in response to SC. It may be that initial cytokines released in response to SC drive T lymphocytes to continue producing RANTES. Alternatively, it may be that a potential regulator of RANTES normally present is inhibited by shark cartilage treatment, allowing RANTES production to remain elevated.

Delineation of cellular responses to specific cell types to SC stimulation was difficult to achieve when using peripheral blood leukocytes as a source of cell enriched cultures. For this reason, a murine macrophage-specific cell line (RAW 264.7) was used as a target representing a single cell type. Experiments using the RAW murine cell line were designed to test whether the active component(s) in SC are capable of stimulating cytokine production irrespective of macrophage lineage. The cytokine response of RAW cells, a murine-derived cell line, upon SC stimulation was a significant finding that indicated that the active component in SC could induce cytokine production in cells derived from more than one mammalian species. The response of a macrophage cell line to SC suggests that in a heterogenous population of cells the monocyte/macrophage is most likely to play a central role in initiating the cytokine/chemokine responses observed in such leukocyte cultures because it represents a specific cell type that is responding directly to SC. What is also obvious from the results using the RAW cells is that the response induced in monocyte/macrophage can occur in the absence of other cell types *in vitro*. It should be

noted, however, that the nature of the cytokine response to SC in RAW cells was significantly different from that of human peripheral blood leukocytes. While cytokine response to SC stimulation occurred early in primary cultured HPBL, the cytokine response of RAW cells took longer to be induced (significant induction of TNF-α took ~12 hours). The result above is not entirely unexpected as a cell line requires time to initiate a response to stimulation while primary cultures represent cells that are primed to respond. Furthermore, unlike HPBL, RAW cells constitutively produced significant baseline levels of TNF-α as seen in their response to medium alone. However, production of TNF-α was up-regulated in SC-stimulated RAW cells and peaked at 18 hours (12 hours for LPS-stimulated RAW cells) after which levels remained relatively stable through 96 hours. Given the TNF-α response of unstimulated RAW cells, the question remains whether the TNF-α response to either SC or LPS represents an up-regulation of the constitutive response or whether it represents a distinct inducible response.

SC- stimulated RAW cells did not produce significant levels of IL-12 and IL-1β, which are produced by HPBL in response to SC. The lack of an IL-12 response to SC is not entirely unexpected as RAW cells do not typically produce high levels of IL-12 unless stimulated with a combination of LPS and IFN-γ under certain experimental conditions. The lack of an IL-1β response suggests that even though significant levels of TNF-α are produced by RAW cells in response to SC, the pro-inflammatory response in this single cell type is attenuated when compared to the pro-inflammatory response observed in

HPBL. Macrophages stimulated with SC did produce significant levels of IL-6 when compared to cultures treated with medium alone, but there was significant variation between samples and production levels were relatively low when compared to LPS. The response of RAW cells to LPS was dramatically different than the response to SC in terms of IL-12, IL-1β, and, particularly, IL-6 production, this provides further evidence that the cytokine induction observed in response to SC is not LPS driven.

The RAW cell line was also used to investigate the effect of SC stimulation on nitric oxide production. Preliminary data (results not shown) from studies using HPBL cultures indicated that SC did not induce a significant amount of inducible nitric oxide in responding cells as measured by the Griess reaction or the iNOS ELISA assay. When the induction of iNOS in the cytoplasm of RAW cells responding to SC was measured microscopically, the results indicated that there is a slight up-regulation in iNOS activation in response to SC. While LPS alone did not induce iNOS production, a combination of LPS and IFN-γ produced significant induction of iNOS in RAW cells when compared to unstimulated cells (medium alone). The RAW cells stimulated with SC also showed moderate up-regulation of iNOS expression. At first, results with RAW cells appear to contradict those obtained with primary cultures of HPBL. However, a plausible explanation might be that the slight up-regulated iNOS response to SC is an initial response of the monocyte/macrophage and is short-lived and was undetectable in heterogenous leukocyte cultures or that the iNOS production by the monocyte population within heterogenous leukocyte culture is masked by increased overall

baseline nitric oxide production in primary cultures. Alternatively, it may be that the difference observed reflects a difference between the two species, since the human iNOS appears to be less inducible than the mouse iNOS response.

Given the results for the RAW cell model, it is possible that the monocyte/macrophage plays a major role in initiating the responses to SC, particularly with regards to TNF-α response. However, it also suggests that another cell, perhaps the neutrophil, might play a role in this initial response as the macrophage response appears limited when using the RAW model (that is a single cell type). It is wise, however, not to draw too many conclusions from this model in terms of leukocyte responses because cell lines can behave significantly different than primary cultures in terms of cytokine production and immune responses. Although it was useful to confirm the response in a single cell type target, one must use caution in extrapolating the results of these experiments to the overall likely response of leukocytes to cartilage stimulation.

For cells to respond to a stimulant, it is understood that some form of interaction of the stimulating substance (i.e., ligand) with a cell surface molecule (i.e., receptor) must occur to initiate a response. It was speculated that there are ligands in shark cartilage extract that bind to receptors on the surface of leukocytes and induce downstream signaling and gene expression responsible for the cellular response (i.e., cytokine production). To determine the role of Toll-like receptors in this recognition process, blocking antibodies to TLR 2 and TLR 4 were employed. When the TLR 2

receptor was blocked prior to stimulation, subsequent cytokine induction was not affected significantly. In contrast, when the TLR 4 receptor was blocked, a significant reduction of cytokine induction was noted in HPBL when compared to unstimulated cultures.

One of the main challenges in investigating the TLR 4 pathway is eliminating the potentially interfering role of LPS, since it is the major agonist for the TLR 4 pathway. In these experiments, LPS was used as the positive control to show that blocking of the receptor abrogated the response to LPS. Initially when LPS (5 μg/ml) was used to stimulate cells, it overwhelmed the system and subtle changes in cytokine production following blocking of the receptor were undetectable. When the concentration of LPS was reduced to 0.1 ng/ml, the blocking of the TLR 4 receptor was sufficient to abolish the TNF-α response in HPBL. Once this positive control was established, the effect of blocking TLR 4 on the cytokine response to SC stimulation was measurable. Blocking of TLR 4 prior to stimulation reduces the TNF-α response in leukocytes by more than 50%. One conclusion that can be drawn from these findings is that the active component in shark cartilage is a TLR 4 agonist. However, since there is residual cytokine production in the presence of blocking antibody, it suggests that there may be additional co-receptors and/or adaptor proteins necessary for efficient stimulation. Furthermore, as discussed earlier, results from anion exchange chromatography showed that there are at least two different active components present in SC-SPE based on their behaviors on the column (i.e. size and charge) and their protein/carbohydrate composition. It may be

that one active component requires TLR4 for leukocyte stimulation while the other works through an independent cell surface recognition pathway. Since one of the components has been identified to be a glycosylated protein, the sugar residues may be at least partly involved in ligand-receptor interactions with TLR 4. However, further study is required to determine the molecular mechanism by which these components initiate cell surface recognition. Deglycosylation of the active component in SC may aid in determining the role of biochemical moieties that directly interact with cell surface receptors.

For the active cytokine-inducing component in shark cartilage extracts to induce cytokine and chemokine production in responding cells, it not only must be recognized at the cellular surface but must subsequently be capable of activating specific signaling cascades that would lead to up-regulation of cytokine gene expression and account for the de novo synthesis of these mediators in responding cells. The involvement of several intracellular signaling transduction pathways in cytokine production in response to SC was investigated using a series of inhibitors to specific protein kinase molecules. Protein kinase signaling is involved in almost every aspect of innate immune responses and is responsible for signaling events that occur following TLR immune recognition and lead to the production of inflammatory cytokines and chemokines (Aderem *et al*., 2000). Protein kinase signaling is highly conserved across widely distributed taxa and it is likely that distantly related taxa share many of the same protein kinase families and their related functions (Manning *et al*., 2002). Therefore, investigating the role of key protein

kinase signaling pathways in SC stimulation is critical to understanding the molecular mechanisms underlying the bioactivity of SC extracts.

The Jun Kinase (JNK) pathway represents one of several key mitogen activated protein (MAP) kinase pathways involved in cytokine gene expression. TNF-α production in HPBL was completely abolished when the JNK pathway was blocked; this suggests that JNK plays a central role in intracellular signaling in response to SC. In the case of the Phosphoinostidol-3 kinase (PI-3) kinase pathway, however, a completely different response was observed. When PI-3 kinase was blocked, there was a significant up-regulation in the amount of TNF-α produced in response to SC. The same was not true for LPS-stimulated cultures. Although blocking PI-3 kinase did not inhibit cytokine production in HPBL, it did not dramatically increase cytokine production either as seen with SC. One of the major roles of PI-3 kinase is in regulating endosomal trafficking, particularly with regards to the recycling of receptors to the cell surface. It may be that blocking the PI-3 kinase pathway increases signaling in response to SC because the receptor remains on the cell surface therefore increasing its initial activation. Alternatively, it might be that there is another regulatory protein that normally attenuates the cellular response to SC that is governed by PI-3 kinase. When PI-3 kinase is inhibited, this regulatory protein is absent and the response to SC is augmented. PI-3 kinase plays a major role as a negative feedback regulator of TLR-dependent inflammatory responses and this may help explain why blocking this kinase up-regulates the cytokine response to SC.

The protein kinase C (PKC) pathway is involved in many different cellular functions, primarily with cell growth, differentiation, and development. When PKC is blocked in HPBL, there is a significant reduction in the response of leukocytes to LPS. The response to SC, however, remains relatively unchanged when PKC is blocked. The results indicate that PKC is not essential for SC-stimulated cytokine production. A further conclusion that can be drawn from this result is that signal transduction pathways evoked in responding cells to LPS and SC are significantly different. Although LPS is used as a positive control in these studies, there are major differences in the cytokine and/or chemokine responses induced in response to LPS and SC. It appears that this is due to different signaling patterns induced in responding cells. PKC may play an important role in LPS stimulation, but appears not to do so in SC stimulation.

Another major signaling pathway involved in the induction of cytokine/chemokine gene expression is another member of the MAPK family of kinase, p38 MAPK. When the p38 MAPK signaling cascade was specifically inhibited, cytokine response following stimulation by both SC and LPS was completely abrogated. Even at low inhibitor concentrations, HPBL did not produce significant amounts of TNF-α in response to SC. These results suggest that p38 like JNK is an important signaling pathway for SC stimulation of leukocytes. Finally, the role of the Raf/MEK/ERK2 MAPK pathway was studied by specifically blocking the Raf 1 molecule. The results showed that blocking the Raf/MEK/ERK2 pathway did not significantly affect the production of TNF-α in response to either SC or LPS. The principal hypothesis in this study was that there would likely be

more than one biochemical pathway involved in the response to shark cartilage. It can be concluded given these data that there are two key MAPK signaling pathways at least partially responsible for the cytokine/chemokine responses to SC observed thus far. Both JNK and p38 MAPK appear to play a central role in SC stimulation of leukocytes. It is likely that there is significant cross-talk between multiple signaling pathways. If the active component in SC is acting as a TLR 4 agonist, one would expect that an additional set of signaling molecules related to TLR signaling could play a role initially and, consequently, up-regulate JNK and p38 MAPK signaling, leading to up-regulation of transcription factors responsible for de novo cytokine gene expression.

This study demonstrates that the stimulation of primary cultures (human leukocytes) and a transformed cell line (murine macrophage RAW cells) with extracts of shark cartilage is characterized by the production of multiple inflammatory cytokines and chemokines. Nuclear factor kappa B is a key transcription factor in the up-regulation of cytokine gene expression during innate immune responses and, specifically, inflammatory responses. Nf-κB is constitutively present in the cytoplasm of cells but remains sequestered with an IKK inhibition complex. It is only when the IKK complex is dismantled (requires phosphorylation signaling event) that Nf-κB is freed and able to translocate to the nucleus of responding cells and initiate gene expression. To determine whether Nf-κB is up-regulated in the cellular response to SC, cytoplasmic and nuclear protein extracts of leukocyte lysates cultured with and without SC were prepared. Since activation of Nf-κB is expected to be a very early event in response to

SC, its presence in both cytoplasmic and nuclear extracts was measured at varying time intervals to follow its translocation. By 30 minutes there is a significant up-regulation in Nf-κB in the cytoplasm of responding cells. At 60 minutes, the amount of Nf-κB in the cytoplasm of responding leukocytes peaks at about 2500 pg/ml before steadily decreasing to very low levels after 4 hours. Therefore, an up-regulation in the decoupling of Nf-κB from its inhibitory IKK complex in the cytoplasm of responding leukocytes can be considered an early event and it can be said that Nf-κB is playing a major role in responding cells.

An important feature of this transcription factor, however, is not just activation in the cytoplasm, but also its translocation into the nucleus where it can exert its regulatory capability and affect cytokine and chemokine gene expression. Therefore, it was critical to test for the presence of Nf-κB in the nuclear extracts of responding cells and compare it to the levels present in the cytoplasm at corresponding time intervals. Not only is Nf-κB freed in the cytoplasm of responding cells, but it does in fact translocate into the nucleus as early as 30 minutes following stimulation. The amount of Nf-κB in the nucleus of responding cells is significantly higher than that observed for cells treated with medium alone. It is also significantly higher than amounts seen in cellular response to LPS, which appears to bring about Nf-κB translocation into the nucleus after 2 hours. The response to SC appears to be extremely early shown by the increase in Nf-κB in the nucleus peaking as early as 30 minutes post stimulation. However, Nf-κB levels peak again, after 2 hours, precisely when levels present in the

cytoplasm are particularly low. This can be explained if one does not rule out the involvement of other transcription factors, particularly since it has been shown that there are at least two signaling pathways involved in the cellular response to SC. However, it can be concluded that Nf-κB is clearly involved in the cytokine response to cartilage extract stimulation.

Results from the three-pronged experimental approach taken to meet the objectives of the study can be summarized as follows. Investigating the nature of the active component(s) in SC extract revealed that there are at least two cytokine-inducing components which are acid stable, but differ somewhat in their biochemical characteristics. One anionic component has been identified to be a small (14-21 kDa) glycoprotein with at least 40% carbohydrate content. Defining the scope of cellular responses induced by SC stimulation involved characterizing the cytokine (i.e., TNF-α, IFN-γ, IL-12, IL-1β, IL-6) and chemokine (i.e., MCP-1, RANTES) response in both primary cultures (HPBL) and a cell line (RAW). In HPBL, the response resembled an inflammatory, Th1 polarized response. The response appears as a sequential, two step response consisting of an initial response (i.e., TNF-α) primarily by monocyte/macrophage cells followed by a secondary response (i.e., IFN-γ) by lymphocytes. The TNF-α response of RAW cells indicated that the cytokine could be induced in the absence of other cell types in another mammalian species, suggesting that in HPBL cultures, the monocyte/macrophage is most likely the initial responding cell. RAW cells could also be stimulated by SC extract to produce iNOS. Elucidating the

molecular mechanisms underlying the activation of cells by SC was an interesting facet of this study. Stimulation of cells appears to engage at least one ligand-receptor interaction with TLR 4, although the role of TLR 2 cannot be ruled out and there are likely to be other proteins involved in the recognition of active components in SC at the cell surface. Initial activation is likely followed by the activation of at least the JNK and p38 MAPK signal transduction pathways resulting in activation, release, and translocation of Nf-κB. Results from the study have also definitively shown that the cellular response to SC is distinct from the response to LPS.

Results from this study not only further our understanding of the effects of a natural product on immunological phenomena, but are significant for other areas of biological research, such as investigations into the effect of natural products on public health, their potential application in clinical medicine, and the ecological impact of shark harvesting from oceans. The potential role of active component(s) in cartilage as an inducer of Th1 polarized response and as TLR 4 agonist are compelling reasons for further study to include an in depth characterization of active component(s). The study also raises the possibility that once the exact nature of these active components is defined, alternative sources may be identified, including biosynthesis.

From a public health point of view, given the complex nature of the gut, one can propose a model by which active component(s) in cartilage that survive the digestive process could potentially induce similar responses in immune cells that line the gastrointestinal tract *in vivo* following oral ingestion of cartilage capsules. Localized inflammatory cytokine production in the gut could lead to gastrointestinal upset as well

as exacerbation of conditions for people who suffer from gastrointestinal disease where the underlying pathology involves inflammation. Beyond the gut, one can see how components of shark cartilage are able to up-regulate distinct biochemical pathways in circulating immune cells so that if active component(s) reached other sites in the body, inflammatory processes could be initiated or worsened, making shark cartilage supplementation a particularly poor choice for people with arthritis and other inflammatory conditions. These study findings suggest that the consumption of shark cartilage is a potentially risky endeavor in the absence of a comprehensive characterization of the active components of cartilage and its immune stimulating mechanisms as they are described in this study. Furthermore, unless those components can be isolated and characterized in the absence of contaminating components, it is unlikely that they can have a clinical application in medicine.

From a conservation and ecological point of view, there is currently very little known about the extent to which the shark cartilage industry is responsible for the global decline of shark numbers and potential loss of species. This industry may represent a critical cause of shark losses by direct harvesting by manufacturers. Alternatively, the sale of shark cartilage provides incentives to commercial fisheries to ignore the problem of shark by-catch (known to be a major cause for shark species losses) because there is monetary gain to be had from such activities from manufacturers of shark cartilage.

In conclusion, this dissertation research study represents the first in-depth study into characterizing the bioactive component(s) of commercial shark cartilage

responsible for its immunomodulating properties and defining the subsequent cellular responses at the molecular level.

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APPENDIX

Extraction Buffers

- 0.5M Acetic acid with 0.03% Toluene pH 4.2: 28.6 ml Glacial Acetic Acid (Sigma) and 300 µl Toluene (Sigma) in 1 L ddH_2O; Filter sterilized.
- 0.5M Hydrochloric acid pH 2: 1N HCl diluted 1:1 with ddH_2O (200 ml); Filter sterilized.
- Phosphate buffered saline stock solution pH 7.4 (10x): 1.44 g Potassium Phosphate (MW 136.1) and 7.95 g Sodium Phosphate (MW 268) and 8 g NaCl (Sigma) in 1 L ddH_2O; Filter sterilized and autoclaved.
- Phosphate buffered saline pH 7.4 (1x): Stock10x solution was diluted 1:10 and Filter sterilized.
- Acetonitrile with 0.1% TFA 50% solution: 25 ml of Acetonitrile (HPLC grade) mixed 1:1 with filter sterilized ddH_2O with 50 µl TFA, total volume 50 ml.
- Acetonitrile with 0.1% TFA 80% solution: 40 ml of Acetonitrile (HPLC grade) mixed with 10 ml filter sterilized ddH_2O with 50 µl TFA, total volume 50 ml.

Chromatography Buffers

- 0.5M Tris-HCl pH 7.5 stock solution: 78.82 g of Tris-HCl (Sigma) diluted in 1 L of ddH_2O, pH adjusted to 7.5; Filter sterilized.

- 0.02M Tris-HCl pH 7.5 equilibration buffer: 20 ml of 0.5M Tris-HCl stock and diluted to 500 ml with ddH_2O; Filter sterilized and autoclaved.
- 0.02M Tris-HCl with 1M NaCl pH 7.5 elution buffer: 29.22 g of NaCl was dissolved in 500 ml of 0.02M Tris-HCl buffer, pH adjusted with 1N NaOH; Filter sterilized and autoclaved.

Electrophoresis Reagents/Buffers

- Acrylamide/bis-acrylamide solution: 300 g acrylamide (Sigma) were added to 8 g methylene bis-acrylamide (Sigma) and dissolved in 1 L of ddH_2O; Filter sterilized.
- 0.75M Tris, 0.01M EDTA resolving buffer pH 8.8: 90.83 g Tris (Sigma) and 2.9 g EDTA (acid form, Sigma) were dissolved in 1 L ddH_2O; pH adjusted drop wise with 1N HCl; Filter sterilized.
- 1M Tris-HCl stacking buffer, pH 6.8: 121.1 g of Tris dissolved in 1 L ddH_2O, pH adjusted drop wise with 1N HCl; Filter sterilized.
- 10% Sodium dodecyl sulphate (SDS) solution
- 1% Ammonium persulphate (BioRad)
- Tetramethylethylenediamine or Temed (BioRad)
- SDS-PAGE Resolving gel 10% (Laemmli): 10 ml of acrylamide-bis-acrylamide solution, 15 ml of 0.75M Tris/EDTA solution pH 8.8, 0.3 ml of 10% SDS solution, 1.7 ml of ddH_2O, 3 ml of 1% ammonium persulphate,

and 50 µl of Temed (added immediately before pouring and after gel is de-gassed for 15 minutes under vacuum pressure)

- SDS-PAGE Stacking gel (Laemmli): 1.5 ml of acrylamide-bis-acrylamide solution, 1 ml of 1M Tris pH 6.8, 80 µl of 10% SDS solution, 5.31 ml of ddH_2O, 0.8 ml of 1% ammonium persulphate, and 25 µl of Temed (added immediately before pouring and after gel is de-gassed for 15 minutes under vaccum pressure)
- SDS-PAGE non-reducing sample buffer 5x: 0.6 ml 1 M Tris-HCl pH 6.8, 5 ml of 50% glycerol in ddH_2O, 2 ml of 10% SDS solution, and 1 ml 1% bromophenol blue (tracking dye) in ddH_2O, was added to 1.4 ml ddH_2O; Filter sterilized and aliquoted to store at -20^{o}C
- SDS-PAGE reducing sample buffer 5x: Add 0.65 ml of 2-mercaptoethanol to 10 ml of sample buffer; Filter sterilized
- PAGE Running buffer 10x (Laemmli): 30.0 g Tris and 144 g Glycine dissolved in 1 L $dd_{H2}O$; alternatively, 10x SDS running buffer was purchased (BioRad)
- SDS-PAGE running buffer 1x (Laemmli): 200 ml PAGE 10x running buffer and 20 ml 10% SDS solution, dilute up to 2 L with ddH_2O
- Electrophoresis gel Coomassie Stain (Laemmli): 1.875 g of Coomassie Brillian Blue (R250) was dissolved in a mixture of 227 ml methanol and 94 ml glacial acetic acid and the volume adjusted with 179 ml of ddH_2O

- Fix/De-stain solution (Laemmli): 100 ml glacial acetic acid, 375 ml methanol, and 25 ml glycerol was added to 500 ml ddH_2O, for a total volume of 1 L
- Passive elution buffer (0.05M Tris-HCl, 0.15M NaCl, 0.001M EDTA pH 7.5)

Cell culture Reagents

- RPMI-1640 culture medium (Gibco) without phenol red: Used as purchased from the manufacturer for washing cells prior to culture
- RPMI supplemented/complete growth medium: RPMI-1640 supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin antibiotic solution
- 3% Dextran solution: 4.5 ml of sterile 20% dextran solution (Sigma) was diluted up to 30 ml in sterile ddH_2O
- 0.2% NaCl solution (hypotonic lysis): 0.2 g of NaCl were dissolved in 100 ml of ddH_2O ; Filter sterilized
- 1.6% NaCl solution with 0.2% dextrose (hypotonic lysis): 1.6 g of NaCl and 0.2 g of dextrose were dissolved in 100 ml of ddH_2O; Filter sterilized
- Trypan Blue reagent, 0.1%: 0.05 g of trypan blue reagent (Sigma) were dissolved in sterile PBS; Filter sterilized
- Lipopolysaccharide (LPS) 5µg/ml: Stock solutions of 1 mg/ml were made up and aliquoted; 5 µl of 1 mg/ml stock were diluted in 995 µl of culture

medium to obtain 5 µg/ml solution used to stimulate cells; alternatively, this second stock was diluted further to yield 0.1 ng/ml concentration

- Phosphate buffers saline pH 7.4 (PBS) with 0.1% BSA: 0.1 g of bovine serum albumin (Sigma) were dissolved in 100 ml sterile PBS
- Wortmannin: Wortmanning ready-made solution (4.28 mg/ml in DMSO) was diluted 1:50 in PBS with 0.1% BSA (0.2 µM); diluted 1:10 in PBS with 0.1% BSA (2 µM)
- BI-78D3: 26 mM stock solution was made by dissolving 5 mg in 500 µl DMSO; 1:100 dilution of stock solution in sterile PBS with 0.1%BSA (0.2 µM) and 1:10 dilution of stock solution in sterile PBS with 0.1%BSA (2 µM)
- Rottlerin: 19 mM stock solution was made by dissolving 10 mg in 1 ml DMSO; diluted for 15 µM and 50 µM concentrations
- SB 202190: 30 mM stock solution was made by dissolving 5 mg in 500 µl DMSO; diluted for 5 µM and 50 µM concentrations
- GW5074: 19 mM stock solution was made by dissolving 5 mg in 500 µl DMSO; diluted 1 µM and 10 µM concentrations
- Hypotonic Lysis Buffer pH 7.4: 0.315 g of Tris-HCl, 0.058 g of NaCl, and 0.061 g of $MgCl_2$ dissolved in 100 ml ddH_2O; Filter sterilized; pH adjusted to 7.4 with

  1 N NaOH

- Cell extraction buffer: To a 5 ml aliquot of cell extraction buffer (Invitrogen), 250 µl of protease inhibitor cocktail and 16 µl of PMSF were added immediately before use

VITA

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PUBLICATIONS AND PRESENTATIONS

Liza Merly, Shabana Simjee, and Sylvia L. Smith (2007) *Induction of inflammatory cytokines by cartilage extracts,* International Immunopharmacology, Volume 7 (3): 383-391.

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