The effects of natural compounds on immune cell activation

The effects of dietary fish oil on *ex vivo* chemokine secretion by murine splenocytes and lichen or cyanobacterial polysaccharides on *in vitro* cytokine secretion and signaling pathways in human monocytes

Guðný Ella Thorlacius

Thesis for the degree of Master of Science
University of Iceland
Faculty of Medicine
School of Health Sciences

HÁSKÓLI ÍSLANDS

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Áhrif íslenskra náttúruefna á virkjun fruma ónæmiskerfisins

Áhrif fiskolíu í fæði á flakkboðaseytun miltisfruma úr músum ex vivo *og fjölsykra úr fléttum og cýanóbakteríum á frumuboðaseytun og boðferla mónócýta úr mönnum* in vitro

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Abstract

The effects of Icelandic natural compounds on immune cell activation: *The effects of dietary fish oil on* ex vivo *chemokine secretion by murine splenocytes and lichen or cyanobacterial polysaccharides on* in vitro *cytokine secretion by human monocytes.*

Various plants and natural products have been used in folk medicine for a long time and their popularity has grown steadily in recent years. Fish oil has been popular among the Icelandic nation and a number of studies have revealed numerous benefits of fish oil consumption for many inflammatory diseases that increasingly plague the Western world. Lichens are quite unique both in physiological structure and their production of secondary metabolites. A few of them have been used in Icelandic folk medicine for centuries. Studies have shown that many polysaccharides from plants and other life-forms can mediate various biological effects.

Given the prevalence of numerous inflammatory diseases, products with immunomodulatory effects are an important research subject. One way to examine the effects of natural products on the immune system is to observe their effects on the innate immune responses of animals (*in vivo/ex vivo*) or to examine their effects directly on cells *in vitro*. The aim of this project was to assess the effects of dietary fish oil on murine splenocyte chemokine secretion *ex vivo*, as well as assessing the effects of polysaccharides from the lichens *Cetraria islandica, Collema glebulentum* and *Collema flaccidum* and the cyanobacteria *Nostoc commune* on the activation of human monocytes *in vitro*.

Mice were fed a fish oil diet or control diet for six weeks and then euthanized and spleens collected. The spleens were passed through strainers to form a single cell suspension and seeded in 96 well plates with or without stimulation (lipopolysaccharide for monocytes/macrophages and antibodies against CD3 and CD28 for T cells) and cultured for 48 hours. The culture supernatants were collected and chemokine concentration measured using ELISA.

Polysaccharides from the lichens or cyanobacteria were purified at the laboratories of Sesselja Ómarsdóttir and Elín Soffía Ólafsdóttir at the Faculty of Pharmaceutical Sciences at the University of Iceland. Human THP-1 monocytes were seeded in 48 well plates and pre-treated with IFN-γ for 3 hours and then stimulated with LPS for 3 or 48 hours. The polysaccharides were added with the IFN-γ (for intracellular events) or the LPS (for secreted molecules).

Unstimulated splenocytes from mice fed the fish oil diet secreted less chemokines than splenocytes from mice fed the control diet, suggesting a lowered basal inflammatory state. There was no difference in the chemokine secretion by stimulated splenocytes from mice in the two dietary groups.

THP-1 monocytes treated with the polysaccharide lichenan from *Cetraria islandica* secreted significantly more of the cytokine TNF-α than THP-1 monocytes cultured without lichenan. THP-1 cells treated with the polysaccharide Cg-5-s1 from *Collema glebulentum* or the polysaccharide Nc-5 from *Nostoc commune* secreted significantly less of the pro-inflammatory cytokine IL-6. The polysaccharides Nc-5, Cg-5-s1 and Cf-3-s2 from *Collema flaccidum* all reduced the secretion of

IL-12p40 compared with the control. The polysaccharides did not affect prostaglandin E_2 secretion, COX-2 or iNOS protein levels, activation of MAP kinases or the transcription factor NF-κB.

These results suggest that the polysaccharides Cg-5-s1, Cf-3-s2 and Nc-5 have anti-inflammatory effects, which may reduce Th1 or Th17 immune responses. The mechanisms behind these effects are being studied.

Ágrip

Áhrif íslenskra náttúruefna á virkjun fruma ónæmiskerfisins: Áhrif fiskolíu í fæði á flakkboðaseytun miltisfruma úr músum* ex vivo *og fjölsykra úr fléttum og cýanóbakteríum á frumuboðaseytun og boðferla mónócýta úr mönnum* in vitro*.

Náttúruefni og náttúruvörur hafa í árarað verið notaðar í alþýðulækningum og hafa vinsældir þeirra vaxið stöðugt á síðustu árum. Fiskolía (Lýsi) hefur notið gífurlega vinsælda hér á landi og hafa rannsóknir leitt í ljós margvíslega kosti fiskolíuneyslu gegn ýmsum kvillum sem verða sífellt algengari í hinum vestræna heimi. Fléttur hafa ákveðna sérstöðu bæði vegna líffræðilegrar uppbyggingar og vegna myndunar á annars stigs efnum, en nokkrar fléttur eiga sér einnig langa sögu í íslenskum alþýðulækningum. Rannsóknir hafa leitt í ljós að fjölsykrur úr ýmsum plöntum og lífverum geti miðlað margvíslegum líffræðilegum áhrifum.

Vegna algengis ýmissa bólgusjúkdóma og tengdra kvilla eru efni sem hafa áhrif á ónæmiskerfið vinsælt rannsóknarefni. Ein leið til að skoða áhrif náttúruefna á ónæmiskerfið er að kanna áhrif þeirra á ósérhæfð ónæmissvör í dýralíkönum (*in vivo/ex vivo*) eða með því að skoða áhrif þeirra beint á frumur *in vitro*. Markmið verkefnisins var að kanna áhrif fiskolíu í fæði á frumuboða- (chemokine) seytun músamiltisfruma *ex vivo*, auk þess að kanna áhrif fjölsykra úr fléttunum fjallagrösum, klappaslembru og hreisturslembru, auk cýanóbakteríunnar *Nostoc commune* á ræsingu manna mónócýta *in vitro*.

Mýs fengu fóður með eða án fiskolíu í sex vikur og var svo fórnað og miltum safnað. Miltunum var sundrað í einfrumulausn og frumunum sáð í 96 holu bakka með eða án örvunar (LPS fyrir mónócýta/makrófaga, mótefnum gegn CD3 og CD28 fyrir T frumur) og ræktaðar í 48 tíma. Þá var floti safnað og styrkur frumuboða mældur með ELISA aðferð.

Fjölsykrur úr fléttum og cýanóbakteríu voru hreinsaðar á rannsóknastofum Sesselju Ómarsdóttur og Elínar Soffíu Ólafsdóttur við Lyfjafræðideild Háskóla Íslands. THP-1 mónócýtum úr mönnum var sáð í 48 holu bakka og þeir meðhöndlaðir með IFN-γ í 3 klst og svo örvaðir með LPS í 3 eða 48 klst. Fjölsykrunum var bætt í ræktirnar ýmist með IFN-γ (fyrir mælingar á innanfrumuboðleiðum) eða LPS (fyrir mælingu á seyttum sameindum).

Óörvaðar miltisfrumur músa sem fengu fiskolíufóður seyttu minna af frumuboðum en frumur músa sem fengu viðmiðunarfæði sem bendir til minni bólgu í grunnástandi. Það var ekki munur á frumuboðaseytun miltisfruma músa úr fæðuhópunum tveimur eftir örvun.

THP-1 frumur meðhöndlaðar með fjölsykrunni lichenan úr fjallagrösum seyttu marktækt meira af frumuboðanum TNF-α en viðmið sem ekki fékk sykru. THP-1 frumur meðhöndlaðar með fjölsykrunni Cg-5-s1 úr klappaslembru eða Nc-5 úr *Nostoc commune* seyttu marktækt minna af bólguboðefninu IL-6. THP-1 frumur meðhöndlaðar með Cg-5-s1, Nc-5 eða Cf-3-s2 úr *Collema flaccidum* seyttu marktækt minna af IL-12p40 en viðmið án sykra. Fjölsykrurnar höfðu hvorki áhrif á prostaglandin E_2 seytun eða magn COX-2 og iNOS próteina né á virkjun MAP kínasa eða umritunarþáttarins NF-κB.

Þessar niðurstöður benda til þess að fjölsykrurnar Cg-5-s1, Cf-3-s2 og Nc-5 hafi bólguhemjandi áhrif á svör THP-1 mónócýta sem gætu dregið úr Th1 eða Th17 ónæmissvörum. Rannsóknir á hvernig þessum áhrifum er miðlað halda áfram.

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AA	Arachidonic acid
AP-1	Activator protein 1
APCs	Antigen presenting cells
BSA	Bovine serum albumin
Cf	*Collema flaccidum*
Cg	*Collema glebulentum*
COX	Cyclooxygenase
DHA	Docosahexaenoic acid
DMEM	Dulbecco's modified Eagle medium
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
eNOS	endothelial nitric oxide synthase
EP	Prostaglandin E_2 receptor
EPA	Eicosapentaenoic acid
ERK	Extracellular signal regulated kinases
FCS	Fetal calf serum
IFN	Interferon
IFNGR	Interferon gamma receptor
IKK	IκB kinase
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRAK	Interleukin-1 receptor-associated kinase
Iκ-B	Inhibitor of kappa B
JAK	Janus kinase
JNK	c-Jun N-terminal kinases
KC	Keratinocyte chemoattractant
LOX	Lipoxygenase
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MAPK	Mitogen activated protein (MAP) kinases
MAPKK	MAP kinase kinase
MAPKKK	MAP kinase kinase kinase
MCP	Monocyte chemotactic protein

MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
MUFA	Monounsaturated fatty acid
NADP	Nicotinamide adenine dinucleotide phosphate
Nc	*Nostoc commune*
NED	*N*-1-napthylethylenediamine dihydrochloride
NF-κB	Nuclear factor-kappa B
NK cells	Natural killer cells
nNOS	neuronal nitric oxide synthase
NO	Nitric oxide
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PGE	Prostaglandin
PI3K	Phosphoinositide 3-kinase
PMA	Phorbol 12-myristate 13-acetate
PRRs	Pattern recognition receptors
PUFA	Polyunsaturated fatty acid
PVDF	Polyvinylidene Fluoride
RA	Rheumatoid Arthritis
RANTES	Regulated upon activation, normal T-cell expressed, and secreted
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SI	Secretion index
STAT	Signal Transducer and Activator of Transcription
TBS	Tris buffered saline
TGF	Transforming growth factor
T_H1/T_H2	T helper cells
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
Treg	T regulatory cells
Ub	Ubiquitination

1 Introduction

The knowledge that certain plants or other life forms in our environment have the power to relief symptoms of illness or increase general well-being has been with us through the centuries. Most of this time we have had a *shoot first, ask questions later* approach when it comes to the use of natural products for our health. If it worked, no further questions were asked. With evolving knowledge of the human body as well as our knowledge in the chemistry and biology of other life forms we started to explore these effects more thoroughly, sometimes isolating the active ingredient and even synthesizing it in order to fully exploit its effects and distributing it to the masses.

As time progressed and the use of pharmaceuticals grew we have realized that in some ways this life-changing discovery can be a double edged sword. With an increased use of pharmaceutical drugs came unwanted side effects and drug resistant strains of pathogens as well as an increased demand for a cure for every disease. Since the best way to wield a double-edged sword is to know how to use it, we turn to research. With various *in vitro*, *in vivo*, *ex vivo* and clinical trials, as well as new ways of isolating, visualizing and measuring the smallest change, we can accumulate an arsenal of knowledge on certain compounds and their effect before using them pharmaceutically. This approach can be used in the search for new, biologically active compounds as well as further research into the functions of already defined pharmaceuticals.

1.1 The immune system

Our bodies have a built in defense system against invading pathogens in the form of our immune system. With growing knowledge we have come to realize the enormous complexity of this invisible armor. The ability to recognize and tackle a threat when needed whilst ignoring it when appropriate is paramount. Most of the time our immune system annihilates threats without a hitch, however when our immune system is overpowered or too slow to tackle an infection or inflammation; we turn to antibiotics, antivirals and anti-inflammatory drugs for assistance.

Most of the pathogens we encounter in our daily life do not get past our first line of defense; our skin and mucosa, and thus pose little threat. Should this first line of defense fail, e.g. through a breach in our outer boundary, we have two distinct yet intricately connected systems to tackle the invading pathogens; the innate immune system and the specific acquired immune system.

1.1.1 The innate immune system

The innate immune system is based on recognizing and annihilating threats based on a few common characteristics shared by most invading microorganisms but not found on our own cells. Among the immune cells keeping up our first line of defense, once invaders make it into our tissues, are macrophages, neutrophils and dendritic cells. With receptors for pathogen-associated molecular patterns (PAMPs) generally known as pattern recognition receptors (PRRs) these cells can tell when they encounter an unwelcome invader. The approach is quite straightforward; a common element shared by most unwelcome invaders of a certain type, for example lipopolysaccharide (LPS, a PAMP) from the bacterial cell wall of gram-negative bacteria, is recognized by the appropriate PRRs on the

immune cell. By this semi-specific binding (not specific to a certain type or strain of bacteria, but specific to a gram-negative bacteria) the cell knows it has encountered a non-self threat and therefore attacks it, in most cases via phagocytosis. This act then causes a cascade of events resulting in the elimination of the pathogen, sometimes with the assistance of the specific acquired immune system (1).

Other weapons of the innate immune system are various soluble proteins, f.ex. the complement system which relies on proteins normally dormant in serum or on the surface of certain cells. Once activated the complement system sets off a cascade of biochemical events resulting in the lysis of invading pathogens and the activation or modulation of other immune responses.

1.1.2 The specific acquired immune system

The specific, acquired immune system (a.k.a. the adaptive immune system) is based on recognizing an invading pathogen through a system of highly specific receptors on the surface of lymphocytes. The binding of these specific receptors to a distinct structure on the pathogen sets off a cascade of events resulting in the destruction of the pathogen via a few different mechanisms, each swifter and more effective than the last.

The cells of the adaptive immune system are divided into T and B lymphocytes. Both arise from a common lymphoid progenitor in the bone marrow (Figure 1). Their names match their place of maturation; B cells mature in the bone marrow and T cells mature in the thymus. Each lymphocyte has an antigen receptor that recognizes a distinct antigen. Lymphocytes that have not run into their antigen are referred to as naïve lymphocytes, and when they have encountered their antigen and become activated they become effector lymphocytes or long-lived memory lymphocytes.

Naïve and memory B lymphocytes express their antigen receptor on their surface; however, when they become activated these antigen receptors are secreted in the form of antibodies. Secreted antibodies bind to antigens on a pathogens surface, either tagging them for destruction by other cells, preventing them from binding to cells or activating the complement system. B cells can detect pathogens directly and generate an antibody response, but for a full activation of their defenses they need to interact with T helper cells. The T cells deal with antigens presented on the surface of cells infected with a pathogen or cells that have recently ingested pathogens and subsequently display foreign peptides on their surface. These interactions between antigen presenting cells (APCs) and T cells are discussed in more detail below in the chapter on cytokines.

1.1.3 Cells of the Immune system

Like all of our blood cells, the cells of the immune system derive from pluripotent, hematopoietic stem cells in the bone marrow (Figure 1). These stem cells can then form two kinds of progenitor cells. One are the lymphoid progenitor cells, later dividing into the T and B cells that govern the responses of the specific acquired immune system or the natural killer (NK) cells functioning in the innate immune system. The NK cells mostly recognize and kill abnormal cells, such as cancer cells or cells infected with intracellular viruses. The other kind of progenitor cells are the myeloid progenitor cells which form all the other blood cells; dendritic cells, granulocytes (neutrophils, eosinophils, basophils), mast cells

and the monocytes and macrophages forming the rest of the arsenal of the innate immune system, as well as the platelets and erythrocytes.

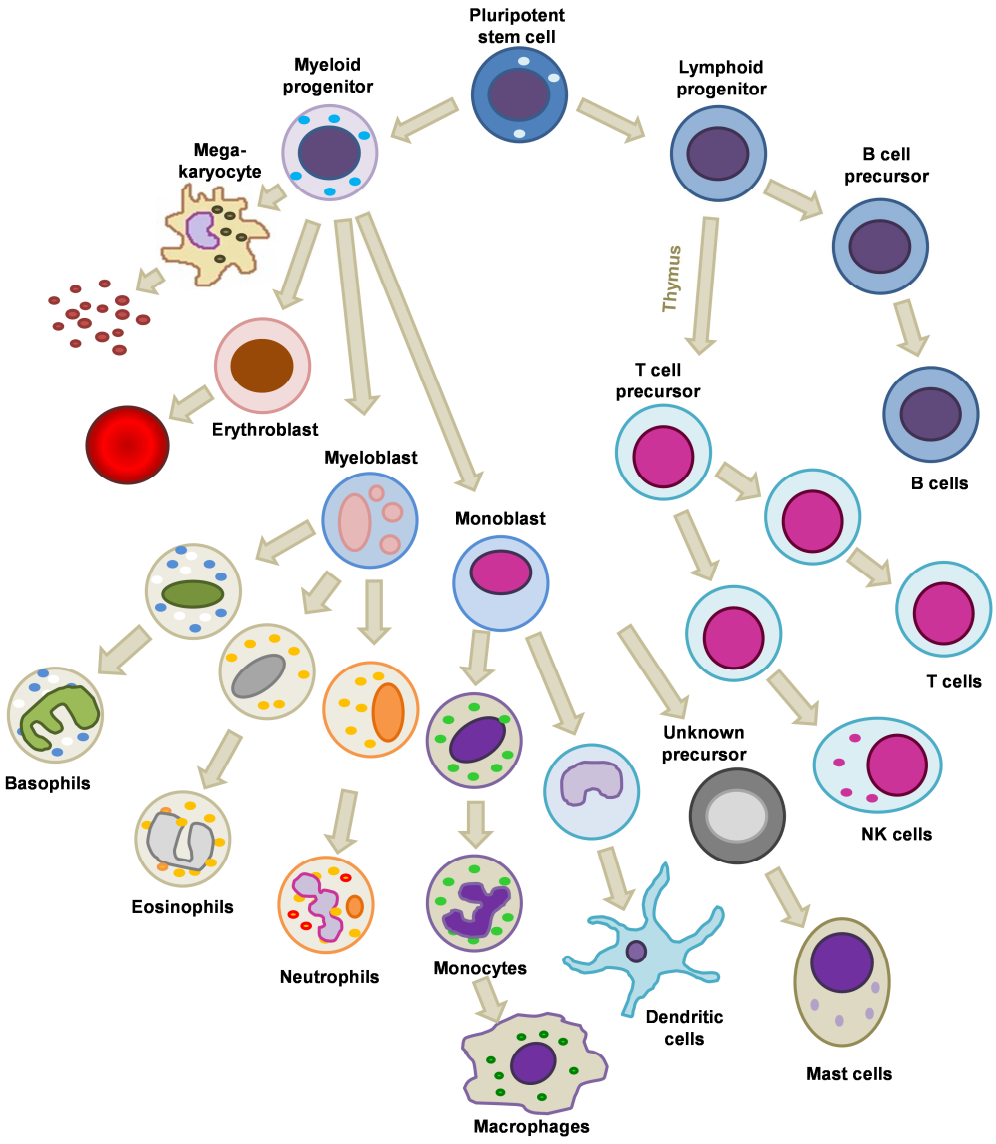

Figure 1. Origin of the cells of the immune system.
The cells of the immune system all derive from a pluripotent hematopoietic stem cell. Pluripotent stem cells form the two progenitor cell types, the myeloid progenitor and the lymphoid progenitor. The lymphoid progenitor cells divide into the T and B lymphocytes of the specific acquired immune system, as well as the NK cells which function in innate immunity. The myeloid progenitor cells however form all other blood cells, including the cells of the innate immune system other than NK cells, as well as the platelets and erythrocytes. Authors image based on (1, 2).

Among the immune cells are the phagocytes; the macrophages and the granulocytes. Monocytes are the precursors of the macrophages and are present in the blood. They leave the blood to become macrophages, which are long-lived phagocytic cells residing in all tissues. There they act as scavengers clearing the body of dead and dying cells and debris. They are the first cells that engulf and destroy microorganisms entering the body. Once a macrophage has taken up a pathogen, it alerts

other immune cells by secreting molecules such as cytokines to attract cells to the origin of the invasion and present lymphocytes with antigens.

The neutrophils are short lived cells that only leave the blood vessels and enter the tissues during an infection or inflammatory reaction. They are very active phagocytes which take up a variety of microorganisms during an infection but the details of their actions will not be discussed here.

Dendritic cells reside in tissues like the macrophages. They are continuously searching the tissues for antigens by phagocytosis and macropinocytosis. Their function is not to eat and kill microorganisms but to find antigens and present them to lymphocytes to activate the specific acquired immune system.

1.1.3.1 THP-1 monocytes

THP-1 cells are a human acute monocytic leukemia cell line first isolated by Tsuchiya et al. in 1980 from a boy with acute monocytic leukemia (3). THP-1 cells are commonly used as monocyte-derived macrophages after treatment with phorbol esters such as phorbol 12-myristate 13-acetate (PMA) (4, 5). THP-1 monocytes are known to respond to LPS stimulation by secreting cytokines (discussed below) and when stimulated with both LPS and cytokines the cells secrete nitric oxide (NO) (6). Using a cell line has several advantages, such as reduced cost and no variation between donors, compared to isolating cells from donor blood. The flaws are that the responses of cultured cell lines may be slightly affected by long-term culture and passage number and thus not completely the same as freshly isolated cells. The general characteristics of THP-1 cells make them a desirable candidate to examine monocyte/macrophage responses in a quick and efficient manner with reduced cost.

1.1.4 Cytokines

The term “cytokine” encompasses a vast array of different proteins with different functions. They can be categorized according to several different attributes both structural and effectual. Effectually cytokines may be sorted by their effects on lymphocytes or by their placement (extracellular or surface bound) or structurally, by size or the presence of certain structural motifs. One subgroup of cytokines is called chemokines (discussed further below) mostly because of their effect on the chemotaxis of cells and chemokines are further divided into different groups based on structural elements. Since it is far outside the scope of this text to review all cytokines or cytokine subgroups, only the relevant cytokines will be discussed here.

Cytokines are small to medium sized proteins which exert an extraordinary amount of different effects on some cell types by binding to specific cytokine receptors with high affinity. From their discovery, cytokines have been shown to play a significant role in practically all physiological processes, from mediating inflammation to organizing cell arrangements in various organs and tissues. Cytokines are key mediators when it comes to immune cell interactions, serving as signal molecules orchestrating drastic adjustments of the general direction of inflammatory or immune reactions. Cytokines can modulate which path is chosen by the immune system when confronted by inflammatory stimulus and thus influence the resulting response and subsequent outcome (1).

As an example of processes influenced by cytokines is when a macrophage runs into a ligand for one of the toll-like receptors (TLRs) on its surface (discussed in detail below), e.g. LPS, the ligand for TLR4. The ligand-receptor binding sets of a signaling cascade resulting in the activation of transcription factors which in turn cause the transcription of the appropriate genes. Some of these genes will be for cytokines serving other important functions in mediating the inflammatory response and among them chemokines secreted to summon other immune cells to the site (Figure 2) (1).

In order to grant arriving cells easier access, the endothelium is affected by cytokines including tumor necrosis factor-α (TNF-α) (see below) secreted by the macrophages or other cells at the inflammatory site to increase vascular permeability as new cells are emerging (1).

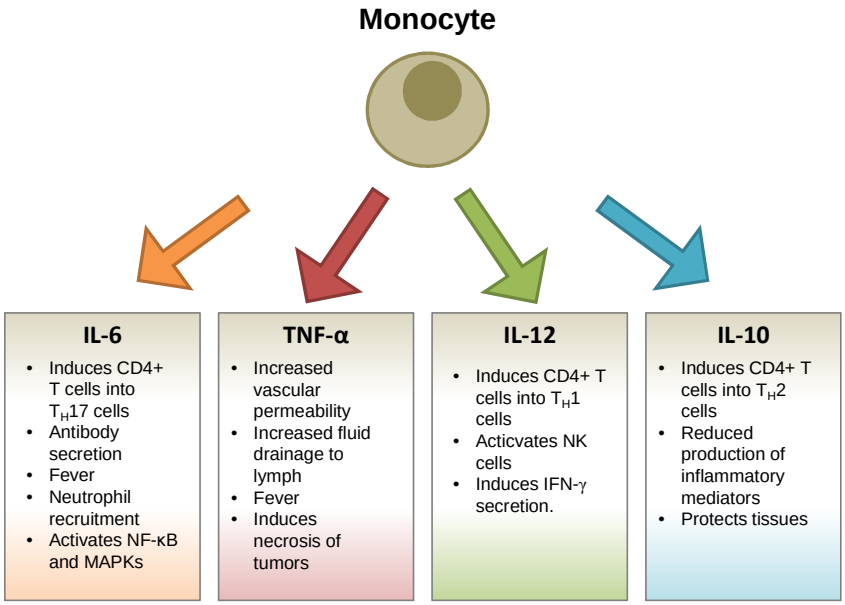

Figure 2. THP-1 monocytes and cytokine secretion.
Monocytes and macrophages secrete an array of cytokines. Among them are these four. These cytokines have various different functions, discussed below. Authors image based on (1, 7).

The activation of naïve T cells is a necessary step in virtually all adaptive immune responses and it is affected by cytokines. Monocytes are activated and differentiated into macrophages when they enter tissues. Toll-like receptors on their surface bind their target and the macrophages (or other APCs) receive signals to express the B7.1 (CD80) and B7.2 (CD86) co-stimulatory molecules on their surface as well as an antigenic peptide presented by the major histocompatibility complex (MHC) class II proteins (1).

When an APC with foreign molecules presented on its surface runs into a naïve $CD4^+$ T cell recognizing that specific antigenic peptide:MHC complex, the T cell receptor is engaged and its CD4 co-receptor is ligated to the MHC molecule as well (Figure 3, left, signal 1). This binding can activate the naïve $CD4^+$ T cell but does not fully stimulate it. If the B7.1 and B7.2 co-stimulatory molecules on the surface of the APC bind to the CD28 molecules on the T cell surface the T cell receives a necessary survival signal (Figure 3, left, signal 2). When the $CD4^+$ T cell has received both the activation and survival signals, an interaction via cytokine secretion takes place (Figure 3, left, signal

3). The cytokines secreted by the APC and neighboring cells dictates what type of effector function the naïve $CD4^+$ T cell acquires (Figure 3, right) (1).

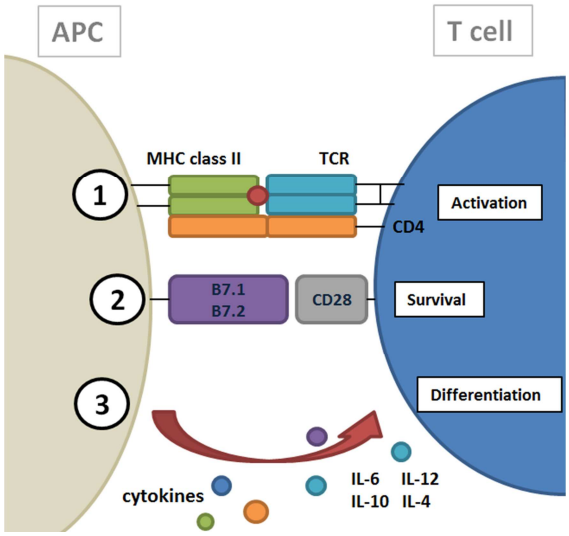

Figure 3. Activation of a naïve $CD4^+$ T cell by an antigen presenting cell.
When an APC displaying a foreign peptide on the MHC class II molecule on its surface finds a $CD4^+$ T cell carrying a T cell receptor recognizing the peptide the T cell receptor is engaged. Should this be followed by a binding of the CD4 co-receptor on the T cell surface it receives an activation signal. This activation signal is insufficient for full activation. Binding of the B7.1 and B7.2 molecules on the APCs surface with the CD28 receptor on the T cell surface provides the second signal. That signal constitutes a survival signal for the T cell. Once the T cell has received both the activation and survival signals it needs a third signal in the form of cytokines (1).

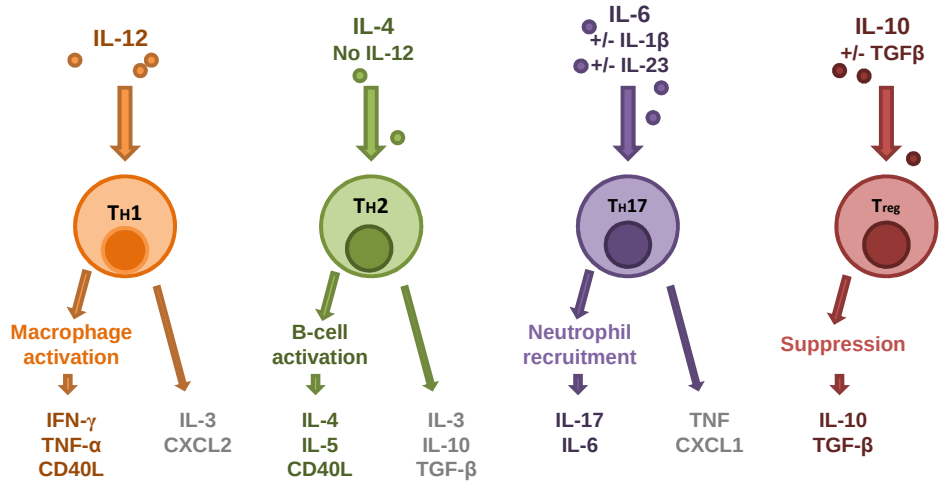

Figure 4. Signal 3 from APCs and their microenvironment to T cells causing differentiation into effector cells and their effector molecules.
Different cytokines cause different effector functions of the $CD4^+$ T cells. The type of effector function chosen then directly dictates the path of the following response making this step extremely important for the subsequent outcome of the infection. The T_H1 cells increase macrophage activation by secreting cytokines such as IFN-γ and TNF-α, causing increased inflammation and tissue infiltration. The T_H2 cells lean toward a gentler B cell activating response with less inflammation and tissue damage. The T_H17 cells participate in the recruitment of neutrophils to promote acute inflammation and the Treg cells suppress the immune response by secreting regulatory cytokines such as IL-10 (discussed in detail below) (1).

In Figure 4 the different $CD4^+$ T cell effector subsets and their effector molecules are listed. The cytokines produced during the $CD4^+$ T cell activation and differentiation is determined by the signals received by the APCs.

1.1.4.1 Interleukin-10

IL-10, sometimes called cytokine synthesis inhibitory factor for its dampening effect on pro-inflammatory cytokine production, is generally characterized as an anti-inflammatory mediator functioning as a tissue protector. It belongs to a family of cytokines named the IL-10 family (8).

IL-10 is produced by all kinds of cells, including most T cell subsets, some B cells, dendritic cells, monocytes and macrophages, NK cells and mast cells among others, but the major cellular source of IL-10 *in vivo* is generally lymphocytes, monocytes and macrophages (9, 10). IL-10 can be produced by APCs by stimulation through Toll like receptors (discussed below) via both mitogen activated protein kinases (MAPKs) and nuclear factor-κB (NF-κB) (both discussed below) (10). IL-10 induction is sometimes initiated in response to pro-inflammatory cytokines (IL-12, IL-6 for example) as a form of negative feedback inhibition to prevent the formation of positive-feedback loops as well as functioning as a tissue-protector (8). It inhibits the production of several pro-inflammatory mediators by LPS stimulated monocytes and macrophages, including IL-12, TNF-α, IL-1β, IL-6 and IL-8 (11).

IL-10 family members exert their effects through two receptors; IL-10R1 and IL-10R2. The binding of IL-10 to its receptor activates the Janus kinase/Signal Transducer and Activator of Transcription (JAK/STAT) signaling pathway (not discussed in detail here) with STAT3 functioning as a key transcription factor in immune cells (8, 10).

1.1.4.2 Interleukin-12

IL-12 is a typical pro-inflammatory cytokine mostly produced by APCs in response to inflammation. It calls forth IFN-γ production in neighboring cells and induction of naïve $CD4^+$ T cells into T_H1 cells (1, 12, 13), see Figure 4. IL-12 functions as a heterodimer of two subunits, a p35 and a p40 unit (shared with IL-23) together making a p70 protein (14). Treatment with LPS and IFN-γ together increases the transcription of the p40 gene and induces high levels of IL-12 secretion from monocytes. IL-12 then causes a positive feedback loop, making it possible for cells to produce large amounts of cytokines in response to inflammatory stimuli (13, 15). IL-10 and prostaglandin E_2 (PGE_2) can inhibit IL-12 production, as well as 1,25 Dihydroxyvitamin D3, NO and various drugs (12).

Although necessary for the clearing of various infections due to viruses, bacteria or parasites, there are numerous T_H1 mediated autoimmune diseases and disorders, including rheumatoid arthritis (RA) and chronic intestinal inflammation in which IL-12 is known to play a significant role, making it an interesting target for possible treatments of such ailments.

1.1.4.3 Interleukin-6

IL-6 is a cytokine important in several processes including nervous system development and inflammation (16). When secreted by macrophages and dendritic cells in response to TLR binding, IL-6 can enhance lymphocyte responses and direct naïve $CD4^+$ T cells towards the T_H17 phenotype (1, 17). IL-6 is produced by T cells, B cells, macrophages, astrocytes, stromal cells, vascular endothelial

cells, smooth muscle cells, and fibroblasts (16) in response to endotoxins (18), viruses (19), cytokines (20, 21) and more. IL-6 binds to the IL-6 receptor and signals through STAT3, MAP kinases and NF-κB (16). Anti-IL-6 therapy is already quite successfully being tested for RA as well as several other diseases (22, 23).

1.1.4.4 Tumor necrosis factor-α

TNF-α is a protein found both in membrane-bound and soluble form. It is functional as a homotrimer of three 17 kDa subunits and was originally found to induce the hemorrhagic necrosis of tumors. It was later shown to function in tumorigenesis, tumor metastasis, viral replication, septic shock, fever and inflammation. Anti TNF-α treatment has emerged as a new treatment for some inflammatory diseases with good results (24).

TNF-α is produced by activated macrophages after the binding of ligands to various PRRs (including TLRs, discussed below) as well as cytokine receptors. It can also be produced by many other types of leukocytes (25). One function of TNF-α is to affect cell-cell junctions in the vascular endothelium to make it easier for cells entering tissues from the bloodstream to pass through the blood vessels, as well as increasing the endothelial cell production of cell-adhesion molecules, all in order to set off a localized infection. TNF-α also facilitates blood clotting to inhibit bacteria from entering the blood stream. These functions of TNFα make it important in septic shock (1).

The TNF-α gene contains binding sites for NF-κB and activator protein-1 (AP-1) among others (26, 27). IFN-γ, IL-1 and IL-2 are cytokines that induce TNF-α production as well as various bacterial products, LPS and whole bacteria (28). Several compounds are known to induce or enhance TNF-α production including PMA (26) and cyclooxygenase (COX) inhibitors such as indomethacin (29). Functional TNF-α binds to two different receptors; p60 and p80 both inducible by IFN-γ (30).

1.1.4.5 IFN-γ

Secreted cytokines such as interferon-γ (IFN-γ) activate newly recruited cells at the site of inflammation. An arriving monocyte binds IFN-γ with its interferon-gamma receptors (IFNGR1 and 2) setting off the JAK/STAT pathway (31). More than 100 genes have been identified as IFN-γ targets, many of whom are involved in early inflammatory responses so this binding can have a drastic effect on protein synthesis. The IFN-γ activation increases surface TLR4 transcription causing an increase in LPS binding and therefore amplifying the resulting response. IFN-γ priming also inhibits TLR4 down-regulation in response to LPS and promotes the production of accessory molecules assisting the TLR4 binding and subsequent signal transductions. LPS induced signaling through TLR4 results in the activation of the transcription factor NF-κB (discussed below) and IFN-γ priming has been shown to both increase NF-κB activation and DNA binding, thus greatly increasing its transcription capabilities (32).

This simplified path of effects caused by IFN-γ signaling on the process of localized inflammation and pathogen-recognition response is just one of the ways cells can affect each other (and themselves) by secreting cytokines.

1.1.5 Chemokines

Chemokines are the largest subgroup of cytokines. Chemokines are small heparin bound proteins (8-10 kDa compared to 10-25 kDa for most other cytokines) originally known for their role in mediating chemotaxis. Cells at the site of inflammation or infection secrete chemokines which bind to the endothelium to form a gradient, strongest at the center and fading as distance increases to attract leukocytes expressing the appropriate chemokine receptors from the circulation (Figure 5) (1, 33, 34). This way, the armada of immune cells responding to a certain stimuli may be controlled by both the expression and secretion of chemokines by nearby cells and the expression of suitable chemokine receptors by the responding cells.

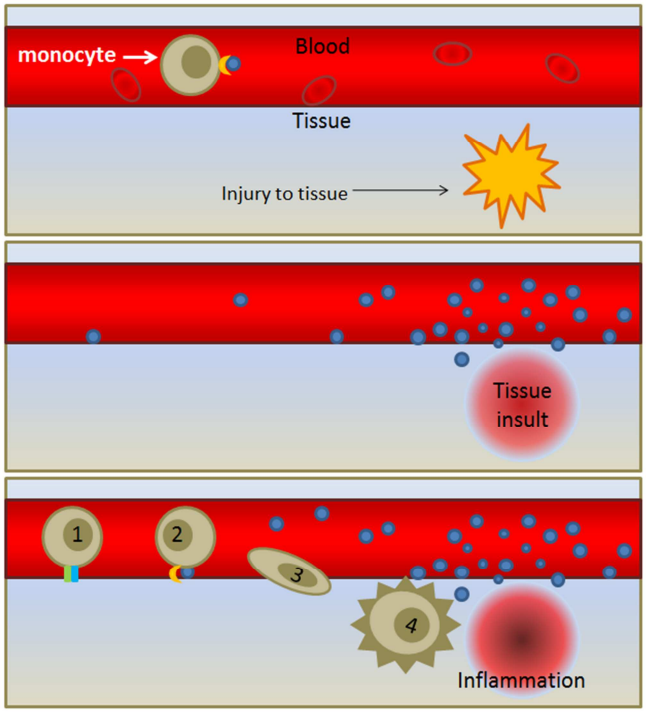

Figure 5. Chemokines and chemotaxis.
When necessary, monocytes (and other immune cells) circulating in the bloodstream can be drawn into tissues by chemokines secreted by the cells at the origin of the cause. After first binding to adhesion molecules on the endothelium (#1) the monocyte finds the chemokines also surface-bound and starts differentiating (#2). It rolls along the endothelium towards the origin of inflammation where it migrates into the tissue (#3) and differentiates into a macrophage (#4). Authors image.

1.1.5.1 Keratinocyte chemoattractant

Keratinocyte chemoattractant, (KC in mice) is better known as IL-8 (or CXCL8) in humans. It is a chemokine produced by monocytes, T cells, neutrophils and NK cells, as well as endothelial cells, fibroblasts and epithelial cells in response to stimuli such as pro-inflammatory cytokines, bacteria, endotoxins and viruses (35). IL-8 binds to CXCR1 and 2 receptors expressed most importantly on neutrophils, but also on monocytes and some lymphocytes (36). It has also been shown to activate neutrophils and work as a chemoattractant for basophils, some eosinophils and T cells. IL-8 has been connected to inflammatory diseases such as Crohn's disease, ulcerative colitis (37), acute respiratory distress syndrome and asthma (35), as well as chronic obstructive pulmonary disease in animals (38).

1.1.5.2 Monocyte chemoattractant protein-1

Monocyte chemoattractant protein 1 (MCP-1) is a chemokine of the CC beta chemokine family. It binds to the CCR2 chemokine receptor on monocytes and triggers their chemotaxis. MCP-1 is secreted by fibroblasts, endothelial cells, vascular smooth muscle cells, monocytes themselves, T cells and other cells that mediate the attraction of cells to sites of inflammation when stimulated with inflammatory cytokines and stress factors. MCP-1 has been found to play a role in several inflammation-related diseases such as atherosclerosis, arthritis (37) and cancer (39, 40).

1.1.5.3 Macrophage inflammatory protein-1α

Macrophage inflammatory protein 1α (MIP-1α, CCL3) is a member of the MIP-1 CC chemokine family of small, structurally related proteins binding to the CC receptor CCR1. It is important for attracting T cells into inflamed tissue, and also serves a function in monocyte, dendritic cell (41) and NK cell migration. MIP-1α is produced by most cells involved in immune responses, such as macrophages, T cells, B cells, neutrophils, dendritic cells, mast cells and NK cells. MIP-1α is generally considered a pro-inflammatory cytokine and has been implemented in several inflammatory diseases and disorders such as asthma, arthritis, pneumonia and psoriasis (42).

1.1.5.4 Regulated upon activation, normal T cell expressed and secreted

Regulated upon activation, normal T cell expressed and secreted (RANTES or CCL5) is a CC type chemokine that mediates the attraction of T cells, dendritic cells, eosinophils, NK cells, mast cells and basophils (43) by binding to the receptors CCR1, 2 and 5 (44). It is produced by many cell types in response to inflammation but especially by macrophages, platelets, eosinophils and fibroblasts, and also endothelial cells, epithelial cells and endometrial cells (43). RANTES has been associated with several diseases such as asthma, atopic dermatitis, colitis and cancer (40). However, the most surprising disease connection came when RANTES was found to have anti HIV activity later found out to be due to the CCR5 chemokine receptor being a co-receptor for HIV (43).

1.1.6 Cell signaling

Receptor-binding on the cell surface triggers a change inside the cell. The impact of this change can be local and quite unremarkable, or set off a multiplying cascade of different events. These events can culminate in a range of consequences; from complete polarization of the cell's purpose to smaller, less significant changes such as the production and/or secretion of other molecules.

Since the human body and most living beings on the planet provide an extreme versatility of different cells, each responding differently, the field of cell signaling is vast and complex and signaling within the field of immunology is certainly no exception. With several different cell types all programmed to mount vast responses to little or very specific offenses, the cells of the immune system provide their own world of signaling events.

1.1.6.1 Toll-like receptors

The invasion of pathogens into our bodies calls forth a fast acting inflammatory response through the activation of receptors on the surface of immune cells such as monocytes, macrophages and dendritic cells. These receptors, named PRRs as mentioned before, bind and recognize foreign molecules as

invaders. Among these receptors are the TLRs, named after the *toll* gene originally discovered in *Drosophila melanogaster*. There are currently 13 known TLRs, binding different PAMPs but very little is known about four of the most recently discovered ones (TLR10-13) as of yet so they will not be discussed any further in this text (45, 46).

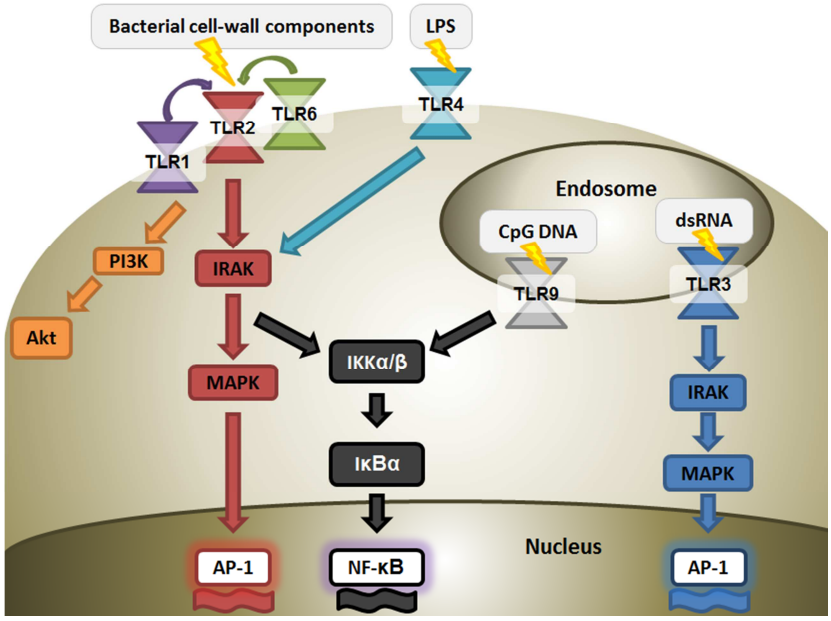

Figure 6. A simplified version of the signaling through TLRs after the binding of bacterial substances.
Common microbial molecules trigger signaling through TLRs 1, 2, 4 and 6 on the cell surface or TLR 3, 4 or 9 in the endosome, causing a signaling cascade through the MAPKs (JNK, p38 and ERK, discussed below), the PI3K and Akt pathway or through IκB kinases (IKKs). Eventually this causes the activation of the transcription factors AP-1 and NF-κB. Authors image based on (47-49).

The TLRs are found both on the surface of cells as well as on the membrane of endosomes. The surface-bound receptors (TLR1, 2, 4, 5 and 6) sense pathogens outside the cell, whereas TLR3, 7, 8 and 9 are found in the endosomes, as well as TLR4 which is found in both places (50). These TLRs bind to various ligands. Bacterial LPS is a TLR4 ligand. It is a component of the cell wall of gram negative bacteria and consists of lipid A domain and a covalently linked polysaccharide or oligosaccharide part (49). It is a potent stimulator of innate immune responses and as Figure 6 shows, it can initiate signaling cascades, through MAPKs (discussed below) resulting in the activation of the transcription factor AP-1 or through a cascade resulting in the activation of NF-κB, (discussed below). Generally, the binding of a TLR-ligand to the receptor can initiate several different pathways involving MAPKs such as p38, c-Jun N-terminal kinases (JNK), or extracellular signal regulated kinases (ERK1/2) as well as phosphoinositide 3-kinase (PI3K) which in turn ultimately activate the transcription factors NF-κB and AP-1 (Figure 6).

These transcription factors in turn initiate the transcription of several cytokines (for example TNF-α, IL-6 and IL-12 (51)), chemokines and various inflammatory mediators causing a cascade of immunological responses (52). Signaling through TLRs also causes an increase in prostaglandin and NO formation, as well as increased production of the enzymes COX and inducible nitric oxide synthase (iNOS) (both discussed below).

1.1.6.2 Mitogen activated protein kinases

As mentioned above, on receiving an activation signal through TLR binding or other inflammatory stimuli, an intracellular signaling cascade is set off in the activated monocyte/macrophage, resulting in the activation of MAPKs (Figure 7). The MAPKs can be divided into three major groups; ERKs, JNKs and p38 MAP kinases. Once activated, MAPKs activate appropriate transcription factors via phosphorylation.

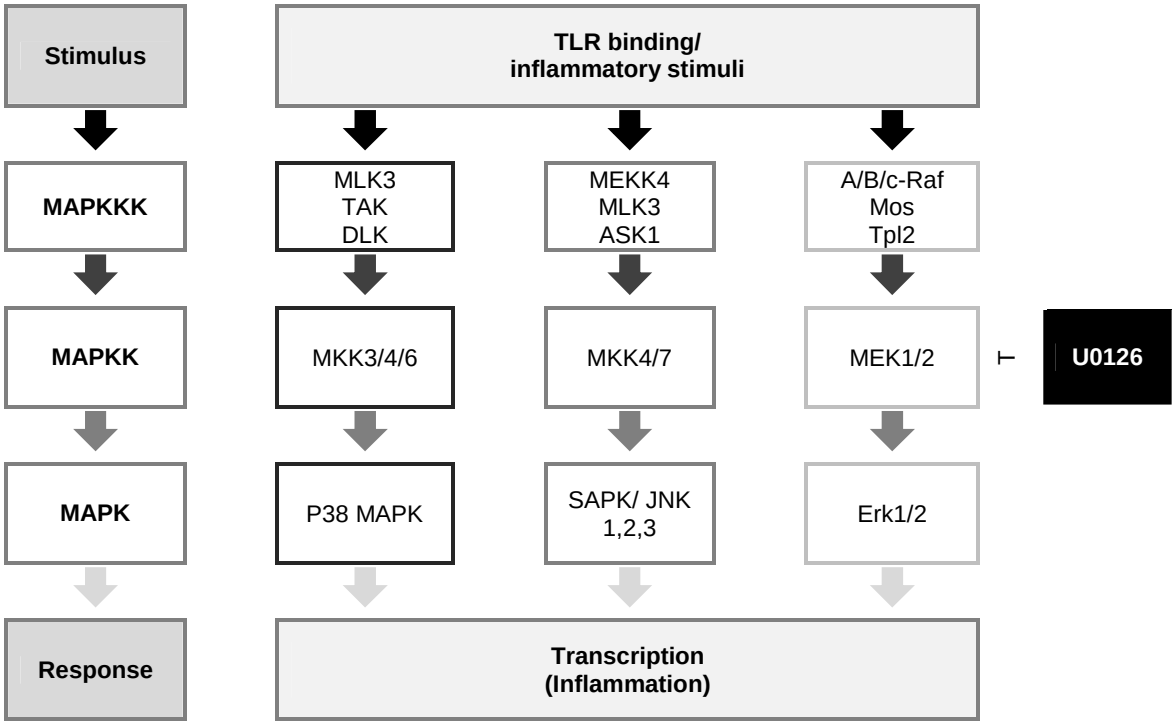

Figure 7. MAP kinase activation.

A simplified model of MAP kinase activation through toll-like receptor binding and/or other inflammatory stimuli (pro-inflammatory cytokine or stress signals). A stimulus activates a MAP kinase kinase kinase (MAPKKK) which in turn activates a MAP kinase kinase (MAPKK) resulting in the activation of a MAP kinase (MAPK) causing nuclear translocation of the kinase and finally transcription factor phosphorylation and activation. All of these pathways can also be activated by growth factors and the ERK pathways can be activated by mitogens. Authors image based on (53-56).

1.1.6.3 Nuclear transcription factor kappa-B

NF-κB is an important transcription factor regulating numerous genes governing cell-signaling, cell growth, apoptosis, cell survival and cellular stress. When it comes to immune cell signaling it controls a large part of the transcription set in motion in response to inflammatory stimuli, therefore making it an intriguing target for various drug treatments (57, 58).

In its inactive state, NF-κB is inhibited by inhibitors of kappa-Bs (IκBs) which bind to the transcription factor and mask the DNA binding and nuclear translocation sequences. During classical NF-κB activation, IKKs (IKKα and/or IKKβ) phosphorylate IκBα, resulting in its ubiquitination and subsequent degradation. This results in the release of an active NF-κB unit in the cytoplasm. The active NF-κB unit is thought to be further phosphorylated or post-transcriptionally modified before

translocating to the nucleus (59). Once the active NF-κB unit is in the nucleus, it binds to κB sites in promoters or enhancers of target genes resulting in their transcription (Figure 8).

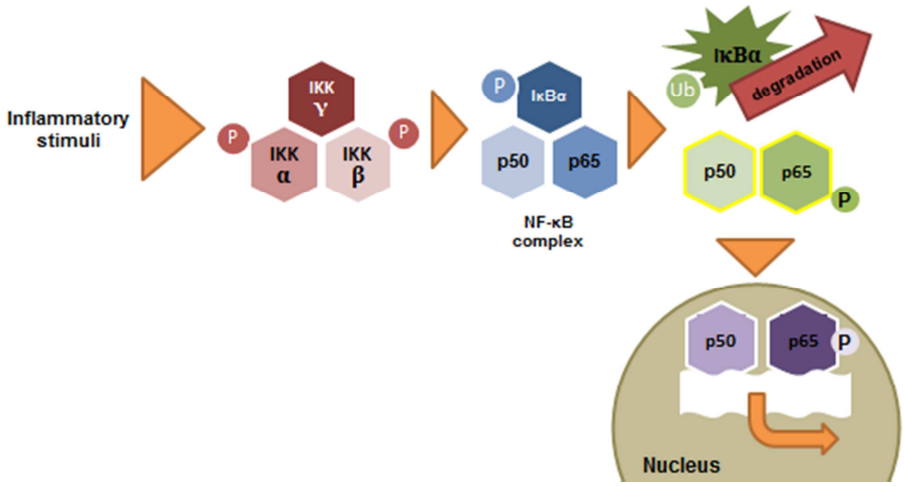

Figure 8. NF-κB activation through IKK phosphorylation of IκBα.
Inflammatory stimuli activate the IKK complex. This results in the phosphorylation and subsequent ubiquitylation of the IκB protein which then leads to its degradation. Degradation of the IκB protein frees the NF-κB in the cytoplasm resulting in phosphorylation, nuclear translocation and the binding of NF-κB to κB sites in the promoters or enhancers of target genes and subsequent transcription (60).

Although widely researched and generally thought of as an important target when it comes to immune modifying drugs, the activation and general control of NF-κB is more complex than the classical activation pathway might suggest and thus any drug treatment would have to fall into just the right place in the pathway to have a useful effect as an anti-inflammatory treatment. Practically all of the steps shown in Figure 8 are subject to inhibition of some sort and many of the proteins involved in the process are thought or known to have alternative motives once a signaling event takes place. For example, IKKα has been found to interact with NF-κB directly and influence its effect on transcription in the nucleus, sometimes resulting in a reduced pro-inflammatory response. Some IκB proteins are also thought to positively regulate transcriptional co-activators, so a simple hindrance to the pathway at some point might actually result in the disruption of a negative-feedback reaction and thus have detrimental effects on the outcome of the inflammatory response (60).

1.1.6.4 Nitric oxide and Nitric oxide synthase

NO is a messenger which can have a protective function when present in low levels to kill invaders or undesired cells, and functioning as a cytotoxin in higher levels, affecting transcription activities and causing DNA damage (61).

In cell types of varying origin, NO is formed via the function of NOS. The reaction involves arginine, O_2 and NADPH being converted into citrulline, $NADP^+$, H_2O and NO (Figure 9). In biological texts, the nitric oxide in question is usually simply termed NO (and will be referred to that way here), but specifically in most cases the molecule in question is the free radical $NO^\bullet$ (as opposed to NO^- or NO^+). The $NO^\bullet$ radical is formed by the reaction in Figure 9 via the actions of three different isoforms of NOS, termed nNOS/NOS1 (n=neuronal), iNOS/NOS2 (i=inducible) and eNOS/NOS3 (e=endothelial). The

three isoforms are coded for on separate genes and function separately. nNOS and eNOS catalyze NO formation in a calcium (Ca^{2+}) dependent manner, whereas iNOS functions calcium-independently (61). The function of NO in the human body is extremely diverse. eNOS and nNOS have more specific functions in selected cell types, however iNOS can be produced in nearly all cells of the body in response to different kinds of stimuli and will be the only one discussed here.

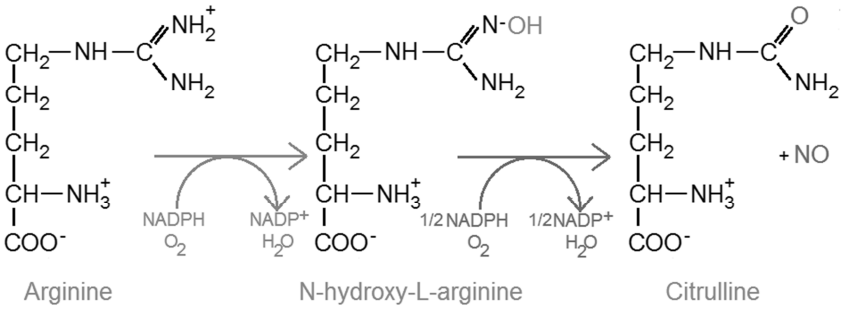

Figure 9. NO generation from arginine, NADPH and oxygen.
NOS uses oxygen in the catalysis of the conversion of arginine to citrulline and NAPDH to $NADP^+$ to form NO. This process is Ca^{2+} dependent for nNOS/NOS1 (in neurons) and eNOS/NOS3 (happening constitutively) but not in iNOS/NOS2 processes (for example in immune cells). NO can then diffuse through the cell membrane without a carrier and enter neighboring cells (61, 62) Authors image based on (63).

The amount of NO present in different cell types and different environments varies enormously, causing some difficulty in determining what concentration should be considered detrimental. As mentioned above, the presence of NO in tissues can have positive effects, and removing it altogether can cause tissue damage and a reduction in antimicrobial activity. However, higher levels of the radical can cause increased tumor growth, inflammation, DNA-damage and impaired cellular function so close regulation of the enzymes governing NO production is key (61).

The NOS enzymes are known to be diversely regulated at all levels from gene transcription to regulation of the enzyme itself, including a feedback inhibition of NOS by NO. iNOS however is thought to be mostly regulated at the transcription level with the exception of a few proteins thought to be able to affect iNOS function (64).

1.1.6.5 Cyclooxygenase, lipids and eicosanoids

Eicosanoids are signaling molecules derived from 20 carbon fatty acids. The eicosanoids prostaglandin, leukotriene, lipoxin and thromboxane, have many different roles in the human body, including initiating inflammation, causing pain, controlling sleep, blood clotting and the formation of hormones. Different eicosanoids have different functions and since the eicosanoids are made from polyunsaturated fatty acids (PUFAs), the availability of PUFAs in cell-membranes directly influences what eicosanoids are formed at each time (Figure 10). The availability of PUFAs in the cell membranes depends on the PUFAs consumed in the diet.

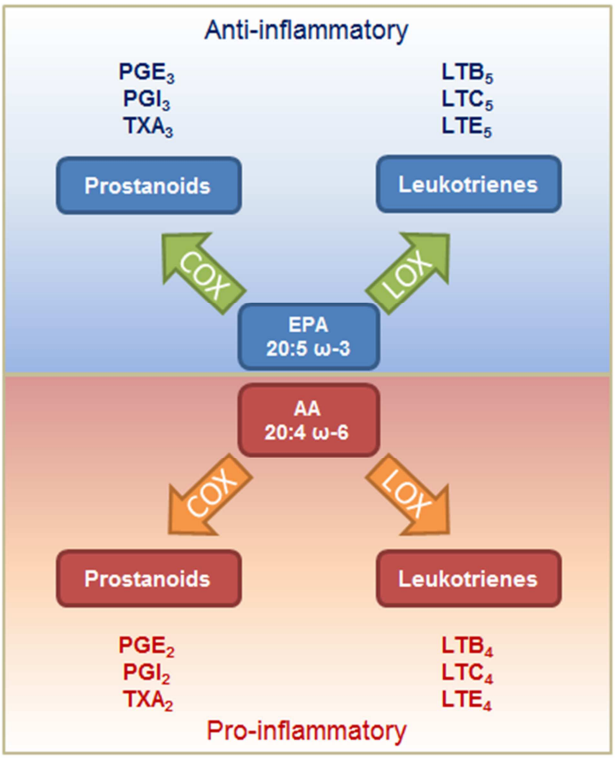

Figure 10. Eicosapentaenoic acid (EPA) and arachidonic acid (AA) derived eicosanoids. A general scheme of the prostanoids and leukotrienes formed by COX and lipoxygenase (LOX). When AA is abundant in the cellular membrane, the COX and LOX enzymes have more access to them and thus more of their products are formed. This causes a rise in pro-inflammatory cellular mediators. Similarly, when EPA is more abundant, anti-inflammatory mediators reign. Picture modified by author from (65).

Of the biologically active eicosanoids, PGE_2 stands out as an extensively researched, multi-active molecule. Like the other prostanoids, PGE_2 is formed when arachidonic acid (AA) is released from the plasma membrane by phospholipases and then metabolized by COX-1, a constitutively expressed "household" enzyme and COX-2, an induced version. COX-2 is not produced under normal circumstances, however, during inflammatory stimuli and chronic inflammation high levels of the enzyme can be measured. Several drugs inhibiting the COX-2 enzyme are used as anti-inflammatory drugs in arthritis and other chronic inflammatory diseases underlining its role in the inflammatory process (66) but some toxic effects of prolonged use of these drugs have indicated other roles for COX or its products (67).

PGE_2 has four subtypes of receptors named $EP_{1\text{-}4}$ (68). PGE_2 production is controlled by inflammatory signaling cascades through activation of the NF-κB transcription factor and the effect of its production then further controlled by the expression of the four different receptors. The four different EP receptor subtypes are each encoded by different genes and they have a wide range of effects, including the facilitation of ovulation, suppression of dendritic cell differentiation, promoting chemical carcinogenesis and mediating joint inflammation in collagen-induced arthritis models (69).

Prostanoids derived from AA are usually pro-inflammatory mediators, however in the case of PGE_2, the effects are sometimes ambiguous. PGE_2 has for example been shown to reduce the levels of TNF-

α secreted by LPS stimulated human whole blood cultures (70) as well as the pro-inflammatory cytokine IL-12 (71) and augment the response of THP-1 cells to some cytokines, steering them towards an anti-inflammatory response (72).

1.2 Polyunsaturated fatty acids

Fatty acids are hydrocarbon chains with a methyl group on one end and a reactive carboxyl group on the other. Fatty acids can have different degrees of saturation, ranging from saturated or monounsaturated to polyunsaturated hydrocarbon chains. Around 20 different fatty acids are available in our diet. N-3 and n-6 fatty acids qualify as essential fatty acids since they cannot be synthesized *de novo* in human (and animal) cells and thus must be obtained from our diet or supplements much like several vitamins. Fish and fish oil are a good source of n-3 PUFAs, whereas n-6 PUFAs are available from most vegetable oils (73).

When fatty acids are ingested they have three possible destinations; oxidation for energy (so called β-oxidation), storage in fatty tissue or incorporation into cell membranes in the form of phospholipids (74). Fatty acids deposited in phospholipids may be elongated and/or further unsaturated to a certain extent. These alterations of PUFAs into other, further unsaturated or longer fatty acids have limitations, and thus the repertoire of available fatty acids depends on the availability of necessary fatty acids in the diet. This repertoire of fatty acids greatly influences the availability of signaling molecules formed in the cells and therefore has the ability to influence signaling cascades within cells and cellular responses (75).

Changes in the consumption of PUFAs in the Western world has reached a point where the average consumption of n-6 PUFAs is up to twenty times greater than the consumption of n-3 PUFAs (75). This change in the n-6/n-3 ratio has been linked to increased incidence of several health issues now much more common in the Western world than other cultural areas where n-3 consumption is greater (for example with the Inuits of Greenland or some areas of Japan where diets consist mainly of fish and/or marine mammals) (76-78). The known positive effects of n-3 PUFAs on the symptoms and severity of various diseases have caused doctors to recommend n-3 PUFA supplements or increased fish consumption, especially for people suffering from chronic inflammation and related diseases such as arthritis, Crohn's disease or other autoimmune diseases or any ailment causing strain on the immune system (78, 79).

When n-6 PUFAs increase in the diet, n-6 PUFA levels in cellular membranes increase resulting in a higher ratio of n-6 PUFAs in phospholipids. This results in more n-6 derived signaling molecules produced, which cause a more severe inflammatory response than the n-3 derivatives (74, 78, 80, 81). Control over this balance between pro- and anti-inflammatory signaling in the body is important for people suffering from chronic inflammatory ailments and can dramatically influence their quality of life. Furthermore, an overall decrease in the basal inflammatory response can be important for preventing inflammation-related diseases in future years (79, 82, 83).

1.2.1 Polyunsaturated fatty acids, cytokines and chemokines

Research into the mechanism behind the anti-inflammatory effects of fish-oil consumption has demonstrated an effect on APCs, including monocytes and macrophages. Dietary supplementation has been shown to affect the ability of human monocytes to induce an inflammatory response (84) and alter the maturation of dendritic cells *in vitro* by affecting peroxisome proliferator-activated receptor-γ (85). Dietary fish oil has also been shown to affect cytokine secretion of both T cells and APCs (86, 87) but less information is available on the effect of dietary fish oil on the chemokine secretion of these cells.

1.3 Natural products

The use of natural products/herbal medicines to prepare pharmaceutical formulations, as complementary and alternative medicine and as dietary supplements has steadily been increasing in popularity over the last sixty years. Natural products can be prepared from plants, fungi, bacteria or animals by different methods and used to for the desired purposes. Different methods of extraction can yield different products from the same source, and these extracts can then be purified by various methods to yield a mixture of only a few similar components or a pure compound. We commonly use countless plants as providers of primary metabolites such as carbohydrates, proteins and fats simply for nutrition. Aside from these generic substances many plants produce so called secondary metabolites. These differ in distribution and function and in some cases, neither is completely known. The known roles of some secondary metabolites in plants range from protecting the plants from predators to attracting insects to carry out fertilization.

In Iceland, natural products have been isolated from a variety of biological sources. Fish oil is commonly extracted from cod liver and widely used as a dietary supplement. Several Icelandic plants and lichens are sources for herbal medicines used throughout Icelandic history.

1.3.1 Natural products and immune function

Many natural compounds have been shown to affect cytokine production. Cinnamaldehyde from the leaves of *Cinnamomum osmophloeum* Kaneh has been shown to reduce the secretion of TNF-α, IL-1 and IL-6 in LPS stimulated human THP-1 monocytes (88). Schisantherin A, a dibenzocyclooctadiene lignan isolated from the fruit of *Schisandra sphenanthera* has been shown to reduce the secretion of TNF-α and IL-6 in murine RAW 264.7 cells stimulated with LPS (89). An extract from *Uncaria tomentosa* or 'Cat's claw' has been shown to increase the secretion of IL-1β while decreasing the secretion of TNF-α in LPS stimulated THP-1 cells (90).

Studies on the immunomodulatory effects of several natural compounds have shed light on a role for MAP kinases as possible mediators of those effects. Demethoxycurcumin, a derivative of curcumin has been shown to reduce the phosphorylation levels of the MAP kinases p38, JNK and ERK in murine N9 microglia cells (91). CKBM, a herbal formula composed of *Panax ginseng*, *Schisandra chinensis*, *Fructus crataegi, Ziziphus jujube* and *Glycine Max* supplemented with processed *Saccharomyces cerevisiae* has been shown to reduce the activation of JNK while increasing the activation of p38 in THP-1 monocytes (92).

The involvement of the transcription factor NF-κB in mediating the effects of natural compounds is a popular research subject. The previously mentioned Cat's claw extract has been shown to reduce the activation of NF-κB in LPS stimulated THP-1 cells (93). Lactoferrin, a glycoprotein found in milk, has been shown to reduce the DNA binding of NF-κB in LPS stimulated THP-1 cells (94).

Several natural compounds have been shown to have an inhibitory effect on iNOS expression and NO production, including Arctigenin from *Arctium lappa L.* seeds (95), Styraxoside A isolated from the stem bark of *Styrax japonica* (96), and ethyl acetate extracts from *Angelica Dahuricae Radix* (97) on RAW 264.7 macrophages and THP-1 cells and many more (98, 99).

Altering the levels of active COX-2 and thus prostaglandin formation is an effect seen in several studies researching the effects of natural compounds on inflammatory responses. As discussed above, altering the prostaglandin formation in cells can have a variety of effects making this subject important for immunomodulatory processes. Caffeic acid phenethyl esters from propolis have been found to reduce both the COX-2 levels and PGE_2 production in LPS stimulated RAW 264.7 cells (100). Isoliquiritigenin, a flavonoid from *Glycyrrhiza uralensis* (Leguminosae) has been shown to reduce COX-2 levels in RAW 264.7 cells (101).

These studies and many more have shown that natural products can affect inflammatory responses in a variety of ways.

1.3.2 Polysaccharides from lichens and cyanobacteria

Lichens are a symbiotic association of fungi and green algae or cyanobacteria or even all three. They can survive in extreme conditions and produce a diverse range of active metabolites, and many are unique in nature (102). Several lichen species have been used in folk medicine all over the world (103) and many lichen extracts have been shown to affect various cell types and functions such as macrophage oxidative burst (104) and macrophage tumoricidal activity (105). Most lichens are rich in polysaccharides and a few lichen polysaccharides have already been tested for immunomodulatory effects (106-108). In general, the search for active compounds from remedies popular in folk medicine is increasingly leaning towards active polysaccharides (109) and the search for ways for polysaccharides from various sources to bind and affect cells is very active (110-112).

1.3.2.1 Cetraria islandica

Cetraria islandica (IS: Fjallagrös, Figure 11) is widespread all over Iceland. Its use in folk medicine has a rich tradition in many countries, most often as an aqueous extract. For example in Turkish folk medicine *Cetraria islandica* has been used to treat various diseases such as hemorrhoids, bronchitis and tuberculosis (113) as well as digestive disorders such as diarrhea and intestinal parasites (114).

Figure 11. *Cetraria islandica* (IS: Fjallagrös).
Image captured by Hörður Kristinsson in Oddsskarð in 1993, floraislands.is.

Several compounds have been isolated from *Cetraria islandica* and some of them have been tested for immunomodulating activity. Among them are the polysaccharides lichenan and isolichenan. When an aqueous extract of *Cetraria islandica* and lichenan were tested for *in vivo* and *in vitro* immunomodulating activity they were shown to direct the maturation of dendritic cells towards an anti-inflammatory phenotype *in vitro* and the extract was able to reduce arthritis in rats (115). Purified protolichesterinic and fumarprotocetraric acids from *Cetraria islandica* were also tested on the dendritic cell model, as well as the polysaccharide isolichenan, but had no effect (115).

1.3.2.2 Collema glebulentum

Collema glebulentum (Figure 12, IS: Klappaslembra) is a jelly lichen consisting of a *Nostoc* cyanobacteria and an *Ascomycota* fungus. It is commonly found on rocky surfaces often among moss.

Figure 12. *Collema glebulentum* (IS: Klappaslembra).
Image captured by Hörður Kristinsson at Mýrar in 1989.

The name ‘jelly lichen’ is derived from the jelly-like texture caused by water absorption by the otherwise dry and brittle lichen.

1.3.2.3 Collema flaccidum

Collema flaccidum (Figure 13, IS: Hreisturslembra) is a jelly lichen consisting of a *Nostoc* cyanobacteria and an *Ascomycota* fungus. It is widespread in Iceland and grows mostly on cliffs and rocks. Glycosides from *Collema flaccidum* have been found to have significant anti-tumor activity in the crown gall tumor inhibition test (116).

Figure 13. *Collema flaccidum* (IS: Hreisturslembra).
Image captured by Hörður Kristinsson at Stafafell in August 1990.

1.3.2.4 Nostoc commune

Nostoc commune is a cyanobacteria or blue-green algae of the *Nostoc* genus. Many cyanobacteria have been shown to produce bioactive compounds with a range of biological effects, and some edible blue-green algae such as *Spirulina* are a popular dietary supplement. Lipids from *Nostoc commune* var *sphaeroides* Kützing have been shown to reduce NF-κB activity, TNF-α, IL-6, iNOS and COX-2 mRNA without affecting viability in Raw 264.7 murine macrophage cells (117) as well as affecting cholesterol metabolism by reducing the levels of sterol regulatory element binding protein 1 and 2 in HepG2 cells (a human hematoma cell line) (118).

2 Aims

Although many natural products are used in folk medicine to alleviate several disorders, their potential mechanisms of action are largely unknown. The aims of this study were:

1. To determine if dietary fish oil has an effect on chemokine secretion by murine splenocytes.
2. To determine if polysaccharides from Icelandic lichens and a cyanobacteria affect THP-1 monocyte function by specifically examining:
    a. Secretion of inflammatory cytokines.
    b. NO and PGE_2 secretion.
    c. Signaling pathways upstream of the cellular production of cytokines, NO and PGE_2.

3 Materials and methods

All animal procedures performed in this project were approved by the Experimental Animal Committee, Ministry for the Environment in Iceland and complied with the National Research Council's Guide for the Care and Use of Laboratory Animals.

3.1 The effects of dietary omega-3 fish oil on mouse splenocyte chemokine secretion

The mouse model described here has been used in our laboratory to examine the effects of dietary fish oil on splenocytes and is part of a larger study where the effect of dietary fish oil on whole-blood, peritoneal fluid, liver and bone marrow are examined.

3.1.1 Experimental animals and diets

Female C57BL/6 mice weighing 18 to 20 g were purchased from Taconic Europe (Ejby, Denmark). Mice were housed five or eight per cage in 45-55% humidity at 23-25℃ with 12 hour light and dark cycles. At arrival, the experimental animals were acclimated for one week before initiating the experiments.

The animals were randomly assigned to one of two diet groups; receiving either a control diet or a fish oil diet for 6 weeks. The composition of the experimental diets is shown in Table 1 and a further breakdown of the fatty acid composition is shown in Table 2. The general composition of the diets was based on the composition of a typical Western diet modified from diets formulated by Monsanto (St. Louis, Missouri, USA) and Research Diets (New Brunswick, New Jersey, USA) by Hildur H. Arnardóttir (as part of her Ph.D. study) and Professor Ingibjörg Harðardóttir in collaboration with Dr. Kevin Fritche (University of Missouri).

The fish oil diet was made by adding 28 g/kg menhaden fish oil (Omega Protein, Reedville, VA) to the basal diet at the expense of the safflower oil (Welch, Holme & Clark CO Inc., Newark, NJ). Arachidonic acid (AA) ethyl ester (Nu-Check-Prep, Elysian, MN) (0.5 g/kg) was added to the control diet to adjust for the AA content in the fish oil diet (Table 1). The fish oil diet contained 1.4% eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA) (see Table 2 for details of the fatty acid composition), corresponding to a human intake of approximately ~3 g/day of EPA + DHA (assuming an energy intake of 2000 kcal/day).

The diets were aliquoted into daily portions and stored under nitrogen at -70℃ to prevent oxidation. The animals were provided fresh food daily and no restrictions were made to their food and water intake. The animals were weighed weekly and there was no difference in weight or weight gain between the two groups.

Table 1. Composition of the control and fish oil (FO) diets.

Ingredient	Control	FO
	g/kg	
Casein	229	229
L-Cystine	3	3
Cornstarch	274	274
Maltodextrin 10	86	86
Sucrose	114	114
Cellulose	57	57
Cocoa Butter	43	43
Linseed Oil	5	5
Palm Oil	60	60
Safflower Oil	32.5	4.5
Sunflower Oil, Trisun	31	31
Fish oil	0	28
20:4 n-6 ethyl ester	0.5	0
Mineral Mix S10026	11	11
Dicalcium Phosphate	15	15
Calcium Carbonate	6	6
Potassium Citrate	19	19
Vitamin Mix V13401	11	11
Choline Bitartrate	2	2
α-Vitamin E Acetate	0.15	0.15
t-BHQ	0.03	0.03

Table 2. Fatty acid composition of the control and fish oil (FO) diets as provided by the manufacturer.

Fatty acid	Control	FO
	g/kg	
10:0	ND	ND
12:0	0.2	0.2
14:0	0.5	2.4
15:0	ND	0.1
16:0	36.8	39.2
16:1	0.1	2.9
18:0	19.5	19.5
18:1	70.1	69.5
18:2 (n-6)	36.2	14.9
18:3 (n-3)	3.4	3.8
20:0	0.7	0.8
20:1	ND	0.4
20:2	ND	0.1
20:3	ND	0.1
20:4 (n-6)	0.6	0.6
20:5 (n-3)	ND	4.0
22:1	ND	0.1
22:4	ND	0.1
22.6 (n-3)	ND	2.5
24:0	ND	0.1
Total saturated FA	57.7	62.5
Total MUFA$^{\theta}$	70.2	72.9
Total PUFA$^{\Phi}$	40.2	26.5
Total (n-6)	36.8	15.6
Total (n-3)	3. 4	10.6
P:S ratio$^{\Psi}$	0.7	0.4
(n-6):(n-3) ratio	10.7	1.5
(n-3):(n-6) ratio	0.09	0.68

$^{\theta}$: MUFA: Monounsaturated fatty acid
$^{\Phi}$: PUFA: Polyunsaturated fatty acid
$^{\Psi}$: P:S ratio: Polyunsaturated vs. saturated ratio

3.1.2 Isolation, separation and culturing of mouse splenocytes

The mice were anesthetized and blood was collected via axillary artery for use in splenic cell culture. The spleens were collected free from connective tissue in DMEM medium (Gibco, Invitrogen) supplemented with penicillin and streptomycin. The spleens were then passed through a 70 µm nylon cell strainer (BD Falcon, Franklin Lakes, NJ) to form a single cell suspension. Red blood cells were

lysed with an ACK lysing buffer (NH_4Cl (8.3 mg/l), $KHCO_3$ (1 mg/l), Na_2EDTA (37 mg/l), pH 7.4) for 5 minutes on ice and the remaining cells were washed, re-suspended in medium and counted using trypan blue staining and a cell counter (Countess ®, Invitrogen). The cell density was adjusted to $1x10^6$ cells/ml and 200 µl/well were added to flat-bottomed 96 well plates.

Serum for the culture was prepared by collecting whole blood from the animals, keeping it at room temperature for 30 minutes before it was centrifuged for 15 minutes at 3000 RPM. The serum was then collected and heat inactivated for 45 minutes at 56℃. After heat inactivation the serum from all animals in the same food group was pooled and 10% v/v was added to the cell culture.

For macrophage stimulation the cells were treated with 2 µg/ml LPS with or without 0.1 M indomethacin. For T cell stimulation the wells were pre-coated with 1 µg/ml antibody against CD3 (αCD3), 30 µl per well, for 45 minutes and then washed off with phosphate-buffered saline (PBS) before adding the suspended cells. The αCD3 treated cells were then further stimulated with 5 µg/ml antibody against CD28 (αCD28).

The cells were stimulated for 48 hours before the plates were centrifuged and the supernatants collected and stored at -70℃ for chemokine measure ments using commercially available enzyme linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, Minnesota, USA) (described below).

3.2 The effects of natural compounds on human monocytes/macrophages

Several compounds and extracts isolated from plants, lichens and a cyanobacteria were tested on the THP-1 human monocyte cell line. Some of the compounds were only tested once or twice and the data from those experiments can be found in appendix B.

3.2.1 Preparation of natural compounds

The preparation of the natural compounds and extracts was not part of this project. The compounds and extracts used were kindly provided by Elín S. Ólafsdóttir and Sesselja Ómarsdóttir and their students at Faculty of Pharmaceutical Sciences as well as other students of Jóna Freysdóttir and Ingibjörg Harðardóttir at the Faculty of Medicine.

3.2.1.1 Lichenan from Cetraria islandica

Cetraria islandica was collected and the lichenan extracted and purified as described in (115).

3.2.1.2 Polysaccharide extract from jelly lichens and Nostoc commune

The polysaccharide Cg-5-s1 from *Collema glebulentum* was isolated by Tanja Veselinovic in her MSc project with an alkali extraction, ethanol precipitations, dialysis and anion exchange chromatography and lyophilization (Figure 14, left).

The *Collema flaccidum* polysaccharide Cf-3-s2 was also isolated by Tanja Veselinovic. The lichen material was extracted with hot water and the polysaccharides precipitated from the supernatant using ethanol and the water soluble fraction Cf-3 further purified by anion-exchange chromatography and dialysis. The polysaccharide fractions were lyophilized (Figure 14, right).

As with the jelly lichens, both an aqueous and an alkali-extraction of *Nostoc commune* were made yielding three aqueous and two alkali extractions. The Nc-5 alkali extract was used for these experiments.

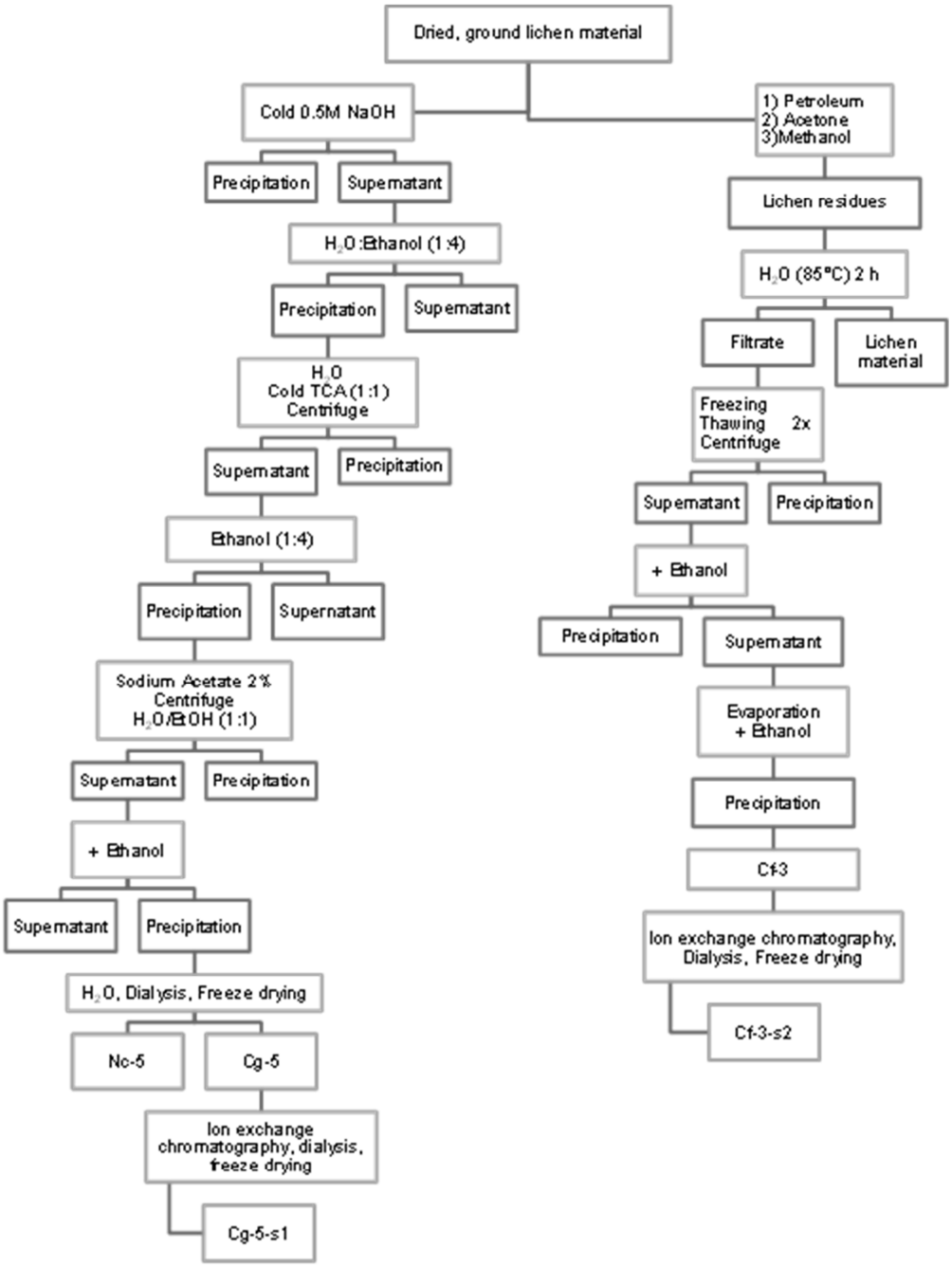

Figure 14. The extraction and purification of polysaccharides from the jelly lichens and *Nostoc commune*.

Dried, ground lichen or cyanobacterial material was either extracted with cold sodium hydroxide for alkaline extractions or with hot water for aqueous extractions. A series of purification steps yields the three polysaccharides used; Nc-5 from *Nostoc commune,* Cg-5-s1 from *Collema glebulentum* and Cf-3-s2 from *Collema flaccidum*.

3.2.2 Cell culture

The human monocytic cell line THP-1 was obtained from DSMZ (The German Collection of Microorganisms and Cell cultures, Braunschweig, Germany) and maintained in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum (FCS, Invitrogen), with penicillin (100 U/ml) and streptomycin (100 µg/ml). The cells were passaged twice a week. Before each passage, the cell concentration was determined using trypan blue staining with a Countess® cell counter. The cells were centrifuged and re-suspended in fresh medium at a concentration of 2.7 - 2.8x10^5 (depending on passage intervals, which were 3 or 4 days) in 75 cm^2 culture flasks. Cell-passages were noted, and each batch only passaged 25 to 28 times maximum.

For use in experiments cells were seeded at a density of 5x10^5 cells/ml in supplemented RPMI 1640 medium in 48 well plates, 1 ml/well.

When the THP-1 cells were used as activated undifferentiated monocytes, the cells were treated with 100 U/ml IFN-γ for three hours. When testing the effects of certain compounds on cytokine secretion, the THP-1 cells were activated with IFN-γ for 3 hours, and then the test compounds were added with the LPS and the cells cultured at 37℃, 5% CO_2 and 100% humidity for 48 hours. Then the plates were centrifuged, supernatants collected and stored at -80℃. When measuring the effects of test compounds on protein activation and expression, the compounds and IFN-γ were added to the cells simultaneously and 3 hours later they were stimulated with LPS for another 3 hours (Figure 15). Description of the development of these methods can be found in appendix A.

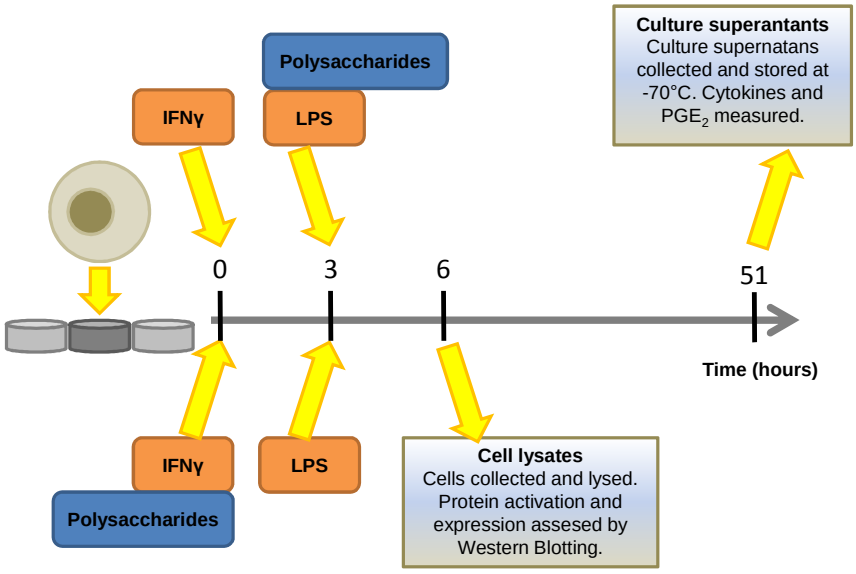

Figure 15. Culturing of THP-1 cells for measuring the effects of natural compounds on cytokine secretion as well as protein activation and expression.
Authors image.

3.2.3 Assays

3.2.3.1 ELISA

ELISA was used to measure mouse chemokines (KC, RANTES, MCP-1 and MIP-1α) as well as human cytokines (IL-6, IL-10, IL-12p40 and TNF-α). DuoSets® from R&D Systems were used according to their instructions.

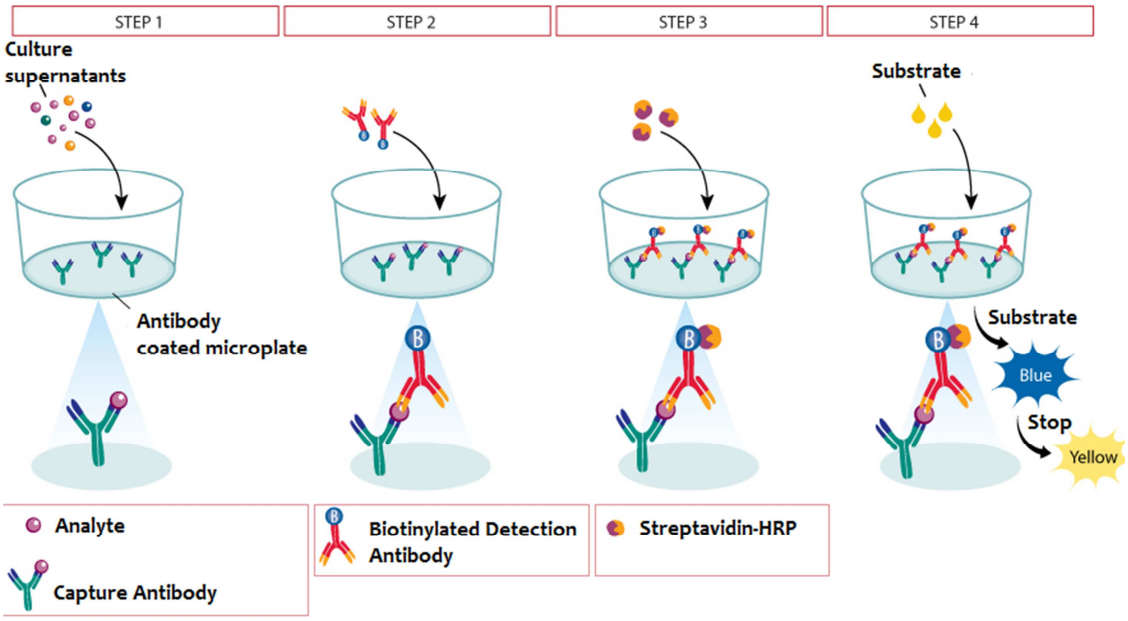

Figure 16. Enzyme linked immunosorbent assay.
The R&D Systems Sandwich ELISA DuoSet® kits are based on first coating a 96 well microplate with an analyte-specific capture antibody and then (step 1) applying culture supernatants or standards to the wells to bind to the immobilized antibodies. Any unbound material is washed away. In step 2 the antibody-bound antigen (the analyte) is bound by another, specific, biotinylated detection antibody. Step 3 is to bind streptavidin-HRP to the secondary antibody. The fourth step involves adding a substrate solution to the wells, forming a blue color in proportion to the amount of analyte present in the sample. The reaction is then stopped with an acidic solution and the absorbance in each well is then measured at 450nm. Image modified from the R&D Systems web-page.

When an R&D DuoSet® sandwich ELISA was performed a flat bottomed polystyrene 96-well MaxiSorp™ plate (Nunc, Roskilde, Denmark) specially modified to bind glycoproteins such as antibodies was coated with an analyte-specific capture antibody by applying the antibody dissolved in PBS and incubating at room temperature away from light overnight. The next day the solution was removed and a blocking solution applied (1% BSA in PBS for the mouse chemokine ELISAs, 1% BSA in PBS with 5% sucrose and 0.5% azide for the human cytokine ELISAs) and incubated for one hour at room temperature away from light. The proteins in the blocking solution then bound to any free space on the plate surface not bound with the capture antibody to avoid unspecific binding by proteins in the sample solution to the plate surface. The plates were then washed three to four times with a wash buffer (PBS with 0.05% Tween-20) and experimental samples added (Figure 16, step 1).

Sample dilution was different for each protein being tested but all samples were diluted in sample buffer (1% BSA in PBS). Seven two-fold dilutions of a standard provided in each DuoSet® was added to the plate and the blank sample for background measurements was sample buffer alone. After incubating the samples away from light at room temperature for two hours, the plate was washed as before. A solution of analyte-specific detection antibody dissolved in sample buffer was then applied to the wells and incubated at room temperature for two hours (Figure 16, step 2). Next the plate was washed again and streptavidin-horse-radish peroxidase (HRP) solution added to the wells (Figure 16, step 3). During a twenty-minute incubation period (at room temperature, away from light) the

streptavidin-HRP bound to the secondary antibody. Unbound Streptavidin-HRP was washed away and a substrate solution added, allowing the HRP to catalyze a reaction, turning the colorless substrate to a blue solution (Figure 16, step 4). This reaction was allowed to continue for about 15-20 minutes before it was stopped by adding 0.18 M sulfuric acid to the wells, turning the solution yellow. The absorbance of each of the sample and standard wells was then measured at 450 nm using a spectrophotometer. A standard curve was made using the known analyte-concentration of the standards and the concentration of analyte in each sample calculated using the equation found by regression calculations of the standard. Each sample was measured in duplicate and the average concentration calculated.

3.2.3.2 Nitric oxide measurements

Since NO is a highly volatile substance it is nearly impossible to measure directly; however, its formation can be measured indirectly using the Griess reaction (Promega, Madison, WI). The Griess reaction is based on the reaction of NO_2^- with sulfanilamide and *N*-1-napthylethylenediamine dihydrochloride (NED) under acidic conditions to form a colored azo product.

Samples or standards (50 µl, 0 to 100 µM, diluted in culture medium like the samples) were pipetted into a 96 well assay-plate and mixed with 50 µl of the sulfanilamide solution (at room temperature). The mixture was then incubated for 5-10 minutes away from light before adding 50 µl of the NED solution and incubating for another 5-10 minutes. Formation of the purple/magenta azo product was then measured immediately at 540 nm using a spectrophotometer with a plate reader.

3.2.3.3 Western blot

After treating the THP-1 cells with IFN-γ and the natural compounds being tested for 3 hours, the cells were stimulated with LPS for another 3 hours. Then cells were centrifuged and the supernatants collected and stored at -80℃. The cells were then washed with ice cold PBS and centrifuged again. Following the protocol provided with the Western immunoblotting antibodies (Cell Signaling Technology, Boston MA) the cells were lysed with the addition of 1X SDS sample buffer (containing 62.5 mM Tris-HCl, 2% w/v SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.01% bromophenol blue). The samples were then sonicated to shear DNA and reduce sample viscosity before heating to 95℃ for 5 minutes. Samples were then centrifuged again before loading onto a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel.

Samples were electrophoresed on 10% SDS-PAGE gels (for MAPKs) or 7.5% gels (for iNOS, COX-2 or NF-κB). Proteins were then blotted onto a polyvinylidene fluoride (PVDF) membrane by 1 hour electrotransfer. After transfer the membranes were washed with Tris-buffered saline (TBS) and then incubated in blocking buffer (1X TBS, 0.1% Tween, 5% w/v non-fat dry milk) for 1 hour at room temperature.

Membranes were then washed three times with TBS-Tween (1X TBS with 0.1% Tween) before incubation with the primary antibodies for phosphorylated or total proteins (where applicable), diluted in primary-antibody dilution buffer (1X TBS, 0.1% Tween and 5% w/v BSA) overnight at 4℃.

Membranes were then washed three times with TBS-Tween before incubation with HRP-conjugated secondary antibody in blocking buffer for 1 hour at room temperature. A LumiGLO® (Cell

Signaling Technology) reagent was used to detect the proteins on the membrane by generating light from the HRP-conjugated secondary antibody (Figure 17). Lastly, the membranes were exposed to X-ray film and the films developed.

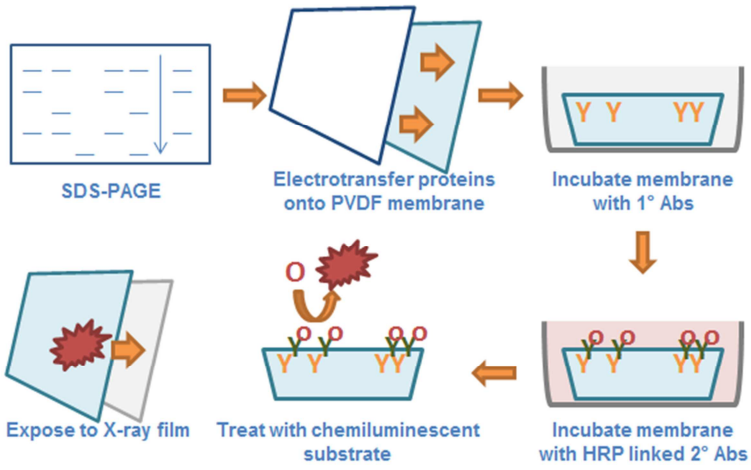

Figure 17. Western blot.
Proteins are electrotransferred from a 7.5-10% SDS PAGE gel onto a PVDF membrane. There they are incubated with primary antibodies specific for the protein in question, then with HRP conjugated secondary antibodies and then a LumiGLO® reagent is used to generate a chemiluminescent signal which is then exposed to X-ray film to generate an image (Authors image).

Next the membranes were stripped using a gentle stripping buffer (1.5% glycine, 0.1% SDS and 1% Tween in ultra-pure water) for 30 minutes at room temperature. The stripping buffer was then discarded and fresh stripping buffer added and the membrane incubated for another 10 minutes. Membranes were then washed twice with TBS before blocking the membranes again with BSA blocking buffer (TBS, 0.1% Tween and 5% BSA). The membranes were then incubated again with primary antibodies for total proteins (where applicable) or household proteins overnight and the process repeated.

The X-ray films exposed to the antibody signal were then scanned with a desktop scanner to digitize the image. The images were then inverted and digitally treated to remove background distortion using ImageJ software developed for processing various types of scientific images. Each lane was then marked for measuring (see Figure 18).

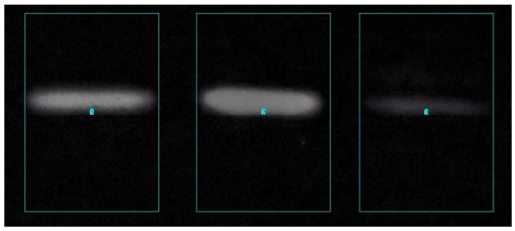

Figure 18. An example of a Western blot image inverted and processed with ImageJ to remove background.
Each lane is marked manually before measuring the intensity of the band. Authors image.

The software measures the intensity of each band in a marked lane. The readout is presented as peaks of various sizes according to the intensity of the bands (see Figure 19).

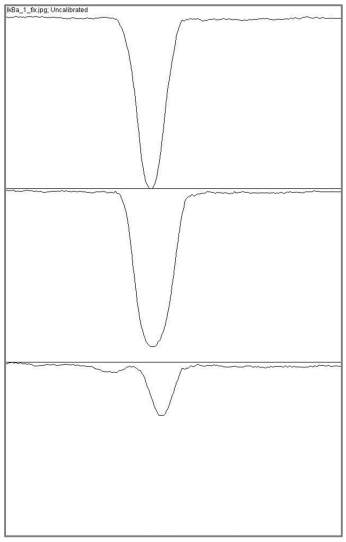

Figure 19. ImageJ readout of the measured intensities for each band in a lane.
Each band in a lane comes as one peak quantifiable individually (Authors image).

3.2.3.4 Prostaglandin E_2 measurements

PGE_2 was measured in culture supernatants after 3 hour IFN-γ treatment followed by LPS and polysaccharide treatment for 48 hours using an EIA kit (Cayman Chemical). Three individual experiments were measured.

The assay is based on a competition between PGE_2 in a sample or standard and a PGE_2-acetylcholinesterase conjugate (tracer) for a limited amount of PGE_2 monoclonal antibody (mAb) (Figure 20). The more PGE_2 there is in the sample or standard, the less room there is for the tracer to bind to the mAb. Pre-coated plates were incubated with samples or standards and tracers and mAb. The mAb then binds to polyclonal goat anti-mouse antibodies bound to the plate. All unbound material was then removed. Since the tracer is conjugated with acetylcholinesterase, addition of the Ellman's reagent (acetylcholinesterase substrate) to the wells caused a reaction resulting in a color change. The color was then measured using a spectrometer and the concentration of PGE_2 in the samples calculated using a standard curve plotted with standards of known concentration.

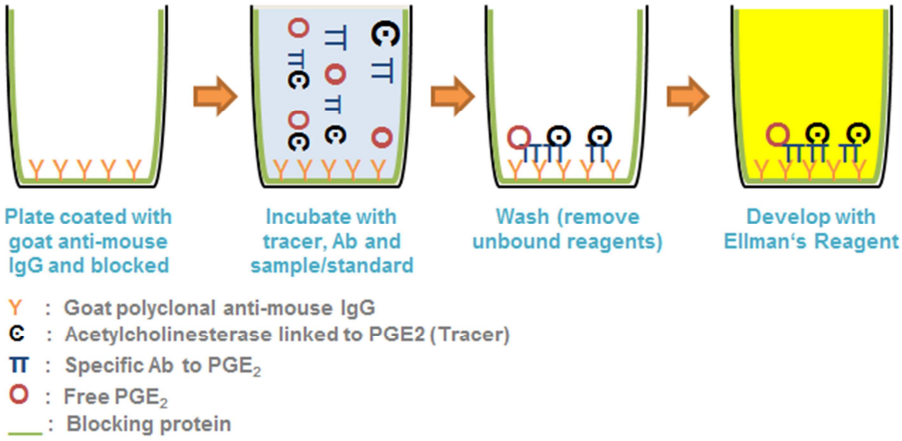

Figure 20. Competitive EIA for PGE_2.

A 96-well plate was coated with goat anti-mouse IgG and blocked with blocking proteins. Then tracer (Acetylcholinesterase linked to PGE_2), PGE_2 specific antibodies and the sample or standard are added to the wells. Unbound reagents are then washed off and the solution developed using Ellman's Reagent (Authors image based on information from Cayman Chemical).

3.2.4 Statistical Analysis

Results were expressed as the mean ± standard error of the mean (SEM) for each treatment group. The results for cytokine concentrations are presented as a secretion index (SI), which is the amount of cytokines secreted by cells treated with polysaccharides divided by the cytokine secretion of cells not treated with polysaccharides. T-test (when only two groups were being compared) or ANOVA, followed by Tukey's post hoc test were used to compare the groups using SigmaStat, with $P \leq 0.05$ being considered significant.

4 Results

4.1 The effects of dietary fish oil on chemokine secretion by total murine spleen cells

Murine splenocytes from mice fed fish oil and mice fed a control diet were isolated and stimulated *ex vivo* with agents affecting either monocytes/macrophages or T cells and their responses analyzed.

4.1.1 The effects of dietary fish oil on LPS stimulated chemokine secretion by murine total spleen cells

The effects of dietary fish oil on the chemokine secretion of total spleen cells in response to LPS was tested (Figure 21).

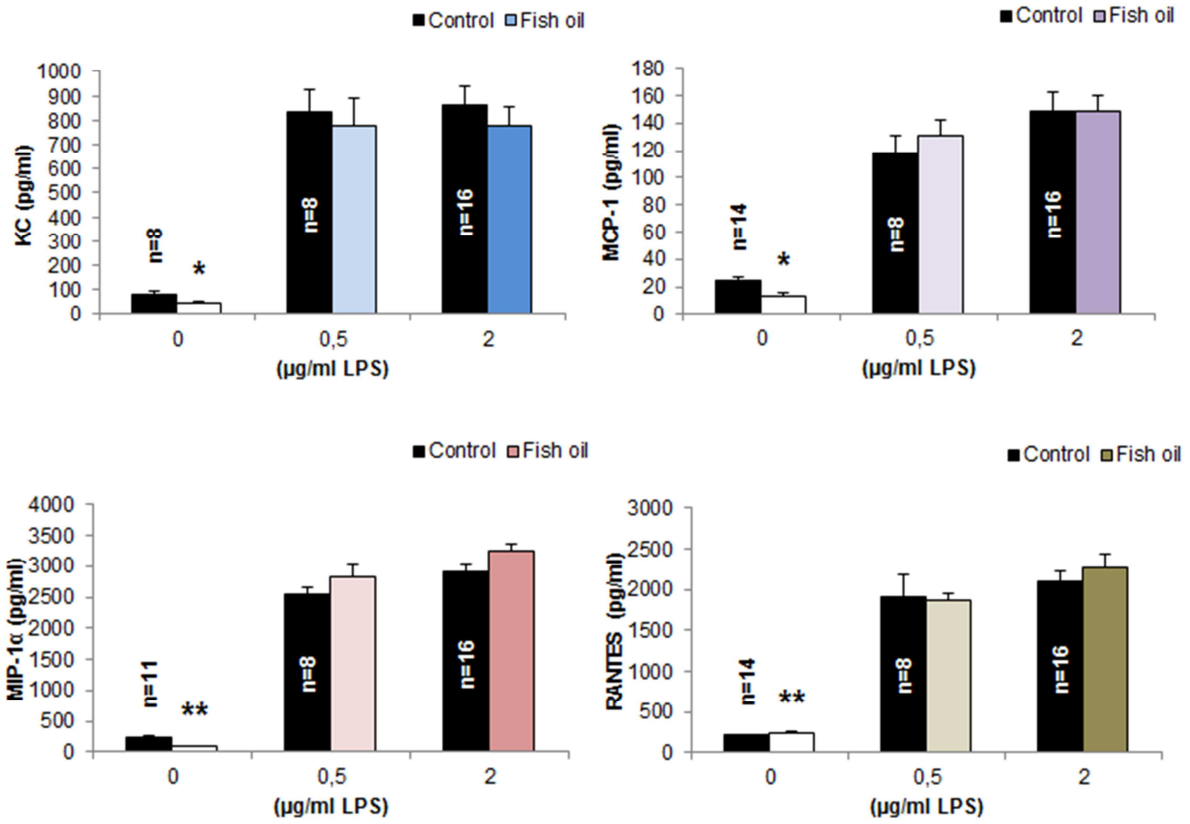

Figure 21. The effects of dietary fish oil on total spleen cells stimulated with LPS on chemokine secretion.
Total spleen cells were seeded into flat bottomed 96 well plates, $1x10^6$ cells/ml, 0.2 ml/well supplemented with heat inactivated serum from animals of the same dietary group. An addition of 0.5 or 2 µg/ml LPS was added to the wells and the plates incubated for 48 hours. The plates were then centrifuged and the culture supernatants collected and stored at -80℃ until measured using an R&D DuoSet ELISA kit for the chemokines. The bars represent a mean value of 8 to 16 mice as indicated. Error bars represent the SEM. Statistically significant difference is indicated with * (P<0.05) or ** (P<0.01).

The dietary fish oil had no statistically significant effect on the chemokine secretion in the LPS stimulated splenocytes in either of the concentrations tested (0.5 and 2 µg/ml). There was, however, a significant reduction in the secretion of KC, MCP-1 and MIP-1α and increase in the secretion of

RANTES by unstimulated splenocytes from mice fed the fish oil diet compared with unstimulated splenocytes from mice fed the control diet.

The same setup was also used to test whether addition of the COX inhibitor indomethacin affected the chemokine secretion by the splenocytes (Figure 22).

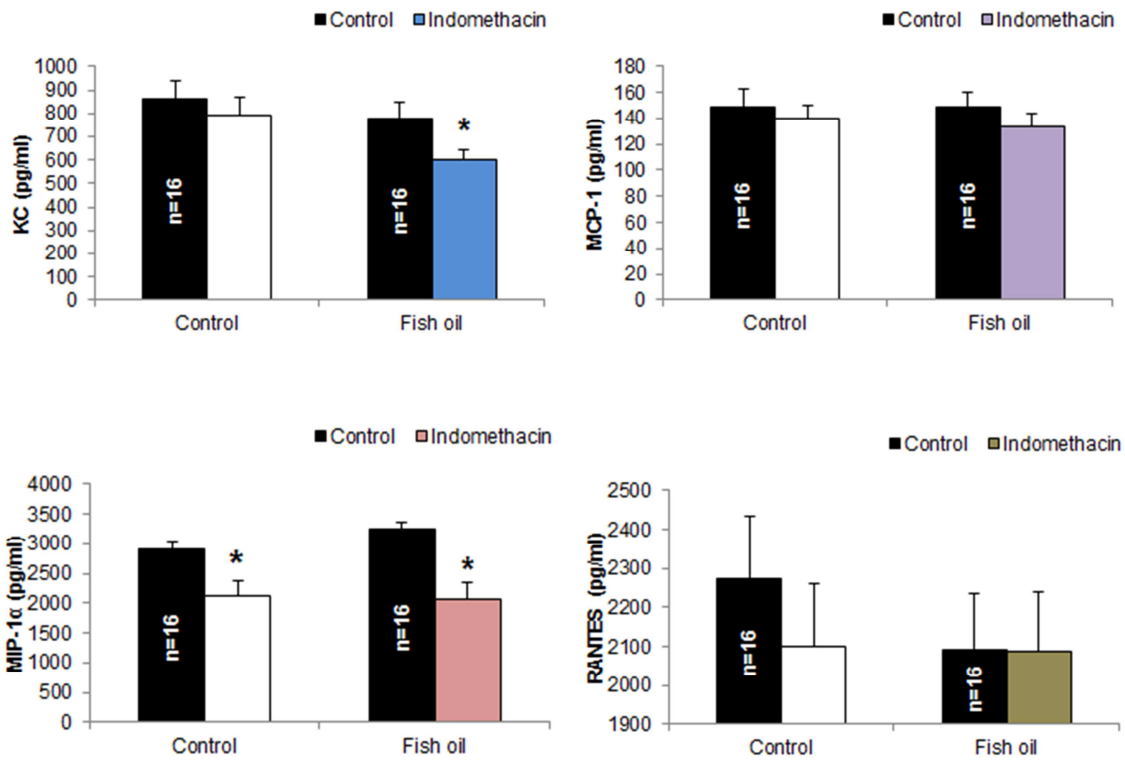

Figure 22. The effects of indomethacin on LPS stimulated murine total spleen cells.
Total spleen cells were seeded into flat bottomed 96 well plates, $1x10^6$ cells/ml, 0.2 ml/well supplemented with heat-inactivated serum from animals of the same dietary group. An addition of 2 µg/ml LPS was added to the wells and the indomethacin added simultaneously. The plates were then incubated for 48 hours. The plates were then centrifuged and the culture supernatants collected and stored at -80℃ until measured using an R&D DuoSet ELISA kit for the chemokines. The bars represent a mean value of 16 mice as indicated. Error bars represent the SEM. Statistically significant difference is indicated with * ($P<0.05$).

Indomethacin significantly decreased the secretion of MIP-1α by splenocytes of both dietary groups, and also the secretion of KC by splenocytes from mice fed fish oil. Indomethacin caused a tendency towards a reduction in RANTES secretion from splenocytes from the control group.

4.1.2 The effects of dietary fish oil on αCD3/αCD28 stimulated chemokine secretion by murine total spleen cells

The effects of dietary fish oil on the chemokine secretion of total spleen cells in response to stimulation through CD3 and CD28 was tested (Figure 23)

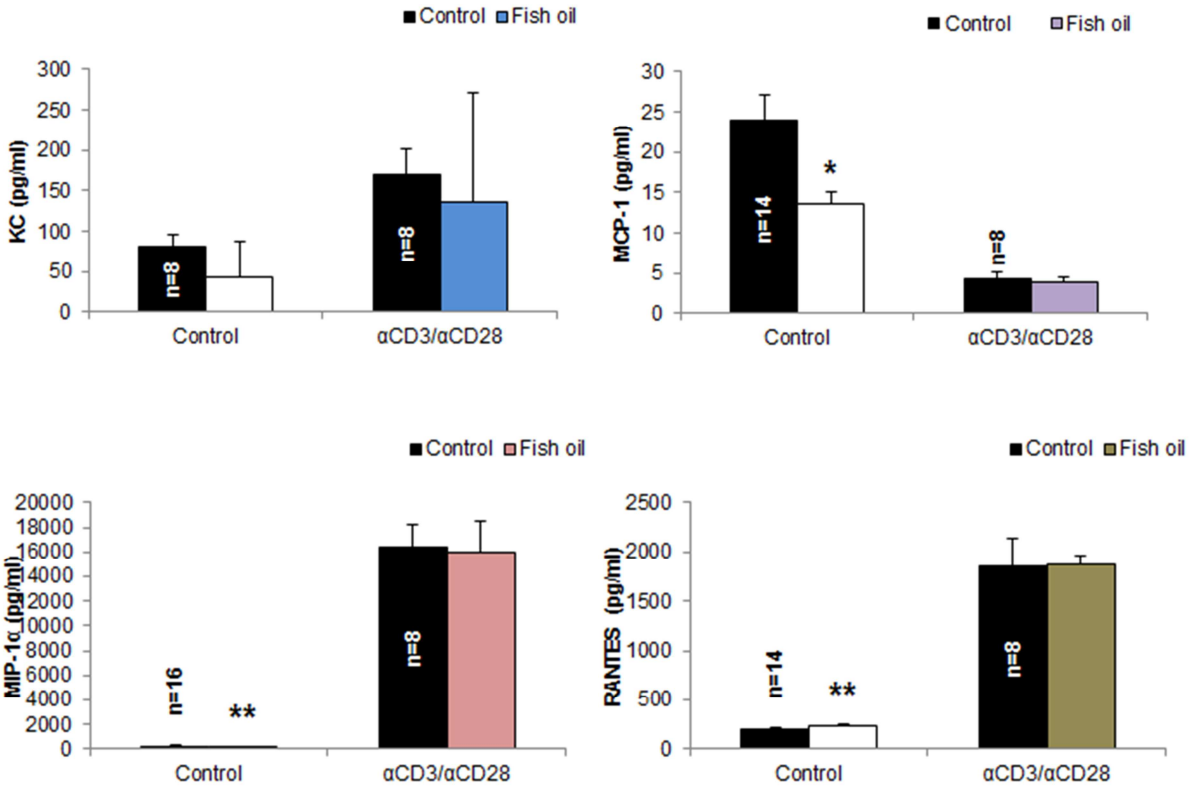

Figure 23. The effects of dietary fish oil on αCD3 and αCD28 stimulated murine splenocytes.
Total spleen cells were seeded into flat bottomed 96 well plates ($1x10^6$ cells/ml, 0.2 ml/well) previously coated with 1 µg/ml αCD3 antibodies. The culture was then supplemented with heat inactivated serum from animals of the same dietary group. An addition of 5 µg/ml αCD28 was added to the wells for co-stimulation. The plates were incubated for 48 hours. The plates were then centrifuged and the culture supernatants collected and stored at -80℃ until measured using an R&D DuoSet ELISA kit for the chemokines. The bars represent a mean value of 8-16 mice as indicated. Error bars represent the SEM. Statistically significant difference is indicated with * ($P<0.05$) or ** ($P<0.01$).

As observed in previous experiments, unstimulated splenocytes from mice fed the fish oil diet secreted less MCP-1 and MIP-1α and increased levels of RANTES compared with unstimulated splenocytes from mice fed the control diet (Figure 23). No difference in chemokine secretion was observed by αCD3/αCD28 stimulated spleen cells between the two dietary groups (Figure 23).

4.2 The effects of extracts from Icelandic lichens and cyanobacteria on THP-1 monocytes

The effects of extracts, fractions and compounds from several plants, lichens and cyanobacteria on cytokine secretion by THP-1 monocytes were analyzed. Many of these biomaterials were only tested once or twice and thus no statistics or clear conclusions can be drawn from their results. A compilation of the background, purification and results from these experiments can be found in Appendix B. The polysaccharides purified from three lichens and a cyanobacteria were tested more extensively and remain the only results presented in the following chapters.

4.2.1 The effects of the polysaccharide lichenan from *Cetraria islandica* on cytokine secretion by THP-1 monocytes

To analyze the effects of lichenan, a polysaccharide purified from the lichen *Cetraria islandica*, on the activation of THP-1 monocytes, their secretion of cytokines was measured.

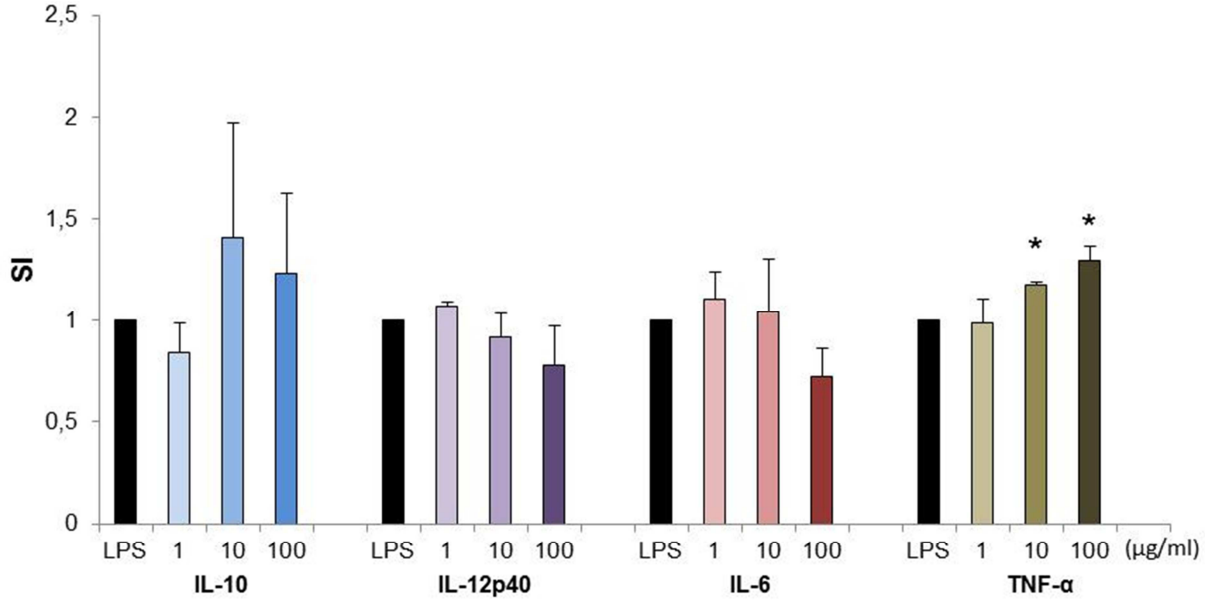

Figure 24. The effects of lichenan, a *Cetraria islandica* polysaccharide, on THP-1 monocyte cytokine secretion.
The THP-1 monocytes were activated for 3 h using IFN-γ and then stimulated with LPS along with the addition of lichenan in different concentrations for 48 hours. A concentration gradient of 1 to 100 µg/ml was tested. Cytokine concentrations were then measured using DuoSet ELISA kits from R&D systems. The bars represent a mean of the secretion index values for 4 different experiments (n=4). Error bars represent the SEM. A significant difference of tested substance vs. LPS control (black bars) is denoted with: * (for $P<0.05$) or ** (for $P<0.01$).

Lichenan significantly increased the TNF-α secretion by THP-1 monocytes at both 10 and 100 µg/ml concentrations after 48 hours of treatment (Figure 24). There was a slight tendency towards a reduction in IL-12p40 and IL-6 secretion but this was not statistically significant.

To shed light on whether these effects were possibly mediated by prostaglandins, indomethacin, a COX inhibitor, was added to the THP-1 culture along with the lichenan (Figure 25). The culturing was otherwise performed as before.

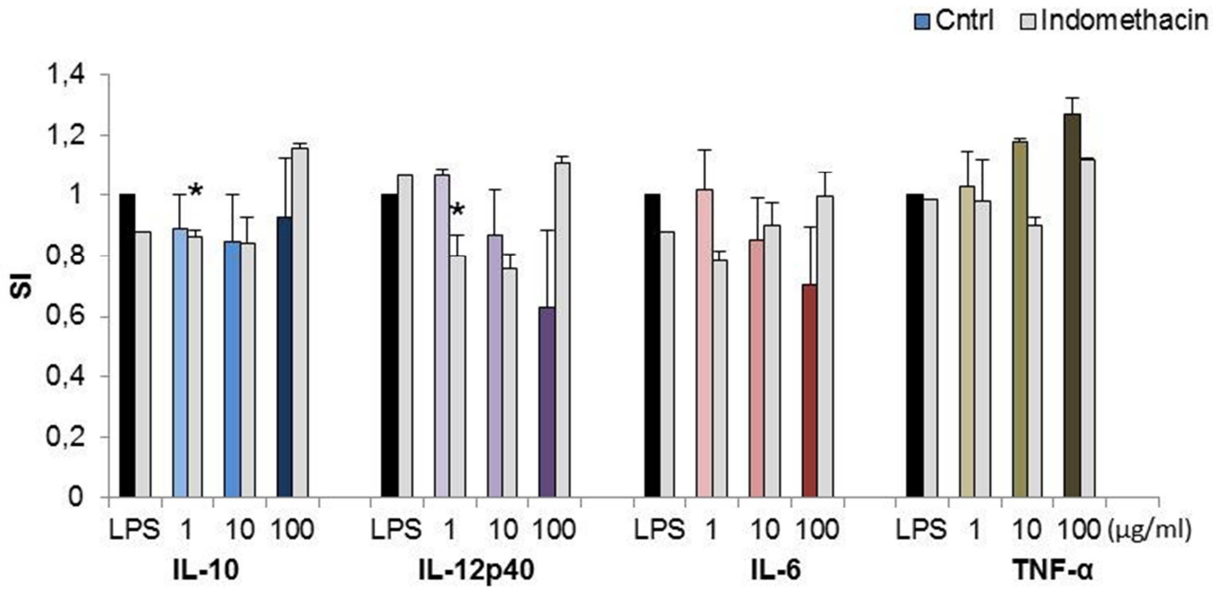

Figure 25. The effect of indomethacin on THP-1 monocytes treated with lichenan.
The cells were pre-treated with IFN-γ for 3 hours as before and then stimulated with 0.75 µg/ml LPS, lichenan in different concentrations with and without indomethacin for 48 hours. As before, a lichenan concentration gradient of 1 to 100 µg/ml was tested. Cytokine concentrations were then measured using DuoSet ELISA kits from R&D systems. The bars represent a mean of the stimulation index values for 2-3 different experiments (the indomethacin was tested on cells treated with 10 µg/ml lichenan in 3 different experiments but only twice with 1 and 100 µg/ml). The two control bars (black and gray) represent the stimulated control (LPS) and a stimulated control treated with indomethacin. Error bars represent the standard error of the mean (SEM). A significant difference of lichenan vs. lichenan and indomethacin is denoted with: * (for $P<0.05$).

Indomethacin significantly lowered IL-12p40 secretion when tested with 1 µg/ml of lichenan (Figure 25). However at the highest tested concentration of lichenan (100 µg/ml) there was a tendency for higher secretion of IL-10, IL-12p40 and IL-6 but this was not significant.

4.2.2 The effects of the polysaccharide Cg-5-s1 from *Collema glebulentum* on THP-1 monocytes

To evaluate the effects of the polysaccharide Cg-5-s1 from *Collema glebulentum* on the activation of THP-1 cells, secretion of cytokines and PGE_2 were measured.

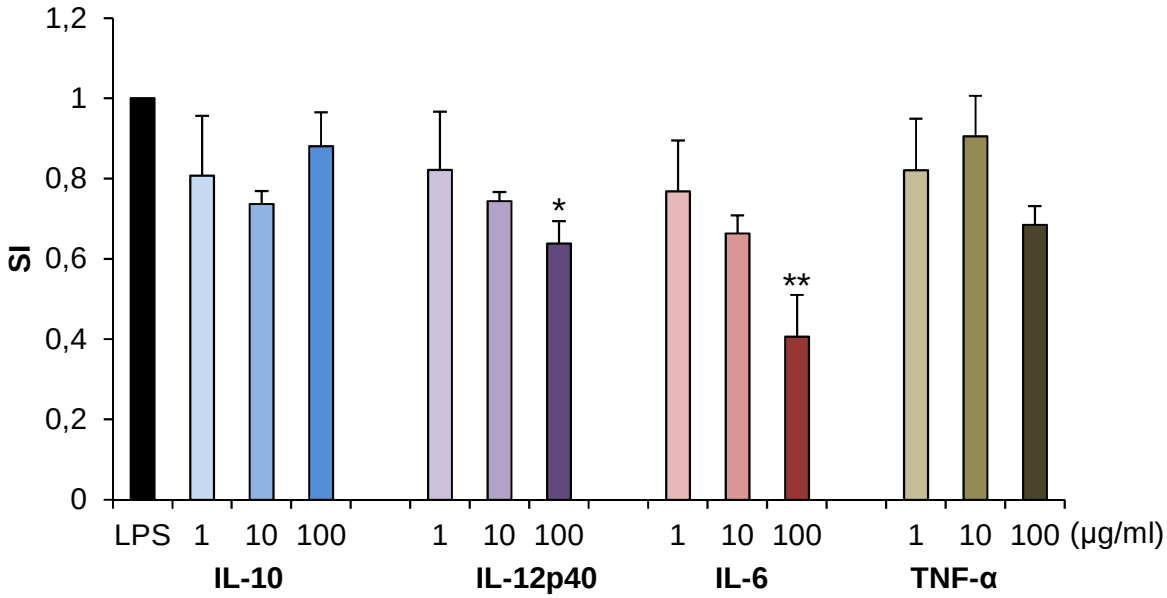

Figure 26. The effects of a *Collema glebulentum* polysaccharide on THP-1 monocyte cytokine secretion.
The THP-1 monocytes were activated for 3 h using IFN-γ and then stimulated with LPS along with the addition of Cg-5-s1 from *Collema glebulentum* in a concentration gradient of 1 to 100 µg/ml. Cytokine concentrations were then measured using DuoSet ELISA kits from R&D systems. The bars represent a mean of the secretion index values for 4 different experiments (n=4). Error bars represent the SEM. A significant difference of tested substance vs. LPS control (black bars) is denoted with: * (for $P<0.05$) or ** (for $P<0.01$).

The highest concentration of the polysaccharide Cg-5-s1 from *Collema glebulentum* caused a statistically significant decrease in IL-12p40 and IL-6 production by the THP-1 cells after 48 hour treatment when compared with THP-1 cells treated with LPS alone (Figure 26).

As with lichenan, an addition of indomethacin to the culture was tested to check for possible prostaglandin involvement.

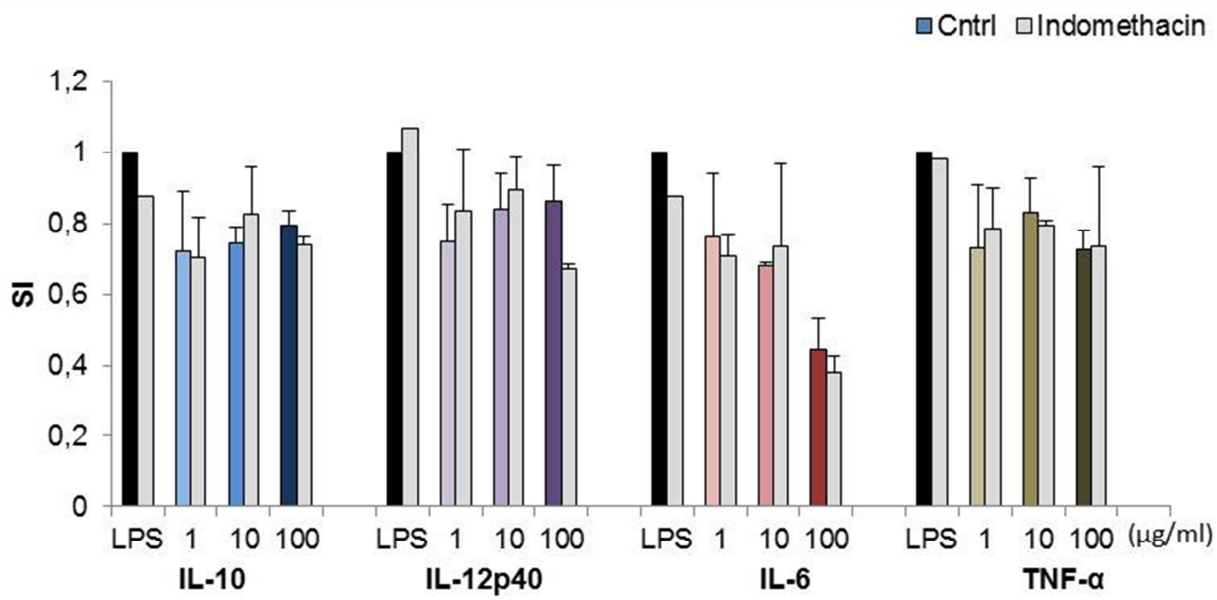

Figure 27. The effect of indomethacin on THP-1 monocytes treated with Cg-5-s1.
The cells were pre-treated with IFN-γ for 3 h as before and then stimulated with 0.75 µg/ml LPS, Cg-5-s1 in a concentration gradient of 1 to 100 µg/ml with or without indomethacin for 48 h Cytokine concentrations were then measured using DuoSet ELISA kits from R&D systems. The bars represent a mean of the secretion index values for 2-3 different experiments (the indomethacin was tested on cells treated with 10 µg/ml of the *Collema glebulentum* polysaccharide in 3 different experiments but only twice with 1 and 100 µg/ml). Error bars represent the SEM. There was no significant difference between samples treated with and the ones treated without the indomethacin.

The indomethacin treatment had no effect on the cytokine secretion by the THP-1 cells when added to cells treated with Cg-5-s1 in all concentrations tested (Figure 27). Secreted PGE_2 was also measured and there was no difference between the untreated and polysaccharide treated THP-1 cells (Figure 28).

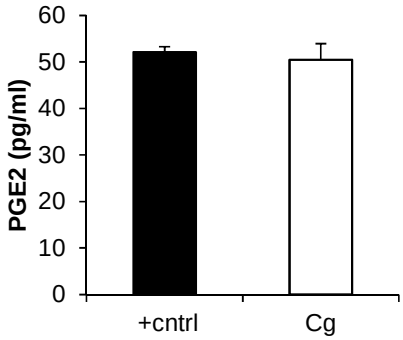

Figure 28. The effect of Cg-5-s1 on PGE_2 secretion.
The cells were treated as described in the Figure 26 legend with the exception that only 100 µg/ml of Cg-5-s1 was tested. The samples were then measured using a PGE_2 EIA kit (Cayman). Bars represent a mean of three experiments (n=3). Error bars represent the SEM. There was no statistically significant difference between the stimulated control and the Cg-5-s1 polysaccharide treated cells.

4.2.3 The effects of the polysaccharide Cf-3-s2 from *Collema flaccidum* on THP-1 monocytes

The effects of the polysaccharide Cf-3-s2 from *Collema flaccidum* on the activation of THP-1 monocytes was tested by measuring the secretion of cytokines and PGE_2.

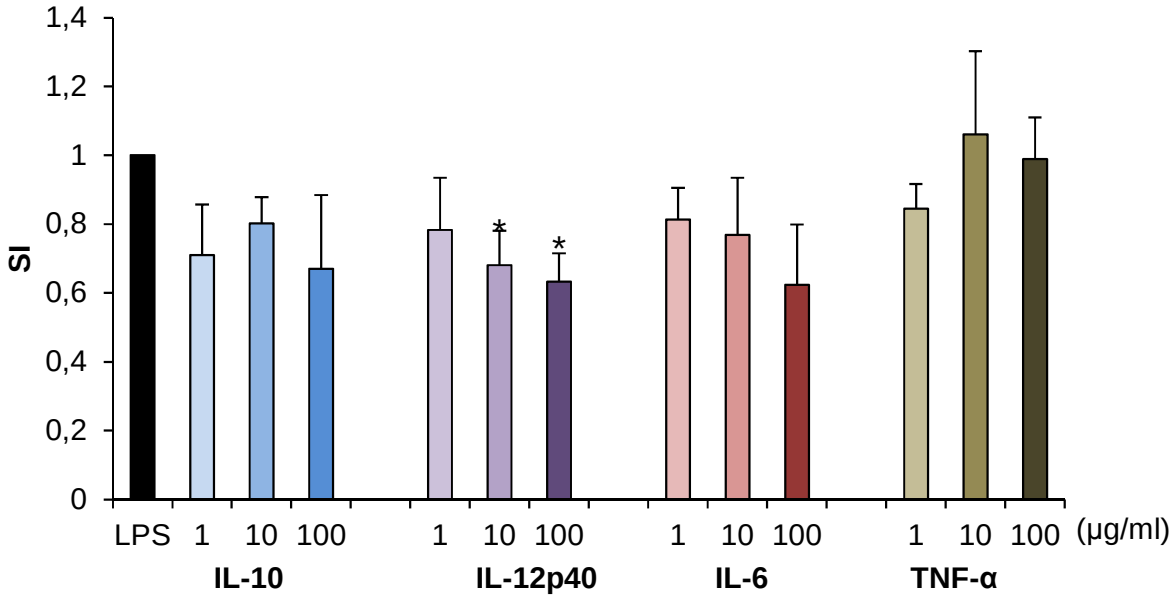

Figure 29. The effects of Cf-3-s2, a *Collema flaccidum* polysaccharide on THP-1 monocyte cytokine secretion.
The THP-1 monocytes were activated for 3 h using IFN-γ and then stimulated with 0.75 µg/ml LPS along with the addition of Cf-3-s2 in a concentration gradient of 1 to 100 µg/ml. Cytokine concentrations were then measured using DuoSet ELISA kits from R&D systems. The bars represent a mean of the secretion index values for 4 different experiments (n=4). Error bars represent the SEM. There was no significant difference in any of the concentrations tested. A significant difference of tested substance vs. LPS control (black bars) is denoted with: * (for $P<0.05$)

After a 48 hour treatment with Cf-3-s2 the THP-1 cells secreted less IL-12p40 than the LPS stimulated control (Figure 29).

As before, indomethacin addition was tested with the Cf-3-s2 treatment of stimulated THP-1 cells to shed light on any possible prostaglandin effects (Figure 30).

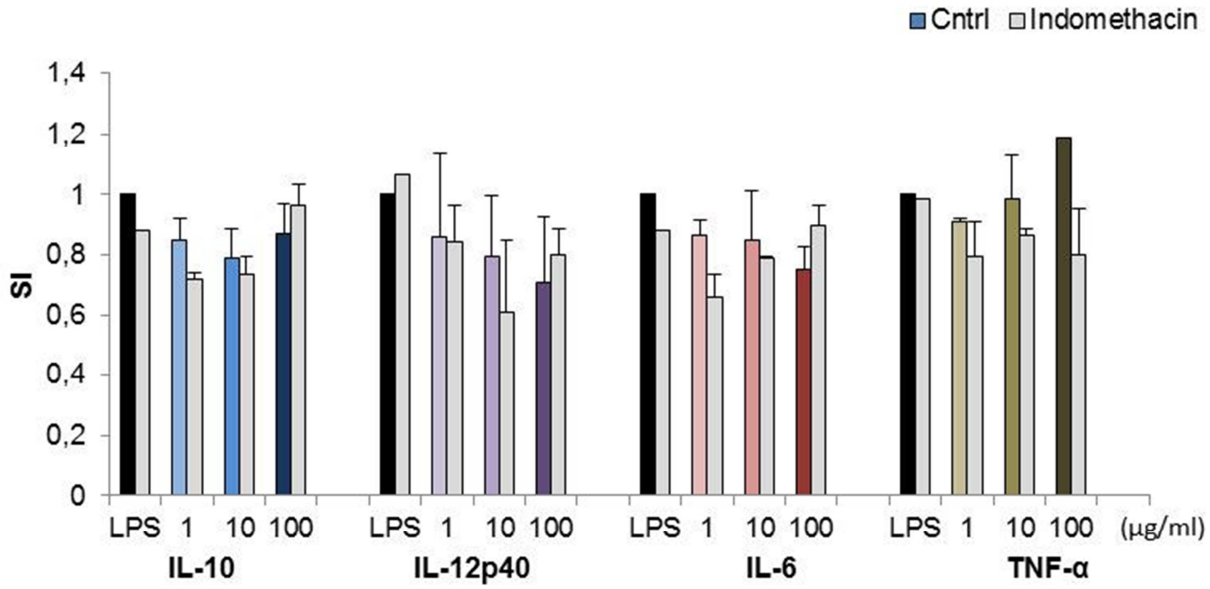

Figure 30. The effect of indomethacin on THP-1 monocytes treated with Cf-3-s2.

The cells were pre-treated with IFN-γ for 3 hours as before and then stimulated with 0.75 µg/ml LPS, Cf-3-s2 at concentrations from,1 to 100 µg/ml with or without indomethacin for 48 hours. Cytokine concentrations were then measured using DuoSet ELISA kits from R&D systems. The bars represent a mean of the secretion index values for 2-3 different experiments (the indomethacin was tested on cells treated with 10 µg/ml Cf-3-s2 polysaccharides in 3 different experiments but only twice with 1 and 100 µg/ml). There was no significant difference between the samples treated with and the ones treated without the indomethacin.

Indomethacin had no statistically significant effect on the cytokine secretion (Figure 30). Cf-3-s2 had no effect on the PGE_2 secretion by the THP-1 cells compared with untreated THP-1 cells (Figure 31).

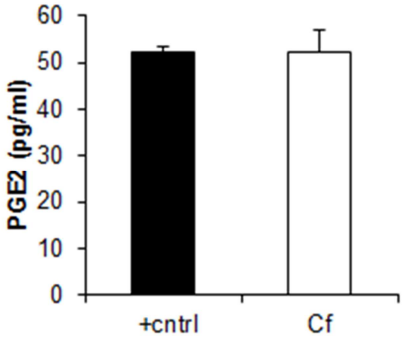

Figure 31. The effect of Cf-3-s2 on THP-1 PGE_2 secretion.

The cells were treated as described in the Figure 29 legend with the exception that only 100 µg/ml of Cf-3-s2 was tested. The samples were then measured using a PGE_2 EIA kit (Cayman). Bars represent a mean of three experiments (n=3). Error bars represent the SEM. There was no statistically significant difference between the stimulated control and the Cf-3-s2 treated cells.

4.2.4 The effects of the polysaccharide Nc-5 from *Nostoc commune* on THP-1 monocytes

To investigate whether the polysaccharide Nc-5 from *Nostoc commune* would affect the activation of THP-1 cells, secretion of cytokines and PGE_2 were measured.

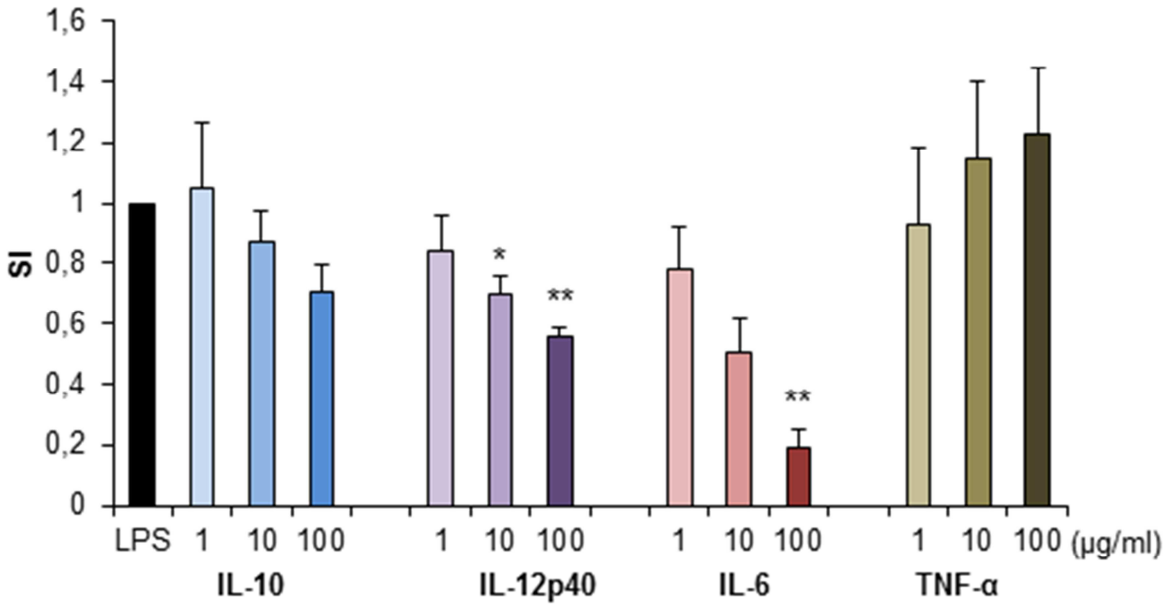

Figure 32. The effects of Nc-5 from *Nostoc commune* on THP-1 monocyte cytokine secretion. The THP-1 monocytes were activated for 3 h using IFN-γ and then stimulated with LPS along with the addition of Nc-5 in a concentration gradient of 1 to 100 µg/ml.. Cytokine concentrations were then measured using DuoSet ELISA kits from R&D systems. The bars represent a mean of the stimulation index values for 4 different experiments (n=4). Error bars represent the SEM. A significant difference of tested substance vs. LPS control (black bars) is denoted with: * (for $P<0.05$) or ** (for $P<0.01$).

The polysaccharide Nc-5 from *Nostoc commune* significantly lowered the secretion of both IL-12p40 and IL-6 when stimulated THP-1 monocytes were treated with 100 µg/ml of the extract (Figure 32). There was a tendency towards less IL-10 secretion as well as an increase in TNF-α secretion but neither reached statistical significance.

The effect of indomethacin treatment on the cytokine secretion of Nc-5 polysaccharide by activated THP-1 cells was analyzed (Figure 33).

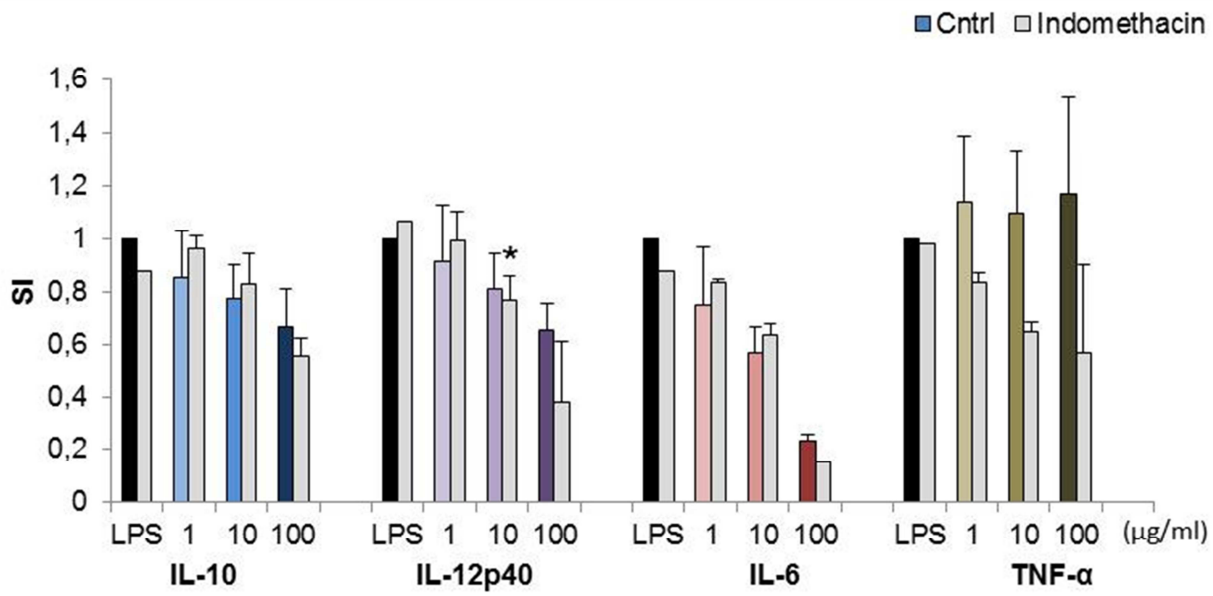

Figure 33. The effect of indomethacin on THP-1 monocytes treated with Nc-5.

The cells were pre-treated with IFN-γ for 3 hours as before and stimulated with 0.75 µg/ml LPS, Nc-5 at concentrations from 1 to 100 µg/ml with or without indomethacin for 48 hours. Cytokine concentrations were then measured using DuoSet ELISA kits from R&D systems. The bars represent a mean of the secretion index values for 2-3 different experiments (the indomethacin was tested on cells treated with 10 µg/ml Nc in 3 different experiments but only twice with 1 and 100 µg/ml). Error bars represent the SEM. A significant difference of the Nc-5 polysaccharides alone vs. Nc-5 and indomethacin is denoted with: * (for $P<0.05$).

Indomethacin had a slight, yet significant effect on the IL-12p40 secretion when the THP-1 cells were treated with 10 µg/ml of Nc-5. The effect of the Nc-5 on secreted PGE_2 was also tested and there was no difference in PGE_2 levels between stimulated THP-1 cells treated with Nc-5 and the THP-1 cells stimulated without the polysaccharide (Figure 34).

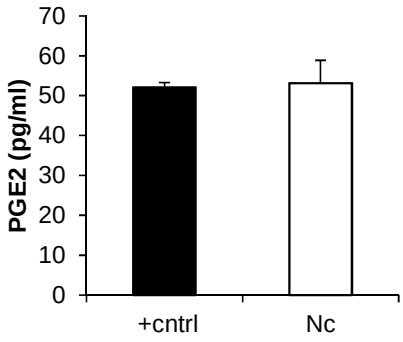

Figure 34. The effect of Nc-5 on THP-1 PGE_2 secretion.

The cells were treated as described in the Figure 32 legend with the exception that only 100 µg/ml of Nc-5was tested. The PGE_2 in the samples was then measured using a PGE_2 EIA kit (Cayman). Bars represent a mean of three experiments (n=3). Error bars represent the SEM. There was no difference between the stimulated control and the Nc-5 treated cells.

4.2.5 The effect of lichen and cyanobacterial polysaccharides on MAP kinase activation

To assess if the polysaccharides affected the activation of MAPKs, Western blotting was used to measure both total and phosphorylated p38, ERK and JNK (Figure 35).

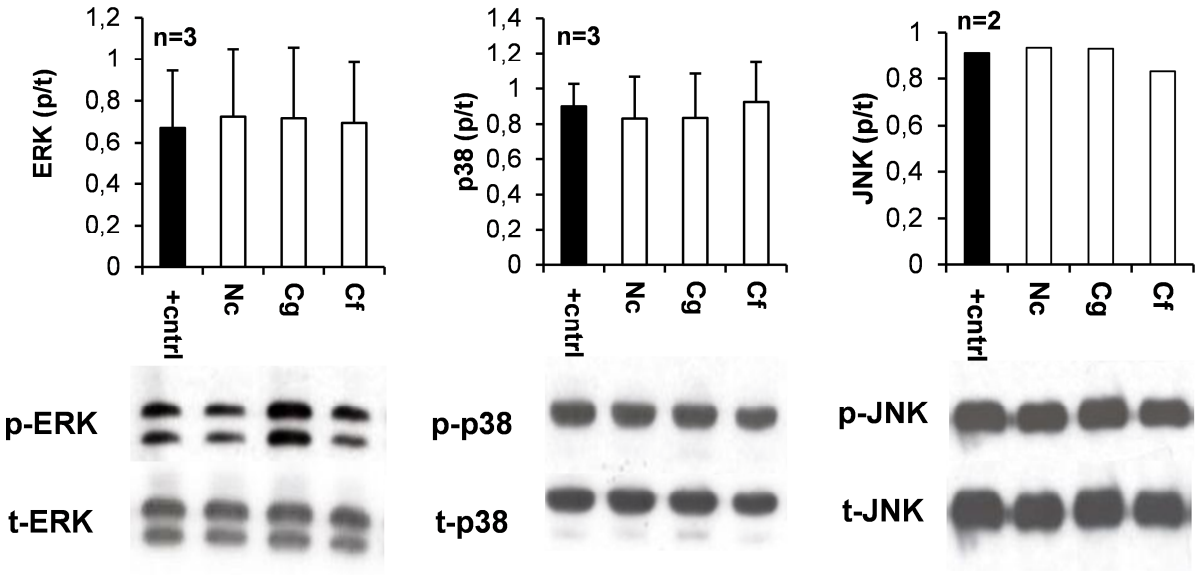

Figure 35. The effects of Nc-5, Cg-5-s1 and Cf-3-s2 on the phosphorylation of MAPKs. THP-1 cells were seeded as usual, pre-treated with polysaccharides and IFN-γ for 3 hours and then stimulated with 0.75 µg/ml LPS for an additional 3 hours. The cells were then spun down and washed with ice cold PBS and lysed in warm sample buffer according to the protocol provided with the antibodies by the manufacturer. The samples were then separated using SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked with non-fat milk, incubated with primary antibodies for phosphorylated MAPKs (p-ERK, p-p38 and p-JNK), washed, incubated with secondary antibodies and detected using LumiGLO® and exposed to x-ray film. The films are then scanned using a standard desktop scanner and band intensities calculated using ImageJ as previously described. The bars represent the mean of the ratio of phosphorylated versus total protein for three experiments (n=3) for ERK and p-38, but only two for JNK. Error bars represent the SEM and the images shown are of one chosen blot for each MAPK. There was no statistically significant difference between the groups when calculated using ANOVA.

There was no difference in the phosphorylation of the MAP kinases in cell lysates from THP-1 cells treated with or without the polysaccharides from *Nostoc commune, Collema glebulentum* or *Collema flaccidum.*

4.2.6 The effects of lichen and cyanobacterial polysaccharides on COX-2 protein

To investigate if the polysaccharides Nc-5, Cg-5-s1 and Cf-3-s2 affected the production of COX-2, the enzyme primarily responsible for catalyzing the production of prostaglandins from AA in monocytes and macrophages in response to inflammatory stimuli, Western blotting was used to measure the total COX-2 concentration in THP-1 cell lysates treated with or without the polysaccharides.

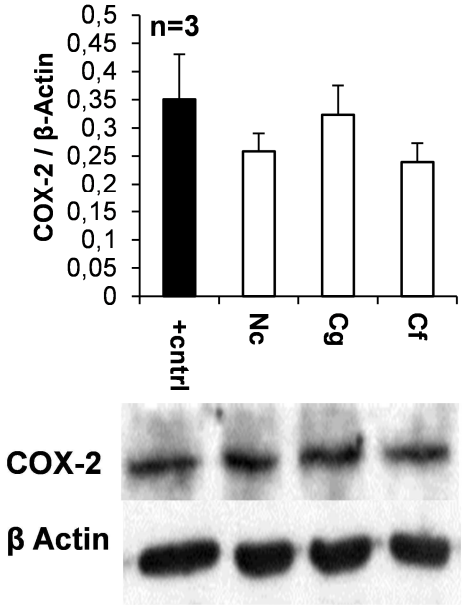

Figure 36. The effects of Nc, Cg and Cf polysaccharides on COX-2 protein in THP-1 cells. Cell culture and lysates were performed as described in the Figure 35 legend. The bars represent the ratio of total COX-2 protein versus the loading control β-Actin, a housekeeping protein, for a total of three different experiments. The error bars represent the SEM. There was no statistical difference between the polysaccharide treatment and the stimulated control.

As seen in Figure 36 there was a tendency towards a decrease in the COX-2/β-Actin ratio for the cells treated with Nc-5, the polysaccharide from *Nostoc commune*, and Cf-3-s2, the polysaccharide from *Collema flaccidum*, but the difference was not statistically significant.

4.2.7 The effects of lichen and cyanobacterial polysaccharides on iNOS protein

To see if the polysaccharides Nc-5, Cg-5-s1 and Cf-3-s2 affected the total iNOS protein, the enzyme responsible for the production of the NO radical ($NO^{\bullet}$) in response to inflammatory stimuli in monocytes and macrophages, Western blotting was used to measure the total iNOS levels in THP-1 cell lysates treated with or without the polysaccharides.

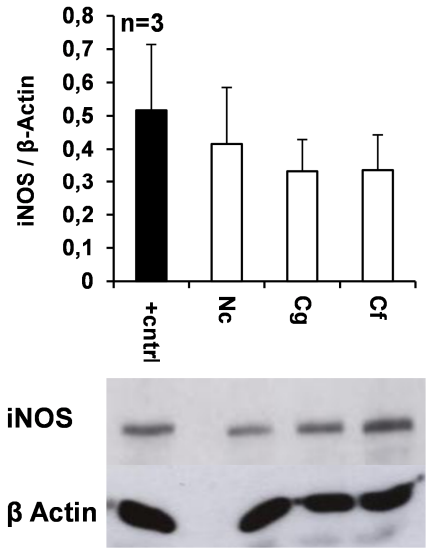

Figure 37. The effects of Nc, Cg and Cf polysaccharides on iNOS protein in THP-1 cells. Cell culture and lysates were performed as described in the Figure 35 legend. The bars represent the ratio of total iNOS protein versus the loading control β-Actin, a housekeeping protein, for a total of three different experiments. The error bars represent the SEM. There was no statistical difference between the polysaccharide treatment and the stimulated control.

As seen in Figure 37 there was a tendency towards lower levels of iNOS in both Cg-5-s1 and Cf-3-s2 treated THP-1 cells compared with THP-1 cell not treated with polysaccharides, however this difference was not statistically significant. Attempts to measure secreted NO in the form of nitrite proved unsuccessful due to insufficient amounts of nitrite in the samples. Further attempts to measure the secreted nitrite will be part of ongoing research on this project in the future.

4.2.8 The effects of lichen and cyanobacterial polysaccharides on NF-κB and associated proteins

To evaluate any involvement of NF-κB and associated proteins in mediating the polysaccharide effects on cytokine secretion, phosphorylated and total NF-κB was measured, as well as its inhibitor (Iκ-Bα) and the inhibitor's kinase (IKKβ).

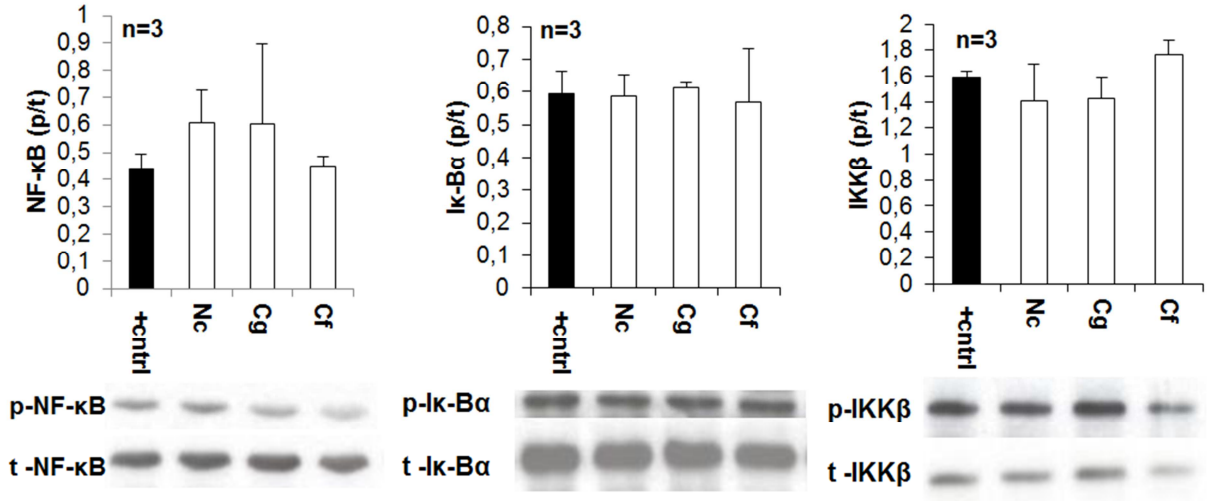

Figure 38. The effects of Nc, Cg and Cf polysaccharides on NF-κB, Iκ-Bα and IKKβ activation. Cell culture and lysates were performed as described in the Figure 35 legend. The bars represent the ratio of phosphorylated proteins versus total proteins for 3 experiments. The error bars represent the SEM. There was no statistical difference between the polysaccharide treatment and the stimulated control for any of the proteins.

The polysaccharides had no effect on the ratio of phosphorylated/total NF-κB, Iκ-Bα or IKKβ (Figure 38).

5 Discussion

5.1 The effects of dietary fish oil on chemokine secretion by total murine spleen cells

Unstimulated splenocytes from mice fed the fish oil diet secreted significantly less KC, MCP-1 and MIP-1α but more RANTES than splenocytes fed the control diet suggesting a difference in the basal chemokine secretion of these cells. As total spleen cells were used, the cell type(s) responsible for the basal chemokine secretion is not known. This difference in basal secretion between splenocytes from mice from the two different dietary groups suggests that under normal (non-inflammatory) conditions there may be reduced secretion of chemokines and subsequently less infiltration of inflammatory cells to the spleen in mice fed fish oil. A generally lowered basal inflammatory status has been suggested as part of the cardioprotective effect of ω-3 PUFA consumption (119).

5.2 The effects of polysaccharides from Icelandic lichens and cyanobacteria on THP-1 monocytes

The increase seen in TNF-α secretion by THP-1 cells cultured with lichenan from *Cetraria islandica* suggests a stimulatory effect on immune responses. That opposes previous results where *Cetraria islandica* extracts had an anti-inflammatory effect on human monocyte-derived DCs (115). However, a tendency towards an increase in IL-10 without affecting IL-12p40 secretion as seen when the THP-1 monocytes were treated with lichenan in the present study suggests an anti-inflammatory effect consistent with previous results (120). When rats with mBSA induced arthritis were treated with s.c. injections of an extract from *Cetraria islandica* the arthritis was reduced, suggesting an anti-inflammatory effect of the extract (115). Further studies on these effects need to be performed before drawing conclusions regarding the effects of lichenan and *Cetraria islandica* extracts on inflammatory responses.

Although *Nostoc commune, Collema glebulentum* and *Collema flaccidum* are similar in nature (containing or being cyanobacteria of the Nostoc genus), both the *Collema glebulentum* polysaccharide Cg-5-s1 and the *Nostoc commune* polysaccharide Nc-5 are from alkaline extractions, while the *Collema flaccidum* polysaccharide Cf-3-s2 is from an aqueous extraction, which may affect which polysaccharides are being extracted from the organisms. The effects Nc-5 and Cg-5-s1 had on both IL-6 and IL-12p40 compared with Cf-3-s2 treatment that only affected IL-12p40 secretion but not IL-6 secretion may indicate a difference in the effects of the polysaccharides obtained by the different extraction methods. Since the cyanobacterial polysaccharide had a similar effect as the polysaccharide from the lichen, one might speculate that the cyanobacterial-contents of the lichen are important for these effects.

The polysaccharides Cg-5-s1 and Nc-5 significantly reduced IL-6 secretion by THP-1 cells. Reducing IL-6 may be beneficial in some inflammatory conditions. Tocilizumab, a monoclonal antibody against the IL-6 receptor is currently approved for use in RA cases where anti TNF-α treatment fails (reviewed in (121)). Clinical trials using this agent have shown that anti-IL-6 therapy is effective as an RA treatment; however some toxic effects of tocilizumab underline the need to find alternative means

of IL-6 suppression. Tocilizumab treatment has also been tested for Crohn's disease and Castelman disease (22). Anti-IL-6 therapy has also been tested for the treatment of some cancers (23).

One *in vitro* investigation that might shed more light on how Nc-5, Cg-5-s1 and Cf-3-s2 affect the direction of immune responses is to test the effects of THP-1 monocytes that have been treated with Cg-5-s1, Cf-3-s2 or Nc-5 on T cell differentiation. The activation and differentiation of naïve T cells is an important function in the control of adaptive immune function. That control is in part facilitated by cytokine secretion by APCs and other cells in the microenvironment of the activated T cells (1). IL-12p40 is a common subunit of IL-12 as well as IL-23 so a reduction in IL-12p40 could mean a reduction in either or both cytokines. The presence of IL-12 can drive naïve $CD4^+$ T cells towards T_H1 effector differentiation whereas its absence can drive the same cells into the more tolerogenic T_H2 or even Treg phenotype (7). IL-6 and IL-23 are both players in driving $CD4^+$ T cells towards the T_H17 phenotype, a known player in autoimmunity (122). Reduced secretion of IL-6 and IL-12p40 by THP-1 cells suggest that Cg-5-s1, Cf-3-s2 and Nc-5 treated THP-1 cells might drive $CD4^+$ T cells towards a more anti-inflammatory phenotype, which could have some relevance in autoimmune disorders.

5.2.1 Involvement of prostaglandins in mediating the effects of lichen and cyanobacterial polysaccharides on THP-1 monocyte cytokine secretion

Involvement of PGs in the effects of lichen and cyanobacterial polysaccharides on cytokine secretion by THP-1 cells was tested using indomethacin, a COX inhibitor and in some cases measurements of secreted PGE_2 and total COX-2 protein. In stimulated THP-1 cells indomethacin had no effect on TNF-α secretion when tested without polysaccharides which was unexpected since others have reported an increase in TNF-α secretion after indomethacin treatment in human monocytes (29, 70).

Indomethacin treatment had a tendency to eradicate the effect lichenan had on the secretion of IL-12p40, IL-6 and TNF-α in the highest polysaccharide concentration tested. Given that indomethacin alone had little effect on TNF-α and IL-12p40 secretion of stimulated THP-1 cells, it is unlikely that lichenan mediates its effects by reducing prostaglandins or COX. However, since adding indomethacin to the cultures along with lichenan had a tendency to affect the cytokine secretion in the opposite direction of lichenan alone, the polysaccharide might be increasing the levels of COX products and indomethacin then reversing that effect. Increased PGE_2 would coincide with reduced IL-12p40 (12) and TNF-α (123) but the levels of PGE_2 and/or other COX targets were not measured in culture supernatants of lichenan treated THP-1 monocytes so the effects of indomethacin have not been verified.

Indomethacin did not alter the effects of Cg-5-s1 or Cf-3-s2 on cytokine secretion, suggesting that either the COX products do not affect the cytokine secretion or that the polysaccharide is already blocking COX, so that the effect of the inhibitor becomes redundant. The first explanation may be correct for Cg-5-s1 as the highest concentration of Cg-5-s1 had no effect on PGE_2 secretion and indomethacin alone did not suppress the cytokines as much as Cg-5-s1. Cg-5-s1 did not affect relative COX-2 protein levels, thus, although COX function can be inhibited without affecting the total protein (29), the cumulative results of these tests suggest that PGs are not involved in the effects of Cg-5-s1.

Adding indomethacin to the THP-1 cells along with Nc-5 had a tendency to decrease TNF-α secrtion compared with that by THP-1 cells stimulated without either indomethacin or Nc-5, whereas Nc-5 had a tendency to increase TNF-α secretion. As these differences are not statistically significant they may be obtained by chance. As indomethacin had no effect on secretion of the either IL-12p40 or IL-6 when added along with Nc-5, prostaglandins are unlikely to be involved in the effect of Nc-5 on these cytokines.

PGs are known to have an effect on the production of inflammatory mediators (29, 68, 124). Treatment with indomethacin inhibits production of all COX targets, as well as affecting the production of PGE_2 receptors in some cell types (125). The scale of inhibition by indomethacin as well as the complexity of the COX targets and their interactions with their receptors, which is outside the scope of this text, make interpretations of indomethacin treatment alone difficult. Nonetheless, the results of indomethacin treatment, such as that seen with lichenan treatment, make further studies into these effects an interesting subject in future studies.

5.2.2 Involvement of MAP kinase activation in mediating the effects of lichen and cyanobacterial polysaccharides on THP-1 monocyte cytokine secretion

Activation of monocytes/macrophages through TLRs results in the phosphorylation of MAP kinases and transcription of several inflammatory mediators such as cytokines (56, 126), COX and iNOS (127). Many publications have reported an effect of natural compound treatment on MAP kinase activation (88, 91, 92) and research on the effects of lichen material on macrophages have shown a link to MAP kinase activation (105).

None of the polysaccharides tested in the present study had an effect on MAP kinase activation compared with the untreated control. This suggests that the effects of the polysaccharides on cytokine secretion are not mediated by alterations in the activation of MAP kinases.

5.2.3 Effect of the lichen and cyanobacterial polysaccharides on iNOS protein

One of the ways many cell types respond to inflammatory stimuli is by expressing iNOS, the inducible version of NOS. The enzyme catalysis the formation of NO (61, 128), which is then secreted. There was a tendency towards reduced iNOS protein levels after treatment with polysaccharides from the jelly lichens, but the difference was not statistically significant. To test if this possible difference in iNOS protein levels would lead to a difference in NO production, attempts were made to measure the secreted NO. NO is a reactive and relatively short lived radical that is metabolized into the stable end products nitrite and nitrate. Measuring NO is usually performed by measuring the amount of nitrite in culture supernatants using the Griess reagent as described in chapter 3.2.3.2. We tested this method but found insufficient nitrite in the culture supernatants to measure. In some cases enzymes are used to convert nitrate to nitrite and then performing the Griess reaction to measure both metabolites. However, the RPMI 1640 medium used for these experiments contains a notable amount of nitrate (0.1 g/l calcium nitrate$\bullet 4H_2O$) making the measurements unreliable once nitrate has been converted to nitrite. Attempts to measure nitrite in the samples using a more sensitive method are currently being performed. The results would indicate if the possible reduction in iNOS protein seen with Cg-5-s1 and

Cf-3-s2 treatment is of relevance. Should these attempts fail, the experiments would need to be repeated using another culture medium not containing nitrate.

5.2.4 Involvement of the NF-κB pathway in mediating the effects of lichen and cyanobacterial polysaccharides on THP-1 monocyte cytokine secretion

NF-κB is a multifaceted transcription factor activated by a cascade of protein phosphorylation as a result of inflammatory stimuli to produce mediators of various inflammatory responses (129). Many natural products have been tested for their effects on NF-κB (reviewed in (130)). The effects of the Cg, Cf and Nc polysaccharides on the activation of NF-κB and its inhibitor Iκ-Bα as well as the inhibitors kinase (IKKβ) were tested; however, no difference was observed in the phosphorylation of the proteins when compared with the total protein levels. As only three experiments were performed and there was a great variation between measurements, they need to be repeated.

5.3 Summary

The polysaccharides Cg-5-s1, Cf-3-s2 and Nc-5 from *Collema glebulentum, Collema flaccidum* and *Nostoc commune* have an effect on cytokine secretion. Although several activation pathways have been analyzed, it has not been possible to determine how these polysaccharides are affecting the cytokine secretion. There is the possibility that the experimental setup may be affecting the results with some practical components overshadowing the real results. However, there may be other mediators that are affected. One route of signaling that still remains unchecked is PI3K which is activated through TLR binding and can cause the transcription of cytokines through Akt and NF-κB. This route of signaling will be tested in further studies.

When contemplating the results presented in this thesis, the complexity of signal transduction must be taken into account (for a review, see (113)). Recent research has suggested that several signaling pathways are activated in repeated cascades, resulting in a kind of oscillatory signal. In many cases, a reduced activation (phosphorylation) of the kinases might be affecting the transcription of proteins, but a general change in the kinetics or duration of the signal might be causing a cumulative effect on the transcription of the cytokines, an effect that would not be seen in changes of protein phosphorylation at any one time-point. Given the number of publications demonstrating direct effects of natural products on transcription or activation of certain intracellular proteins and signaling cascades measured at different time points, this option is an unlikely explanation for the lack of effects seen on signaling cascades in the present study, but perhaps not impossible.

A possible explanation for not finding pathways responsible for the reduced cytokine secretion may be that the effects of the polysaccharides are not at the level of cytokine production, but that the secretion of the cytokines is altered by the polysaccharides. That would cause an accumulation of the cytokines within the cells resulting in reduced secretion. Such a mechanism is discussed in (131). Intracellular cytokine staining of the cells would reveal whether this is the case or not.

6 Conclusion

Decreased chemokine secretion by unstimulated splenocytes from mice fed a fish oil diet suggests that during a quiescent situation, fish oil consumption reduces the secretion of inflammatory mediators by spleen cells, suggesting a lower baseline inflammation. However, during an inflammatory response the mice receiving the fish oil diet are fully capable to respond by chemokine secretion in a similar manner as the mice in the control group.

The polysaccharides Nc-5, Cg-5-s1 and Cf-3-s2 reduced the secretion of IL-12p40 and/or IL-6. Both cytokines affect the differentiation of inflammatory T cells into T helper cell subsets (T_H1 and T_H17 cells). It is therefore interesting to test how the polysaccharide-treated THP-1 cells affect T cell activation and especially how they affect experimental inflammatory diseases. By co-culturing $CD4^+$ T cells with polysaccharide-treated monocytes it is possible to examine whether this change in cytokine secretion may affect the T helper cell phenotype. Altering T cell differentiation towards a tolerogenic phenotype can have important protective effects in some autoimmune diseases, as well as the general outcome of inflammatory responses.

It was not possible to determine how the polysaccharides Cg-5-s1, Cf-3-s2 and Nc-5 affected the cytokine secretion by the THP-1 cells, as they did not mediate changes in the phosphorylation of MAP kinases or the transcription factor NF-κB. The mechanism by which these polysaccharides affect the cytokine secretion of THP-1 cells is hence still unknown.

The next steps will be to repeat the measurements of total iNOS levels and measure the secreted nitric oxide to assess any biological significance of altered iNOS production. Further attempts to elucidate the mechanism behind the altered cytokine secretion after polysaccharide treatment will include measuring members of the PI3K-Akt pathway as well as testing the effects of the polysaccharides on intracellular events at other time points to evaluate any effects on the kinetics of signal transduction on cytokine secretion.

Other possibilities would include measuring intracellular cytokines to assess if there is an accumulation of cytokines inside the THP-1 cells that results in reduced secretion of the cytokines as well as measuring the effects of the polysaccharides on surface markers and surface receptor binding.

References

1. Murphy K, Travers P, Walport M. Janeway's Immunobiology. Seventh ed: Garland Science; 2008.

2. Burmeister G-R, Pezzutto A. Color Atlas of Immunology. Stuttgart

New York: Thieme; 2003.

3. Tsuchiya S, Yamabe M, Yamaguchi Y, Kobayashi Y, Konno T, Tada K. Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). International Journal of Cancer. 1980;26(2):171-6.

4. Auwerx J. The human leukemia cell line, THP-1: a multifacetted model for the study of monocyte-macrophage differentiation. Experientia. 1991 Jan 15;47(1):22-31.

5. Tsuchiya S, Kobayashi Y, Goto Y, Okumura H, Nakae S, Konno T, et al. Induction of maturation in cultured human monocytic leukemia cells by a phorbol diester. Cancer Res. 1982 Apr;42(4):1530-6.

6. Daigneault M, Preston JA, Marriott HM, Whyte MK, Dockrell DH. The identification of markers of macrophage differentiation in PMA-stimulated THP-1 cells and monocyte-derived macrophages. PLoS One. 2010;5(1):e8668.

7. Notley CA, Ehrenstein MR. The yin and yang of regulatory T cells and inflammation in RA. Nat Rev Rheumatol. 2010 Oct;6(10):572-7.

8. Ouyang W, Rutz S, Crellin NK, Valdez PA, Hymowitz SG. Regulation and Functions of IL-10 Family Cytokines in Inflammation and Diseases. Annu Rev Immunol. 2010 Apr 5.

9. Sabat R, Grutz G, Warszawska K, Kirsch S, Witte E, Wolk K, et al. Biology of interleukin-10. Cytokine Growth Factor Rev. 2010 Oct;21(5):331-44.

10. Saraiva M, O'Garra A. The regulation of IL-10 production by immune cells. Nat Rev Immunol. [10.1038/nri2711]. 2010;10(3):170-81.

11. Lim W, Ma W, Gee K, Aucoin S, Nandan D, Diaz-Mitoma F, et al. Distinct role of p38 and c-Jun N-terminal kinases in IL-10-dependent and IL-10-independent regulation of the costimulatory molecule B7.2 in lipopolysaccharide-stimulated human monocytic cells. J Immunol. 2002 Feb 15;168(4):1759-69.

12. Sinigaglia F, D'Ambrosio D, Panina-Bordignon P, Rogge L. Regulation of the IL-12/IL-12R axis: a critical step in T-helper cell differentiation and effector function. Immunol Rev. 1999 Aug;170:65-72.

13. Ma X, Chow JM, Gri G, Carra G, Gerosa F, Wolf SF, et al. The interleukin 12 p40 gene promoter is primed by interferon gamma in monocytic cells. J Exp Med. 1996 Jan 1;183(1):147-57.

14. Lee SM, Suen Y, Qian J, Knoppel E, Cairo MS. The regulation and biological activity of interleukin 12. Leuk Lymphoma. 1998 May;29(5-6):427-38.

15. Wang IM, Contursi C, Masumi A, Ma X, Trinchieri G, Ozato K. An IFN-gamma-inducible transcription factor, IFN consensus sequence binding protein (ICSBP), stimulates IL-12 p40 expression in macrophages. J Immunol. 2000 Jul 1;165(1):271-9.

16. Hirano T. Interleukin 6 in autoimmune and inflammatory diseases: a personal memoir. Proc Jpn Acad Ser B Phys Biol Sci. 2010;86(7):717-30.

17. Kimura A, Kishimoto T. IL-6: regulator of Treg/Th17 balance. Eur J Immunol. 2010 Jul;40(7):1830-5.

18. Medzhitov R, Horng T. Transcriptional control of the inflammatory response. Nat Rev Immunol. [10.1038/nri2634]. 2009;9(10):692-703.

19. Cheung CY, Poon LL, Lau AS, Luk W, Lau YL, Shortridge KF, et al. Induction of proinflammatory cytokines in human macrophages by influenza A (H5N1) viruses: a mechanism for the unusual severity of human disease? Lancet. 2002 Dec 7;360(9348):1831-7.

20. Beyaert R, Cuenda A, Vanden Berghe W, Plaisance S, Lee JC, Haegeman G, et al. The p38/RK mitogen-activated protein kinase pathway regulates interleukin-6 synthesis response to tumor necrosis factor. EMBO J. 1996 Apr 15;15(8):1914-23.

21. Navarro S, Debili N, Bernaudin JF, Vainchenker W, Doly J. Regulation of the expression of IL-6 in human monocytes. J Immunol. 1989 Jun 15;142(12):4339-45.

22. Venkiteshwaran A. Tocilizumab. MAbs. 2009 Sep-Oct;1(5):432-8.

23. Trikha M, Corringham R, Klein B, Rossi JF. Targeted anti-interleukin-6 monoclonal antibody therapy for cancer: a review of the rationale and clinical evidence. Clin Cancer Res. 2003 Oct 15;9(13):4653-65.

24. Simsek I. TNF inhibitors - new and old agents for rheumatoid arthritis. Bull NYU Hosp Jt Dis. 2010;68(3):204-10.

25. Kruglov AA, Kuchmiy A, Grivennikov SI, Tumanov AV, Kuprash DV, Nedospasov SA. Physiological functions of tumor necrosis factor and the consequences of its pathologic overexpression or blockade: mouse models. Cytokine Growth Factor Rev. 2008 Jun-Aug;19(3-4):231-44.

26. Economou JS, Rhoades K, Essner R, McBride WH, Gasson JC, Morton DL. Genetic analysis of the human tumor necrosis factor alpha/cachectin promoter region in a macrophage cell line. J Exp Med. 1989 Jul 1;170(1):321-6.

27. Liu H, Sidiropoulos P, Song G, Pagliari LJ, Birrer MJ, Stein B, et al. TNF-alpha gene expression in macrophages: regulation by NF-kappa B is independent of c-Jun or C/EBP beta. J Immunol. 2000 Apr 15;164(8):4277-85.

28. Mannel DN, Falk W. Optimal induction of tumor necrosis factor production in human monocytes requires complete S-form lipopolysaccharide. Infect Immun. 1989 Jul;57(7):1953-8.

29. Ulcar R, Peskar BA, Schuligoi R, Heinemann A, Kessler HH, Santner BI, et al. Cyclooxygenase inhibition in human monocytes increases endotoxin-induced TNF alpha without affecting cyclooxygenase-2 expression. Eur J Pharmacol. 2004 Oct 6;501(1-3):9-17.

30. Pandita R, Pocsik E, Aggarwal BB. Interferon-[gamma] induces cell surface expression for both types of tumor necrosis factor receptors. FEBS Letters. 1992;312(1):87-90.

31. Hu X, Ivashkiv LB. Cross-regulation of signaling pathways by interferon-gamma: implications for immune responses and autoimmune diseases. Immunity. 2009 Oct 16;31(4):539-50.

32. Schroder K, Hertzog PJ, Ravasi T, Hume DA. Interferon-gamma: an overview of signals, mechanisms and functions. J Leukoc Biol. 2004 Feb;75(2):163-89.

33. Moser B, Letts GL, Neote K. Chemokine Biology - Basic Research and Clinical application. Parnham PMJ, editor: Birkhauser Verlag; 2006.

34. Coelho AL, Hogaboam CM, Kunkel SL. Chemokines provide the sustained inflammatory bridge between innate and acquired immunity. Cytokine & Growth Factor Reviews. [Review]. 2005;16:553-60.

35. Mukaida N. Pathophysiological roles of interleukin-8/CXCL8 in pulmonary diseases. Am J Physiol Lung Cell Mol Physiol. 2003 Apr;284(4):L566-77.

36. David J, Mortari F. Chemokine receptors A brief overview. Clinical and Applied Immunology Reviews. 2000;1:105-25.

37. MacDermott R, Sanderson IR, Reinecker H-C. The Central Role of Chemokines (Chemotactic Cytokines) in the Immunopathogenesis of Ulcerative Colitis and Chron's Disease. Inflammatory Bowel Diseases. 1998;4(1):54-67.

38. Gangur V, Birmingham NP, Thanesvorakul S. Chemokines in health and disease. Veterinary Immunology and Immunopathology. 2002;86(3-4):127-36.

39. Melgarejo E, Medina MÁ, Sánchez-Jiménez F, Urdiales JL. Monocyte Chemoattractant protein-1: A key mediator in inflammatory processes. The International Jorunal of Biochemistry & Cell Biology [serial on the Internet]. 2008.

40. O'Hayre M, Salanga CL, Handel TM, Allen SJ. Chemokines and cancer: migration, intracellular signalling and intercellular communication in the microenvironment. Biochem J. 2008 Feb 1;409(3):635-49.

41. Bachmann MF, Kopf M, Marsland BJ. Chemokines: more than just road signs. Nature reviews. 2006 February 2006;6:159-64.

42. Maurer M, von Stebut E. Macrophage inflammatory protein-1. Int J Biochem Cell Biol. 2004 Oct;36(10):1882-6.

43. Levy JA. The unexpected pleiotropic activities of RANTES. J Immunol. 2009 Apr 1;182(7):3945-6.

44. Danese S, Gasbarrini A. Chemokines in inflammatory bowel disease. Journal of Clinical Pathology. 2005;58:1025-7.

45. Akira S, Takeda K. Toll-like receptor signalling. Nat Rev Immunol. [10.1038/nri1391]. 2004;4(7):499-511.

46. Ostuni R, Zanoni I, Granucci F. Deciphering the complexity of Toll-like receptor signaling. Cell Mol Life Sci. 2010 Dec;67(24):4109-34.

47. Trinchieri G, Sher A. Cooperation of Toll-like receptor signals in innate immune defence. Nat Rev Immunol. [10.1038/nri2038]. 2007;7(3):179-90.

48. Zhang Y, Dong C. Regulatory mechanisms of mitogen-activated kinase signaling. Cell Mol Life Sci. 2007 Nov;64(21):2771-89.

49. Alexander C, Rietschel ET. Bacterial lipopolysaccharides and innate immunity. J Endotoxin Res. 2001;7(3):167-202.

50. Barton GM, Kagan JC. A cell biological view of Toll-like receptor function: regulation through compartmentalization. Nat Rev Immunol. 2009 Aug;9(8):535-42.

51. Krakauer T. Molecular Therapeutic Targets in Inflammation: Cyclooxygenase and NF-κB. Current Drug Targets - Inflammation & Allergy. [Article]. 2004;3(3):317-24.

52. Serbina NV, Jia T, Hohl TM, Pamer EG. Monocyte-mediated defense against microbial pathogens. Annu Rev Immunol. 2008;26:421-52.

53. Dong Z, Bode AM. Dialogue between ERKs and JNKs: friendly or antagonistic? Mol Interv. 2003 Sep;3(6):306-8.

54. Kaminska B. MAPK signalling pathways as molecular targets for anti-inflammatory therapy--from molecular mechanisms to therapeutic benefits. Biochim Biophys Acta. 2005 Dec 30;1754(1-2):253-62.

55. Murphy LO, Blenis J. MAPK signal specificity: the right place at the right time. Trends Biochem Sci. 2006 May;31(5):268-75.

56. Raman M, Chen W, Cobb MH. Differential regulation and properties of MAPKs. Oncogene. 2007 May 14;26(22):3100-12.

57. Hoffmann A, Levchenko A, Scott ML, Baltimore D. The IkappaB-NF-kappaB signaling module: temporal control and selective gene activation. Science. 2002 Nov 8;298(5596):1241-5.

58. Aggarwal BB, Sethi G, Baladandayuthapani V, Krishnan S, Shishodia S. Targeting cell signaling pathways for drug discovery: an old lock needs a new key. J Cell Biochem. 2007 Oct 15;102(3):580-92.

59. Basak S, Hoffmann A. Crosstalk via the NF-kappaB signaling system. Cytokine Growth Factor Rev. 2008 Jun-Aug;19(3-4):187-97.

60. Ghosh S, Hayden MS. New regulators of NF-[kappa]B in inflammation. Nat Rev Immunol. [10.1038/nri2423]. 2008;8(11):837-48.

61. Paradise WA, Vesper BJ, Goel A, Waltonen JD, Altman KW, Haines GK, et al. Nitric oxide: perspectives and emerging studies of a well known cytotoxin. Int J Mol Sci. 2010;11(7):2715-45.

62. Nelson D, Cox M. Lehninger Principles of Biochemistry, Fourth Edition: W. H. Freeman; 2004.

63. Aktan F. iNOS-mediated nitric oxide production and its regulation. Life Sciences. 2004;75(6):639-53.

64. Alderton WK, Cooper CE, Knowles RG. Nitric oxide synthases: structure, function and inhibition. Biochem J. 2001 Aug 1;357(Pt 3):593-615.

65. Lee S, Gura KM, Kim S, Arsenault DA, Bistrian BR, Puder M. Current Clinical Applications of ω-6 and ω-3 Fatty Acids. Nutrition in Clinical Practice. 2006 August 2006;21:323-41.

66. Tilley SL, Coffman TM, Koller BH. Mixed messages: modulation of inflammation and immune responses by prostaglandins and thromboxanes. J Clin Invest. 2001 Jul;108(1):15-23.

67. Rouzer CA, Marnett LJ. Non-redundant functions of cyclooxygenases: oxygenation of endocannabinoids. J Biol Chem. 2008 Mar 28;283(13):8065-9.

68. Bos CL, Richel DJ, Ritsema T, Peppelenbosch MP, Versteeg HH. Prostanoids and prostanoid receptors in signal transduction. Int J Biochem Cell Biol. 2004 Jul;36(7):1187-205.

69. Sugimoto Y, Narumiya S. Prostaglandin E receptors. J Biol Chem. 2007 Apr 20;282(16):11613-7.

70. Miles EA, Allen E, Calder PC. In vitro effects of eicosanoids derived from different 20-carbon fatty acids on production of monocyte-derived cytokines in human whole blood cultures. Cytokine. 2002 December 2002;20(5):215-23.

71. van der Pouw Kraan TC, Boeije LC, Smeenk RJ, Wijdenes J, Aarden LA. Prostaglandin-E2 is a potent inhibitor of human interleukin 12 production. J Exp Med. 1995 Feb 1;181(2):775-9.

72. Cheon H, Rho YH, Choi SJ, Lee YH, Song GG, Sohn J, et al. Prostaglandin E2 augments IL-10 signaling and function. J Immunol. 2006 Jul 15;177(2):1092-100.

73. Meyera BJ, Mannb NJ, Lewisc JL, Milliganc GC, Sinclairb AJ, Howea PRC. Dietary Intakes and Food Sources of Omega-6 and Omega-3 Polyunsaturated Fatty Acids. Lipids. 2003 April 2003;38:391-8.

74. Surette ME. The science behind dietary omega-3 fatty acids. The Canadian Medical Association Journal. 2008;178(2):177-80.

75. Yaqoob P, Calder PC. Fatty acids and immune function: new insights into mechanisms. The British Journal of Nutrition. 2007 Oct;98, Suppl 1:S41-S5.

76. Bjerregaard P, Mulvad G, Pedersen HS. Cardiovascular Risk Factors in Inuit of Greenland. International Journal of Epidemiology. 1997;26(6):1182-90.

77. Lands WEM. Dietary Fat and Health: The Evidence and the Politics of Prevention. Annals of the New York Academy of Sciences. 2005;1055:179-92.

78. Simopoulos AP. Omega-3 Fatty Acids in Inflammation and Autoimmune Diseases. Journal of the American College of Nutrition. [Review]. 2002;21(6):495-505.

79. Calder PC. n-3 Fatty Acids, Inflammation, and Immunity-Relevance to Postsurgical and Critically ill Patients. Lipids. 2004 Dec 2004;39(12):1147-61.

80. Jho DH, Cole SM, Lee EM, Espat NJ. Role of Omega-3 Fatty Acid Supplementation in Inflammation and Malignancy. Integrative Cancer Therapies. 2004;3(2):98-111.

81. Calder PC. n-3 Polyunsaturated fatty acids, inflammation, and inflammatory diseases. The American Journal of Clinical Nutrition. 2006;83:1505S-19S.

82. Calder PC. Polyunsaturated fatty acids and inflammation. Biochemical Society Transactions. 2005;33(2):423-7.

83. Sijben JW, Calder PC. Differential immunomodulation with long-chain n-3 PUFA in health and chronic disease. Proceedings of the Nutrition Society. 2007;66:237-59.

84. Luu N-T, Madden J, Calder PC, Grimble RF, Sherman CP, Chan T, et al. Dietary Supplementation with Fish Oil Modifies the Ability of Human Monocytes to Induce an Inflammatory Response. The Journal of Nutrition. 2007;137(12):2769-74.

85. Zapata-Gonzalez F, Rueda F, Petriz J, Domingo P, Villarroya F, Diaz-Delfin J, et al. Human dendritic cell activities are modulated by the omega-3 fatty acid, docosahexaenoic acid, mainly

through PPAR(gamma):RXR heterodimers: comparison with other polyunsaturated fatty acids. J Leukoc Biol. 2008 Oct;84(4):1172-82.

86. Petursdottir DH, Hardardottir I. Dietary fish oil decreases secretion of T helper (Th) 1-type cytokines by a direct effect on murine splenic T cells but enhances secretion of a Th2-type cytokine by an effect on accessory cells. Br J Nutr. 2008 Aug 5:1-7.

87. Petursdottir DH, Hardardottir I. Dietary fish oil increases the number of splenic macrophages secreting TNF-alpha and IL-10 but decreases the secretion of these cytokines by splenic T cells from mice. J Nutr. 2007 Mar;137(3):665-70.

88. Chao LK, Hua KF, Hsu HY, Cheng SS, Lin IF, Chen CJ, et al. Cinnamaldehyde inhibits pro-inflammatory cytokines secretion from monocytes/macrophages through suppression of intracellular signaling. Food Chem Toxicol. 2008 Jan;46(1):220-31.

89. Ci X, Ren R, Xu K, Li H, Yu Q, Song Y, et al. Schisantherin A exhibits anti-inflammatory properties by down-regulating NF-kappaB and MAPK signaling pathways in lipopolysaccharide-treated RAW 264.7 cells. Inflammation. 2010 Apr;33(2):126-36.

90. Allen-Hall L, Cano P, Arnason JT, Rojas R, Lock O, Lafrenie RM. Treatment of THP-1 cells with Uncaria tomentosa extracts differentially regulates the expression if IL-1beta and TNF-alpha. J Ethnopharmacol. 2007 Jan 19;109(2):312-7.

91. Zhang L, Wu C, Zhao S, Yuan D, Lian G, Wang X, et al. Demethoxycurcumin, a natural derivative of curcumin attenuates LPS-induced pro-inflammatory responses through down-regulation of intracellular ROS-related MAPK/NF-kappaB signaling pathways in N9 microglia induced by lipopolysaccharide. Int Immunopharmacol. 2010 Mar;10(3):331-8.

92. Chan AS, Yip EC, Yung LY, Pang H, Luk SC, Pang SF, et al. Immuno-regulatory effects of CKBM on the activities of mitogen-activated protein kinases and the release of cytokines in THP-1 monocytic cells. Biol Pharm Bull. 2005 Sep;28(9):1645-50.

93. Allen-Hall L, Arnason JT, Cano P, Lafrenie RM. Uncaria tomentosa acts as a potent TNF-alpha inhibitor through NF-kappaB. J Ethnopharmacol. 2010 Feb 17;127(3):685-93.

94. Haversen L, Ohlsson BG, Hahn-Zoric M, Hanson LA, Mattsby-Baltzer I. Lactoferrin down-regulates the LPS-induced cytokine production in monocytic cells via NF-kappa B. Cell Immunol. 2002 Dec;220(2):83-95.

95. Zhao F, Wang L, Liu K. In vitro anti-inflammatory effects of arctigenin, a lignan from Arctium lappa L., through inhibition on iNOS pathway. J Ethnopharmacol. 2009 Apr 21;122(3):457-62.

96. Yun KJ, Min BS, Kim JY, Lee KT. Styraxoside A isolated from the stem bark of Styrax japonica inhibits lipopolysaccharide-induced expression of inducible nitric oxide synthase and cyclooxygenase-2 in RAW 264.7 cells by suppressing nuclear factor-kappa B activation. Biol Pharm Bull. 2007 Jan;30(1):139-44.

97. Kang OH, Lee GH, Choi HJ, Park PS, Chae HS, Jeong SI, et al. Ethyl acetate extract from Angelica Dahuricae Radix inhibits lipopolysaccharide-induced production of nitric oxide, prostaglandin E2 and tumor necrosis factor-alphavia mitogen-activated protein kinases and nuclear factor-kappaB in macrophages. Pharmacol Res. 2007 Apr;55(4):263-70.

98. Villa FA, Lieske K, Gerwick L. Selective MyD88-dependent pathway inhibition by the cyanobacterial natural product malyngamide F acetate. European Journal of Pharmacology. 2010;629(1-3):140-6.

99. Hatziieremia S, Gray AI, Ferro VA, Paul A, Plevin R. The effects of cardamonin on lipopolysaccharide-induced inflammatory protein production and MAP kinase and NFkappaB signalling pathways in monocytes/macrophages. Br J Pharmacol. 2006 Sep;149(2):188-98.

100. Jung WK, Choi I, Lee DY, Yea SS, Choi YH, Kim MM, et al. Caffeic acid phenethyl ester protects mice from lethal endotoxin shock and inhibits lipopolysaccharide-induced cyclooxygenase-2 and inducible nitric oxide synthase expression in RAW 264.7 macrophages via the p38/ERK and NF-kappaB pathways. Int J Biochem Cell Biol. 2008;40(11):2572-82.

101. Kim JY, Park SJ, Yun KJ, Cho YW, Park HJ, Lee KT. Isoliquiritigenin isolated from the roots of Glycyrrhiza uralensis inhibits LPS-induced iNOS and COX-2 expression via the attenuation of NF-kappaB in RAW 264.7 macrophages. Eur J Pharmacol. 2008 Apr 14;584(1):175-84.

102. Oksanen I. Ecological and biotechnological aspects of lichens. Appl Microbiol Biotechnol. 2006 Dec;73(4):723-34.

103. Schooley J. Introduction to Botany. Albany: Tim O'Leary; 1997.

104. Santos LC, Honda NK, Carlos IZ, Vilegas W. Intermediate reactive oxygen and nitrogen from macrophages induced by Brazilian lichens. Fitoterapia. 2004 Jul;75(5):473-9.

105. Choi HS, Yim JH, Lee HK, Pyo S. Immunomodulatory effects of polar lichens on the function of macrophages in vitro. Mar Biotechnol (NY). 2009 Jan-Feb;11(1):90-8.

106. Olafsdottir ES, Ingolfsdottir K. Polysaccharides from lichens: structural characteristics and biological activity. Planta Med. 2001 Apr;67(3):199-208.

107. Olafsdottir ES, Omarsdottir S, Paulsen BS, Wagner H. Immunologically active O6-branched (1-->3)-beta-glucan from the lichen Thamnolia vermicularis var. subuliformis. Phytomedicine. 2003 May;10(4):318-24.

108. Omarsdottir S, Freysdottir J, Olafsdottir ES. Immunomodulating polysaccharides from the lichen Thamnolia vermicularis var. subuliformis. Phytomedicine. 2007 Feb;14(2-3):179-84.

109. Chan GC, Chan WK, Sze DM. The effects of beta-glucan on human immune and cancer cells. J Hematol Oncol. 2009;2:25.

110. Shao BM, Dai H, Xu W, Lin ZB, Gao XM. Immune receptors for polysaccharides from Ganoderma lucidum. Biochem Biophys Res Commun. 2004 Oct 8;323(1):133-41.

111. Shao BM, Xu W, Dai H, Tu P, Li Z, Gao XM. A study on the immune receptors for polysaccharides from the roots of Astragalus membranaceus, a Chinese medicinal herb. Biochem Biophys Res Commun. 2004 Aug 6;320(4):1103-11.

112. Schepetkin IA, Quinn MT. Botanical polysaccharides: Macrophage immunomodulation and therapeutic potential. International Immunopharmacology. 2006;6(3):317-33.

113. Gülçin I, Oktay M, Küfrevioglu ÖI, Aslan A. Determination of antioxidant activity of lichen Cetraria islandica (L) Ach. Journal of Ethnopharmacology. 2002;79(3):325-9.

114. Jónsson J. Lítil ritgjörð um nytsemi nokkurra íslenzkra jurta Reykjavík: Einar Þórðarson; 1880.

115. Freysdottir J, Omarsdottir S, Ingolfsdottir K, Vikingsson A, Olafsdottir ES. In vitro and in vivo immunomodulating effects of traditionally prepared extract and purified compounds from Cetraria islandica. Int Immunopharmacol. 2008 Mar;8(3):423-30.

116. Rezanka T, Dembitsky VM. The colleflaccinosides, two chiral bianthraquinone glycosides with antitumor activity from the lichen Collema flaccidum collected in Israel and Russia. Nat Prod Res. 2006 Aug;20(10):969-80.

117. Park YK, Rasmussen HE, Ehlers SJ, Blobaum KR, Lu F, Schlegal VL, et al. Repression of proinflammatory gene expression by lipid extract of Nostoc commune var sphaeroides Kutzing, a blue-green alga, via inhibition of nuclear factor-kappaB in RAW 264.7 macrophages. Nutr Res. 2008 Feb;28(2):83-91.

118. Rasmussen HE, Blobaum KR, Park YK, Ehlers SJ, Lu F, Lee JY. Lipid extract of Nostoc commune var. sphaeroides Kutzing, a blue-green alga, inhibits the activation of sterol regulatory element binding proteins in HepG2 cells. J Nutr. 2008 Mar;138(3):476-81.

119. Adkins Y, Kelley DS. Mechanisms underlying the cardioprotective effects of omega-3 polyunsaturated fatty acids. The Journal of Nutritional Biochemistry. 2010;21(9):781-92.

120. Omarsdottir S, Olafsdottir ES, Freysdottir J. Immunomodulating effects of lichen-derived polysaccharides on monocyte-derived dendritic cells. Int Immunopharmacol. 2006 Nov;6(11):1642-50.

121. Woodrick R, Ruderman EM. Anti-interleukin-6 therapy in rheumatoid arthritis. Bull NYU Hosp Jt Dis. 2010;68(3):211-7.

122. Weaver CT, Harrington LE, Mangan PR, Gavrieli M, Murphy KM. Th17: An Effector CD4 T Cell Lineage with Regulatory T Cell Ties. Immunity. 2006;24(6):677-88.

123. Shinomiya S, Naraba H, Ueno A, Utsunomiya I, Maruyama T, Ohuchida S, et al. Regulation of TNF[alpha] and interleukin-10 production by prostaglandins I2 and E2: studies with prostaglandin

receptor-deficient mice and prostaglandin E-receptor subtype-selective synthetic agonists. Biochemical Pharmacology. 2001;61(9):1153-60.

124. Takayama K, García-Cardena G, Sukhova GK, Comander J, Gimbrone Jr. MA, Libby P. Prostaglandin E2 Suppresses Chemokine Production in Human Macrophages through the EP4 Receptor. The Journal of Biological Chemistry. 2002 November 15th;277(46, November 15th):44147-54.

125. Fujino H, Chen XB, Regan JW, Murayama T. Indomethacin decreases EP2 prostanoid receptor expression in colon cancer cells. Biochem Biophys Res Commun. 2007 Aug 3;359(3):568-73.

126. Song C-H, Lee J-S, Lee S-H, Kyu L, Kim H-J, Park J-K, et al. Role of Mitogen-Activated Protein Kinase Pathways in the Production of Tumor Necrosis Factor-α, Interleukin-10, and Monocyte Chemotactic Protein-1 by *Mycobacterium tuberculosis* H37Rv-Infected Human Monocytes. Journal of Clinical Immunology. 2003;23(3):194-201.

127. Schindler JF, Monahan JB, Smith WG. p38 pathway kinases as anti-inflammatory drug targets. J Dent Res. 2007 Sep;86(9):800-11.

128. Moshage H. Nitric oxide determinations: much ado about NO.-thing? Clin Chem. 1997 Apr;43(4):553-6.

129. Lawrence T. The nuclear factor NF-kappaB pathway in inflammation. Cold Spring Harb Perspect Biol. 2009 Dec;1(6):a001651.

130. Luqman S, Pezzuto JM. NFkappaB: a promising target for natural products in cancer chemoprevention. Phytother Res. 2010 Jul;24(7):949-63.

131. Low PC, Misaki R, Schroder K, Stanley AC, Sweet MJ, Teasdale RD, et al. Phosphoinositide 3-kinase delta regulates membrane fission of Golgi carriers for selective cytokine secretion. J Cell Biol. 2010 Sep 20;190(6):1053-65.