Immunomodulatory effects of a heteroglycan from the cyanobacterium *Nostoc commune* on THP-1 monocytes

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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 heteróglýcans úr blágrænþörungnum *Nostoc commune* á ónæmissvör THP-1 mónócýta

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Abstract

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For centuries, natural products have been used to promote health. However, only recently have scientists started to search for their exact effects on biochemical pathways. Today's research is focused on increasing that knowledge and finding novel natural products with beneficial effects. Icelandic lichens have been used in folk medicine to alleviate conditions such as inflammatory diseases. These effects could be mediated by polysaccharides as polysaccharides from lichens have been shown to have immunomodulatory effects. The cyanobacterium *Nostoc commune* is common in lichen symbiosis and is known to produce complex polysaccharides. However, the immunomodulatory effects of polysaccharides from *Nostoc commune* have not been explored.

The aim of the current study was to determine the effects of a heteroglycan (Nc-5-s) from *Nostoc commune* on the inflammatory response in human THP-1 monocytes and how the effects are mediated on an intracellular level. THP-1 monocytes were primed with IFN-γ for 3 h and then stimulated with lipopolysaccharide for one (activation of intracellular signaling molecules), 24 (surface marker expression) or 48 h (cytokine secretion). Nc-5-s was added simultaneously with either IFN-γ or LPS. Concentration of the cytokines TNF-α, IL-6, IL-8, IL-10 and IL-12p40 in the medium was determined by ELISA. Expression of the surface molecules TLR4, TLR2, CD14, CD40, CD54 and CD86 was assessed using flow cytometry. Activation of MAP kinase, PI3K/Akt, NF-κB and JAK/STAT pathways was determined using Western blotting. DNA binding by the AP-1 transcription factor was determined with a TransAM® DNA binding assay.

THP-1 monocytes stimulated with LPS in the presence of Nc-5-s secreted less IL-6 and more TNF-α and IL-8 than THP-1 monocytes stimulated without Nc-5-s. Nc-5-s did not affect expression of any of the surface molecules tested. Nc-5-s decreased LPS-induced phosphorylation of the ERK1/2 kinase, without affecting phosphorylation of other MAP kinases. Nc-5-s also decreased phosphorylation of the Akt kinase in the PI3K/Akt pathway. Although Nc-5-s did not affect breakdown of IκBα, there was a tendency towards a decrease in translocation of NF-κB to the nucleus when the cells were stimulated in the presence of Nc-5-s. Nc-5-s did not affect LPS-induced phosphorylation of STAT1 nor phosphorylation or DNA binding of c-fos, a component of the AP-1 transcription factor.

Taken together, the results show that Nc-5-s differently affects different pro-inflammatory cytokines. Hence, the overall effect of Nc-5-s on the inflammatory response in THP-1 monocytes could not be considered as either anti- or pro-inflammatory. The effects of Nc-5-s on cytokine secretion by THP-1 monocytes may be mediated through the ERK1/2, PI3K/Akt and/or the NF-κB pathway.

Although the ability of Nc-5-s to reduce IL-6 secretion by THP-1 monocytes indicates that it may be able to reduce a Th17 mediated autoimmune response and favor Treg differentiation in autoimmune diseases, these effects may be counteracted by the increase in TNF-α and/or IL-8 secretion.

Ágrip

Áhrif heteróglýcans úr blágrænþörungnum *Nostoc commune* á ónæmissvör THP-1 mónócýta

Gegnum aldirnar hafa ýmis náttúruefni verið notuð í heilsueflandi tilgangi. Þrátt fyrir það eru vísindamenn aðeins nýlega farnir að leggja vinnu í að skilja raunverulega líffræðilega virkni slíkra efna. Rannsóknir í dag miða að því að auka þá vitneskju sem og að finna ný náttúruefni með heilsubætandi virkni. Fléttur úr íslenskri náttúru hafa löngum verið nýttar við náttúrulækningar til að lina bólgusjúkdóma sem og aðra sjúkdóma. Mögulega má eigna fjölsykrum úr fléttunum þessi áhrif og hefur verið sýnt fram á að fjölsykrur úr fléttum og sambýlislífverum þeirra geti haft áhrif á ónæmiskerfið. Blágrænþörungurinn *Nostoc commune* er algengur í fléttusambýlum og er þekktur fyrir að framleiða og seyta flóknum fjölsykrum. Ónæmisfræðileg áhrif fjölsykra úr *Nostoc commune* hafa hins vegar ekki verið könnuð áður.

Markmið þessarar rannsóknar var að ákvarða ónæmisfræðileg áhrif heteróglýcansins Nc-5-s úr *Nostoc commune* á ónæmissvör THP-1 mónócýta og hvernig áhrifunum er miðlað innan frumunnar. THP-1 mónócýtar voru næmdir með IFN-γ í 3 klst og í kjölfarið örvaðir með inneitri (LPS) í eina (virkjun kínasa og innanfrumuboðferla), 24 (tjáning yfirborðssameinda) eða 48 klst (seyting bólguboðefna). Nc-5-s var bætt út í frumuræktir samhliða annað hvort IFN-γ eða LPS. Styrkur frumuboðanna TNF-α, IL-6, IL-8, IL-10 og IL-12p40 í æti var ákvarðaður með ELISA aðferð. Tjáning yfirborðssameindanna TLR4, TLR2, CD14, CD40, CD54 og CD86 var könnuð í frumuflæðisjá. Virkjun MAP kínasa, PI3K/Akt, NF-κB og JAK/STAT boðferla var ákvörðuð með Western blot aðferð. DNA bindigeta AP-1 umritunarþáttarins var mæld með TransAM® DNA bindingar prófi.

THP-1 mónócýtar örvaðir með LPS í návist Nc-5-s seyttu minna af IL-6 og meira af TNF-α og IL-8 en THP-1 mónócýtar sem örvaðir voru án Nc-5-s. Nc-5-s hafði engin áhrif á tjáningu þeirra yfirborðssameinda sem kannaðar voru. Nc-5-s dró úr LPS-örvaðri fosfæringu ERK1/2 MAP kínasans án þess að hafa áhrif á aðra MAP kínasa. Nc-5-s dró einnig úr fosfæringu Akt kínasa PI3K/Akt boðferilsins. Þrátt fyrir að Nc-5-s hafi ekki dregið úr niðurbroti IκBα hindrans, var tilhneiging til flutnings færri NF-κB sameinda inn í kjarna þegar frumurnar voru örvaðar í viðurvist Nc-5-s en þegar þær voru örvaðar án hans. Nc-5-s hafði ekki áhrif á LPS-örvaða fosfæringu STAT1 umritunarþáttarins né heldur á fosfæringu eða DNA bindingu c-fos hluta AP-1 umritunarþáttarins.

Samantekið sýna þessar niðurstöður að Nc-5-s hefur sértæk áhrif á seytingu mismunandi bólgumiðlandi frumuboða. Því var ekki hægt að skilgreina heildaráhrif Nc-5-s sem bólguhvetjandi eða bólguletjandi. Áhrifum Nc-5-s á frumuboðaseytingu THP-1 mónócýta er mögulega miðlað í gegnum ERK1/2, PI3K/Akt og/eða NF-κB boðleiðirnar.

Þrátt fyrir að geta Nc-5-s til að minnka seytingu THP-1 mónócýta á bólguboðefninu IL-6 bendi til þess að fjölsykran geti dregið úr Th17 miðluðu sjálfsofnæmissvari og ýtt undir sérhæfingu T frumna í átt að Treg frumugerð í bólgusjúkdómum, er hætt við að aukning í TNF-α og IL-8 geti unnið gegn þeim áhrifum.

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Declaration of contribution

Ástríður Ólafsdóttir performed all the experimental work included in this thesis, except for some of the measurements of the effects of Nc-5-s on cytokine secretion by LPS stimulated THP-1 monocytes that were conducted by former M.Sc. student Guðný Ella Thorlacius. Her experiments were repeated by Ástríður Ólafsdóttir and the results were pooled. The results by Guðný Ella Thorlacius contributed the following: IL-6, n=4; TNF-α, n=2; IL-10, n=4; IL-12p40, n=5.

Ástríður Ólafsdóttir, Ingibjörg Harðardóttir and Jóna Freysdóttir all contributed to the interpretation of the results and along with Guðný Ella Thorlacius contributed to writing of the manuscript, titled "*A heteroglycan from the cyanobacterium Nostoc commune affects inflammatory cytokine release and reduces phosphorylation of ERK1/2 in human monocytic THP-1 cells*" that is currently in preparation and will be included in the final printed version of this thesis.

Elín Soffía Ólafsdóttir and Sesselja Ómarsdóttir provided the heteroglycan Nc-5-s which was isolated and characterized by them and their co-workers.

List of abbreviations

ANOVA	Analysis of variance
AP-1	Activating protein 1
APCs	Antigen presenting cells
BCAP	B-cell adaptor for PI3K
BSA	Bovine serum albumin
CLRs	C-type lectin receptors
CRE	cAMP response element
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular regulated kinase
FCS	Fetal calf serum
GAS	Gamma activated site
HRP	Horseradish peroxidase
HRP	Horse radish peroxidase
ICAM	Intracellular adhesion molecule
IFN	Interferon
IFN-γR	Interferon γ receptor
IKK	IκB kinase
IL	Interleukin
IL-10R	Interleukin 10 receptor
IL-6R	Interleukin 6 receptor
ISRE	Interferon stimulated response element
IκB	Inhibitor of κB
JAK	Janus kinase
JNK/SAPK	c-Jun N-terminal kinase/stress-activated protein kinase
LBP	LPS binding protein
LFA	Lymphocyte function associated antigen
LPS	Lipopolysaccharide
Mal	Myd88 adapter-like
MAPK	Mitogen activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPKK kinase

MEK	MAPK/ERK kinase
MFI	Mean fluorescence intensity
MFI	Mean fluorescence intensity
Myd	Myeloid differentiation factor
Nc	Nostoc commune
NEMO	NF-κB essential modifier
NF-κB	Nuclear factor κB
NIR	Near-infrared
NK cells	Natural killer cells
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PI3K	Phosphoinositide 3-kinase
PMA	Phorbol 12-myristate 13-acetate
PRR	Pattern recognition receptors
RA	Rheumatoid arthritis
RT	Room temperature
SEM	Standard error of the mean
SI	Secretion index
SOCS	Suppressor of cytokine signaling
STAT	Signal transducer and activator of transcription
TBS	Tris buffered saline
Th	T helper cell
TIR	Toll-interleukin-1 receptor
TIRAP	TIR domain-containing adapter protein
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRE	TPA response element
Treg	Regulatory T cell

1 Introduction

For centuries people have used plants and other organisms, found in their immediate environment, to promote health and cure – or alleviate – disease. In the olden days, little or nothing was known about the actual health effects of herbal teas, plant extracts or other natural products. People gathered knowledge from their trials and errors in everyday life and knowing that something had a good effect on them was enough. Nobody asked why. It did not matter.

Today, however, much effort is put into understanding the precise effects of drugs and compounds on our bodies. With increasing scientific knowledge comes an increasing demand for new health promoting products and drugs that affect us in a controlled and known way. At the same time, with new diseases and pathogens emerging, novel compounds are needed to cure and fight illness. In an effort to discover new biologically active compounds, systematic screening of the effects of natural products is taking place in the scientific community. The present study is a contribution to this screening as well as to the understanding of how natural products affect cells of our bodies.

Since the immune system is responsible for protecting our body against infection while at the same time protecting the body from various inflammation-mediated diseases, testing the effects of natural products on its function can provide valuable and useful information on fighting diseases.

1.1 The immune system

The immune system is a large complex network of biological components that all work together to defend our bodies against pathogens, repair damage, destroy abnormalities and clear dead cells, as well as foreign or unwanted material. It requires tight regulation to maintain homeostasis and it must react only when needed and to a desired degree. A weakened immune system that does not respond properly can make the organism susceptible to infections and diseases. On the other hand, an unwanted or too intense response can be harmful and the immune system can turn against its own host. Such an uncontrolled immune response can result in disease, tissue damage and death. The key to maintaining homeostasis is for all immune cells to know exactly what is self and what is non-self. Other than regulating itself, the immune system as a whole must fulfill tasks such as recognizing pathogens, trigger an effector response and then induce a memory of the response to utilize in case of another infection with the same pathogen.

The immune system is divided into two parts; the innate and the adaptive immune systems. Both of these systems are indispensable and it is important to note that despite the distinction between the two systems, they are highly connected and one could not operate without the other.

All cells of the immune system arise from the same pluripotent hematopoietic stem cells in the bone marrow. These give rise to two kinds of progenitor cells; lymphoid and myeloid. The myeloid progenitor cells give rise to dendritic cells, monocytes/macrophages, neutrophils, eosinophils, mast cells and basophils, as well as the erythrocytes (red blood cells) and the platelet-producing cells called megakaryocytes. This lineage of cells comprises most of the cells that are classified within the innate

immune system. However, before a pathogen can possibly encounter some or all of these cell types, it needs to get past the first barriers of the innate immune system, i.e. the skin and mucosal epithelia. The skin and mucosal epithelia form a physical and chemical barrier that very few microorganisms get past in the first place. Those who do, are met with the next line of defense, namely molecules and cells that set off an elaborate innate immune response. These immune responses include recognition of common microbial structural motifs called pathogen-associated molecular patterns (PAMPs), phagocytosis by macrophages, and cytokine and chemokine secretion, to name a few. Cytokines and chemokines signal and attract other cells to the infected tissue and activate the complement system. The complement system consists of plasma proteins that can coat and help to destroy pathogens, as well as act as chemoattractants.

The lymphoid progenitor cells give rise to natural killer (NK) cells, T cells, B cells and dendritic cells. The NK cells are considered to be a part of the innate immune system since they do not recognize a specific antigen. The ability to react to a specific antigen is one of the main characteristics of B and T cells, the most important cells of the adaptive immune response. The adaptive immune system gets its name from the fact that it is developed during the whole lifetime of an organism. When B cells recognize an antigen, they differentiate into antibody-secreting plasma cells, but when T cells encounter an antigen they differentiate into activated effector T cells. The last of the lymphoid cell linage are the dendritic cells. As mentioned, these can also arise from myeloid progenitors, which is more common. Dendritic cells link the innate and adaptive immune responses. This is mainly because they can recognize pathogens via PAMPs and after digesting a pathogen, they can show the antigen to T cells that then will set off an adaptive immune response. Dendritic cells, being professional antigen presenting cells (APCs) are therefore highly important and versatile cells (1, 2).

1.1.1 Monocytes

As mentioned in chapter 1.1, monocytes are cells of the innate immune system. They circulate in the bloodstream and when they migrate into tissues they differentiate into macrophages. Both monocytes and macrophages are phagocytes, but monocytes are smaller, have fewer organelles and do not have pseudopodia. Being of the myeloid lineage, monocytes and dendritic cells arise from the same precursor cell (1, 2). However, monocytes can also directly differentiate into so-called inflammatory dendritic cells under inflammatory and/or infectious conditions (Figure 1) (3).

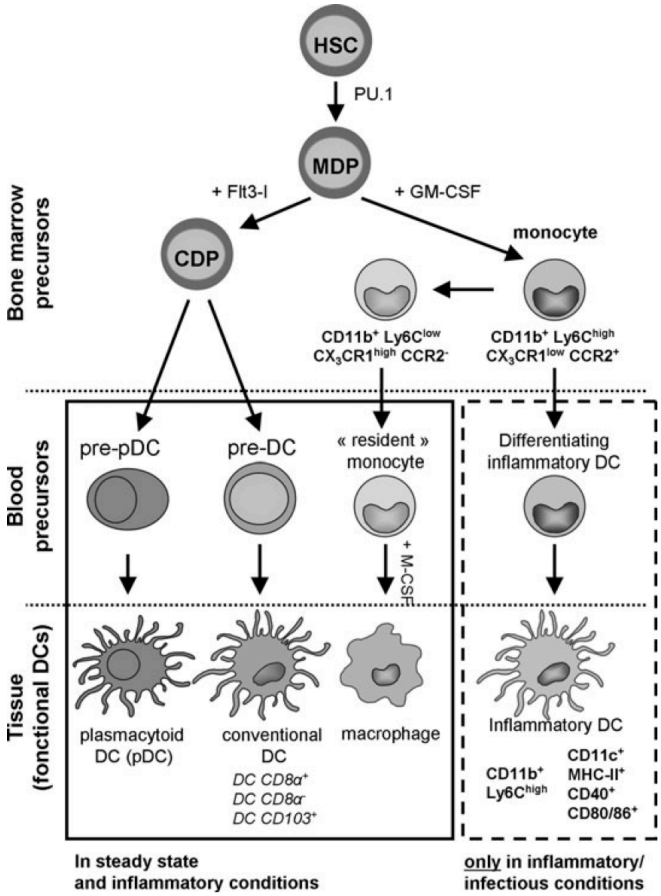


Figure 1. Monocytes can give direct rise to both macrophages and inflammatory dendritic cells (3).

1.1.2 THP-1 monocytes

THP-1 monocytes are a human acute monocytic leukemia cell line derived from cells isolated from a male patient in 1980 (4). The cells maintain their monocytic characteristics in culture. They can be used as undifferentiated monocytes or differentiated into macrophages by treating them with phorbol 12-myristate 13-acetate (PMA) (5). The THP-1 cell line is a popular tool for studying modulation of inflammation and the effects of various compounds on the innate immune system (6-8). Using cell lines rather than primary cells saves time and reduces cost.

THP-1 cells are characterized by an early arrest in differentiation. Markers that are expressed at high levels on mature monocytes, such as CD14, are therefore expressed at very low levels in THP-1 monocytes (9). Undifferentiated THP-1 cells do not respond sufficiently to lipopolysaccharide (LPS) alone, compared with monocytes isolated from human blood. When mature monocytes isolated from human blood were stimulated with LPS, they secreted Tumor necrosis factor α (TNF-α), Interleukin (IL)-6, IL-8, IL-10 and IL-1β. THP-1 cells only secreted TNF-α, IL-1β and little IL-8 (10). The insufficient response of THP-1 cells to LPS is because of their low CD14 expression. Therefore they need to be primed with Interferon (IFN)-γ to up-regulate this co-receptor (11).

1.2 Cellular signaling

The study of cellular communication is a large and complex field. Cells need to constantly communicate to do everything from maintaining basic functions to respond to changes in their environment. Different cell types utilize distinct signaling mechanisms that can either be unrelated or differ by one simple step or feature. Highly similar signaling pathways can lead to completely unrelated responses, all depending on where the original signal came from, what type of cell is receiving it, which other signals the cell is receiving and more.

1.2.1 TLR signaling

As mentioned in chapter 1.1, many molecules of the innate immune system can recognize certain classes of microbial patterns that are unique to microbes and cannot be found in association with other organisms. Pattern recognition is an efficient way to quickly bind and clear the body of unwelcome pathogens. The molecules involved in pattern recognition are the pattern recognition receptors (PRRs), which can be soluble or membrane bound. Toll-like receptors (TLRs) are trans-membrane signal receptor proteins that recognize various PAMPs (2). Toll receptor was first identified in *Drosophila* and found to be important for immune response against fungal infections (12). A mammalian homologue of the *Drosophila* Toll receptor was identified and shown to induce inflammatory gene expression (13) and has now been identified as one of more than ten mammalian TLRs, namely TLR4. To date, 13 members of the mammalian TLR family have been identified. TLR1-10 are expressed in humans, but not TLR11-13. Some TLRs are therefore species specific but most are highly conserved in mammals (14). TLRs can be extra- or intracellular. TLR7-9 and TLR3 are found in endosomal and lysosomal membranes, while TLR1-2, TLR5-6 and TLR10 are sequestered in the plasma membrane (14-16). TLR4 can be found both extra- and intracellular, as it can be endocytosed upon binding to its ligand (17).

TLRs recognize different ligands. TLR4 binds bacterial LPS, while TLR5 binds flagellin. TLR2 is essential for recognizing microbial lipopeptides and it dimerizes with TLR1 or TLR6 and thereby distinguishes between di- and triacyl lipopeptides. DNA and RNA are ligands of the intracellular TLRs (14, 15). A defined agonist or function has not been defined for TLR10 (16).

Given the diversity of the TLRs and their ligands, their triggering and downstream pathways lead to the production of various molecules that play an important role in inflammation. Dysregulation of TLR signaling is connected to inflammatory diseases, cancer and more. These receptors and their downstream effectors are therefore attractive targets for drug discovery (18).

1.2.1.1 TLR4 signaling

LPS induces an immune response and triggers inflammation through TLR4. Stimulation of TLR4 by LPS is one of the most extensively studied immune interactions and bacterial endotoxin has also been extremely well characterized. If the signal becomes excessive, it can lead to systemic inflammation and sepsis (19).

LPS is a component of the outer membrane of Gram negative bacteria. Typically, LPS consists of three structurally different parts, but the composition can vary slightly between different bacteria. Closest to the bacterial cell membrane is a hydrophobic part called LipidA. Next there is a core oligosaccharide and finally a polysaccharide named the O-antigen (1, 20). The part recognized by cells of the immune system is LipidA and some pathogens have evolved a modified LipidA structure in order to evade the innate immune response of their host (21). TLR4 does not recognize LipidA directly but uses three accessory proteins, namely MD-2, LPS-binding protein (LBP) and CD14 to be able to initiate a response to LPS. MD-2 associates with TLR4 within the cell and is important both for the trafficking of TLR4 to the cell surface as well as for the recognition of LPS. LBP can pick up LPS that has become detached from the bacterial membrane and transfers it to CD14, a surface bound protein present on monocytes/macrophages and dendritic cells. CD14 then transfers the LPS on to TLR4/MD-2 and an intracellular signal is initiated when MD-2 recognizes LPS and TLR4 oligomerizes. TLR4 is responsible for transducing the signal inside the cell, through Toll-interleukin-1 receptor (TIR) domains that come into contact with each other during oligomerization. The TIR oligomer recruits adaptor proteins to bridge the way to appropriate kinases and signaling molecules that then carry the signal on all the way to transcription factors in the nucleus. TLR4 signaling can occur either dependent or independent of the myeloid differentiation factor 88 (Myd88) adaptor protein and most likely, TIR recruits adaptors of both pathways through the same sites. The Myd88 dependent pathway is responsible for pro-inflammatory cytokine expression while the Myd88 independent pathway is responsible for the expression of type I IFNs. Only the Myd88 dependent pathway will be discussed here. In Myd88 dependent signaling, the TIR domains of TLR4 recruit an adaptor known as TIR domain-containing adapter protein (TIRAP), also called Myd88 adapter-like (Mal) (23). TIRAP makes a bridge for Myd88, that can then activate several pathways, including the mitogen activated protein kinase (MAPK) pathways, the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway and the phosphoinositide 3-Kinase (PI3K) / Akt (also called Protein kinase B) pathway (1, 22) (see Figure 2).

1.2.1.2 MAP kinases

There are three different types of MAP kinases; extracellular regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), also known as the stress-activated protein kinase (SAPK), and p38. LPS can activate all of these kinases and does so through certain cascades, called MAP kinase cascades. These typically consist of three kinases. The first is the MAPK kinase kinase (MAPKKK), which is activated by kinases downstream of Myd88. Next there is MAPK kinase (MAPKK) and lastly, ERK1/2, SAPK/JNK and p38 MAP kinases. Each of them has their own distinct MAPKK and MAPKKK. An activated MAP kinase can affect dozens of other cytosolic proteins and many nuclear transcription factors, all depending on the biological setting (23).

1.2.1.2.1 ERK1/2

ERK1/2 is activated by its MAPKKK, c-raf, and by its MAPKK called MAPK/ERK kinase 1/2 (MEK1/2). It is well known that LPS causes activation of ERK1/2 in monocytes and macrophages (23). If MEK1/2 is inhibited by the commercially available inhibitor U0126 which causes a reduction in the secretion of several inflammatory cytokines such as TNF-α, IL-1 and more. This suggests that ERK1/2 plays a role in inflammatory cytokine release that is independent of the other two MAP kinases (23).

Under inflammatory conditions, ERK1/2 causes phosphorylation and activation of c-fos, which is a part of the activating protein 1 (AP-1) transcription factor. Together with c-jun, c-fos makes up one form of AP-1. AP-1 regulates several early transcriptional processes after extracellular stimulation of cells. When c-fos is not phosphorylated, it is unstable both on mRNA and protein level. ERK1/2 phosphorylation of c-fos at ser374 in the C-terminus is very important for LPS-induced cytokine response. It causes c-fos to stabilize for hours and primes it for further activation by ERK1/2 phosphorylation at other sites in the transactivation domain (24-26). Phosphorylation of c-fos is crucial for AP-1 DNA binding activity. AP-1 can be made up of c-jun homodimers, but the transcriptional activity of a c-fos/c-jun dimer is 25 times greater (27). Dimers of c-fos and c-jun bind to DNA sequences called TPA response element (TRE) and cAMP response element (CRE) and thereby contribute to the initiation of transcription of various genes (28).

Finally, ERK1/2 can also affect nuclear processes and gene transcription by entering the nucleus itself. There, it can interact with a large number of substrates, direct DNA interactions and affect chromatin remodeling (29).

1.2.1.2.2 SAPK/JNK and p38

The MAPKKK MEKK1/4 and the MAPKK MKK4/7 are responsible for activation of SAPK/JNK. The two isoforms of this kinase are responsible for the phosphorylation and activation of c-jun of the AP-1 transcription factor, as well as many others, depending on the type of signal being conducted (23).

P38 kinase is activated by the MAPKKK PKR and the MAPKK MKK3/6. Like ERK1/2 and SAPK/JNK, it can phosphorylate many downstream kinases and transcription factors, including AP-1 (23, 30).

1.2.1.3 PI3K/Akt pathway

In addition to activating MAP kinases, signaling through TLRs can also lead to activation of a pathway commonly known as the PI3K/Akt pathway. The PI3K/Akt pathway plays an important role in many different cellular settings and regulates cellular proliferation, survival and other cellular functions. However, not much is known about TLR mediated activation of PI3K and its downstream effector, Akt, compared to other inflammatory signaling pathways. The subject has been extensively studied in recent years but the role of the pathway in inflammatory settings is still controversial and depends greatly on the type of cells being activated (31, 32). Many studies show that in monocytes,

macrophages and dendritic cells, the PI3K/Akt pathway serves to negatively regulate pro-inflammatory cytokine production (31, 33-36). Other studies, however, suggest a pro-inflammatory role for PI3K by the PI3K/Akt mediated effects on NF-κB phosphorylation and transcriptional activity (37, 38).

The most recent report on the TLR4 mediated stimulation of the PI3K/Akt pathway indicates that PI3K is activated through a recently discovered TLR adaptor protein called B cell adaptor for PI3K (BCAP) (31). BCAP is expressed in B-cells, monocytes/macrophages and NK cells and is a negative regulator of inflammation. Other reports suggest that PI3K is activated through simultaneous interactions of many proteins, ultimately docking the p85 subunit of PI3K to TLR4 and thereby activating the signaling cascade (39). PI3K subsequently activates the Akt kinase (also known as protein kinase B (PKB)) and Akt further affects series of targets leading to the previously mentioned diverse transcriptional outcomes (31, 32).

1.2.1.4 NF-κB pathway

TLR4 mediated activation of the transcription factor NF-κB is one of the best known intracellular inflammatory signaling pathways. NF-κB was first discovered 27 years ago and has been extensively studied since (40).

NF-κB is a dimeric transcription factor. In mammals there are five NF-κB family members; p65, RelB, C-Rel, p105 and p100 (precursors of p50 and p52). Dimers of these regulate various genes involved in immune responses. In TLR signaling, the most common NF-κB is the one that is made up of p65 and p50. NF-κB can become activated by TLR4 stimuli, through a pathway commonly called the canonical pathway where Myd88 is the adaptor protein. Through a series of steps, Myd88 activates a complex containing a regulatory component called NF-κB essential modifier (NEMO) and two kinases called IκB kinase (IKK)α and IKKβ. When these kinases become activated, they phosphorylate inhibitor of κB (IκB)α. IκBα is a regulatory protein that keeps NF-κB dimers sequestered in the cytoplasm when no stimuli is present. Upon phosphorylation of IκBα, it is ubiquitinated and degraded, leaving the NF-κB dimers free to translocate into the nucleus and bind to their DNA binding sites (41, 42).

1.2.1.5 Cytokines

As a result of the transcription factor activation described above, caused by LPS stimulation via TLR4, many inflammatory genes are transcribed. Among them are the genes of pro- and anti-inflammatory cytokines, important for regulating the inflammatory response by monocytes, macrophages and dendritic cells. Cytokines are small molecules secreted by activated cells, and these small molecules further affect target cells by binding to their matching receptors. LPS-induced cytokine secretion by human monocytes and macrophages has been well characterized (43).

1.2.1.6 IL-6

IL-6 is a pro-inflammatory cytokine, secreted by a variety of cell types such as monocytes, macrophages, B cells and T cells. IL-6 also plays a role in many other types of responses than inflammation (44), but these will not be discussed further here. LPS stimulation enhances IL-6 production by monocytes and by binding to its IL-6 receptor (IL-6R) on target cells such as neutrophils, T cells and other monocytes or macrophages, it can have an array of different effects (1, 44, 45). IL-6 is very important for the regulation of T cell differentiation and induces the production of T helper (Th)17 cells while inhibiting the differentiation of regulatory T cells (Tregs). IL-6, therefore, contributes greatly to a state of inflammation (1, 45) and targeting IL-6 and its receptor in order to treat chronic inflammatory diseases is a very relevant approach. IL-6 inhibitors are already in use for treatment of rheumatoid arthritis (RA) (46, 47).

In the promoter region of the IL-6 gene there are several control elements. Binding sites for both AP-1 and NF-κB are present, among many others (44).

1.2.1.7 IL-8

IL-8, also called CXCL8, is an inflammatory chemokine that primarily serves to attract and activate neutrophils to the site of inflammation and activate them. Stimulation of monocytes with LPS causes secretion of IL-8. Other cells, such as endothelial cells and fibroblasts can also secrete this cytokine. IL-8 binds to two receptors called CXCR1 and CXCR2. High levels of IL-8 have been found in body fluids of patients with many inflammatory diseases. The IL-8 gene promoter region contains binding sites for both AP-1 and NF-κB, among others. NF-κB and AP-1 must act together for IL-8 transcription to occur (43, 48).

1.2.1.8 IL-10

IL-10 is most commonly known for its function as an anti-inflammatory cytokine. It is synthesized by activated monocytes, macrophages and T cells, especially Tregs, as well as other cell types (49). IL-10 exerts its anti-inflammatory effects by inhibiting the production of pro-inflammatory cytokines by activated monocytes and macrophages. In this way, it can help limit tissue damage that is caused by inflammation (1, 43, 50). IL-10 is also able to down-regulate the expression of co-stimulatory molecules such as CD86 on the surface of macrophages and thereby decreasing their ability to support adaptive immunity activation. IL-10 signals to other cells via its IL-10 receptors 1 and 2 (IL-10R1 and IL-10R2). The two receptors signal through the intracellular janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway (see chapter 1.2.1.11) and one of the target genes for IL-10 is the suppressor of cytokine signaling 3 (SOCS3) which has the ability to negatively regulate immune responses (43).

Synthesis of IL-10 depends mainly upon activation of the Myd88 independent pathway, through the (STAT) 3 transcription factor. However, IL-10 gene transcription upon LPS stimulation can also be

promoted by other transcription factors such as AP-1 (43), and the IL-10 gene promoter region contains an AP-1 binding site (51).

1.2.1.9 IL-12

IL-12 is a well known pro-inflammatory cytokine. IL-12 functions as a heterodimer commonly called IL-12p70, since it is made up of disulfide-linked 35 kDa (p35) and 40 kDa (p40) subunits. Each of the subunits is coded for by unrelated genes on separate human chromosomes and are therefore regulated independently. IL-12p70 secretion is controlled by the regulation of IL-12p40 transcription. The IL-12 promoter region contains an NF-κB binding site, among others (43, 52). IL-12 shares the p40 subunit with another newly identified cytokine, called IL-23 that also is a member of the IL-12 family. The expression of p35 and p40 is up-regulated following infection and stimulation with components such as LPS (43).

IL-12 is mostly secreted by stimulated APCs. It directs naive T cells to differentiate into mature Th1 cells and thereby promotes the inflammatory response (1). With inflammation being a required and normal response to infections, IL-12 plays a very important role in normal, balanced immune functions. However, unregulated IL-12 secretion often plays a role in Th1-mediated autoimmune diseases (43, 52). Recently, it has also become clear that the newly identified IL-23 is a major factor in RA and other inflammatory diseases (53).

1.2.1.10 TNF-α

TNF-α is an extensively studied pro-inflammatory cytokine. It exists both as a soluble and a membrane bound protein and is secreted mainly by monocytes, macrophages and T cells in response to many different inflammatory stimuli. Other cells, such as mast cells and B cells are also capable of secreting TNF-α. TNF-α is involved in an array of different cellular responses such as inflammation, cell growth regulation, differentiation and many more. It signals to its target cells via TNF-α receptors 1 and 2. The TNF-α gene is under the control of many regulatory sites and contains binding sequences for both AP-1 and NF-κB (43, 54).

TNF-α is involved in the disease mechanisms of a series of inflammatory diseases, such as RA and psoriasis. Drugs that target TNF-α are available for treatment of these inflammatory conditions and have shown overall good results (43, 55). However not all patients respond well to this treatment and other solutions, such as IL-6 inhibitors, are needed for these patients (56).

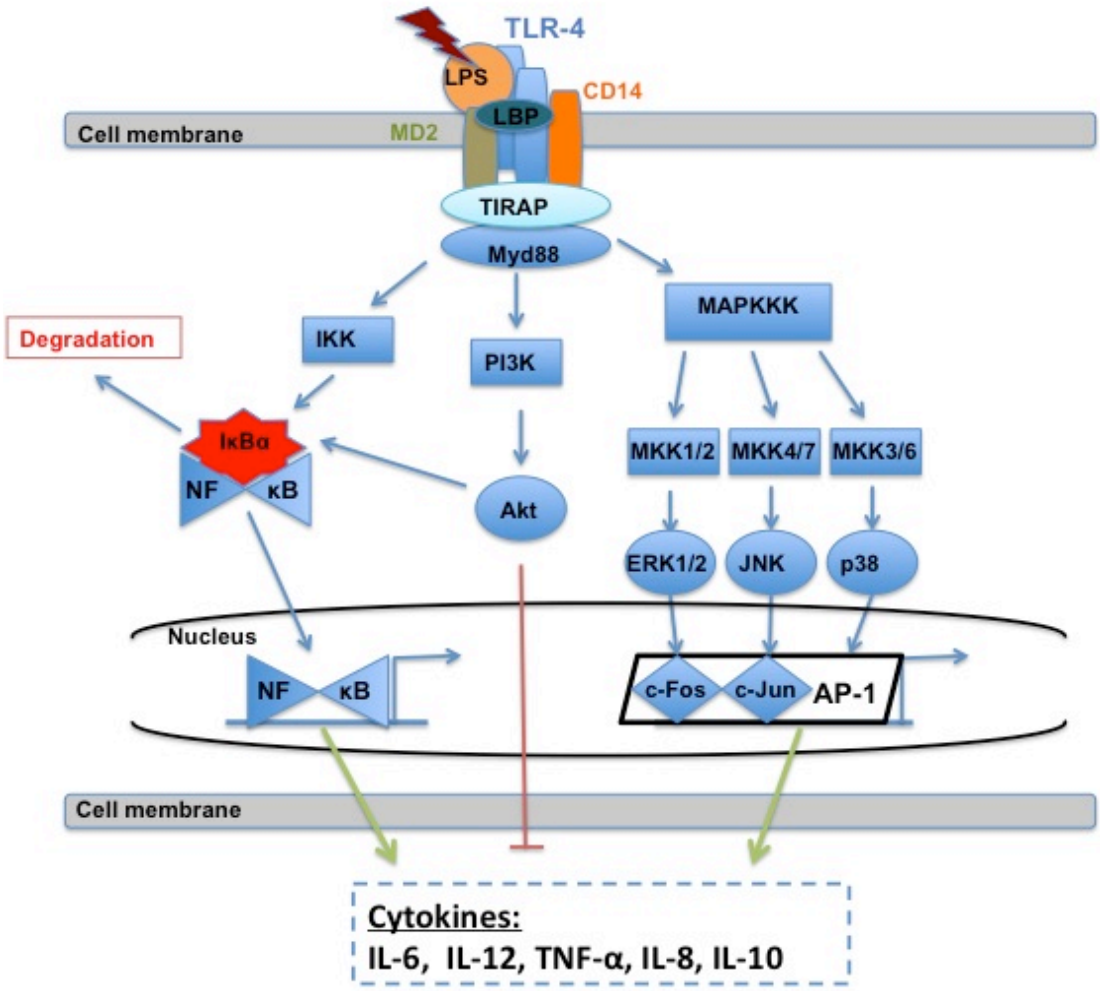


Figure 2. An overview of TLR4 signaling following LPS binding.

A simplified overview of the pathways activated downstream of TLR4 after LPS binds to its receptor. Through the TIRAP adaptor protein and Myd88, the NF-κB, PI3K/Akt and MAPK signaling pathways are activated by phosphorylation. Activation of these pathways further leads to regulation of transcription factor activation, finally resulting in the appropriate secretion of inflammatory cytokines. Authors image based on (14, 22, 23, 31, 32).

1.2.1.11 IFN-γ signaling

IFN-γ is a cytokine secreted by NK cells and T cells and is important for macrophage activation (1). To a lesser extent, B cells, NKT cells and APCs can also secrete IFN-γ (57). IFN-γ signals through a heterodimeric receptor, interferon-γ receptor (IFN-γR), and sets off a well characterized intracellular signaling cascade known as the JAK/STAT pathway. JAKs are associated with the IFN-γR and when IFN-γ binds to the receptor, the JAKs trans-phosphorylate each other. The activated JAKs then phosphorylate the transcription factor STAT1 in the cytosol, making STAT1 capable of dimerizing and translocating to the nucleus (58). STAT1 then causes transcription of inflammatory genes by binding to gamma activated sites (GAS) (59) or, to a lesser extent, to the interferon stimulated response element (ISRE) (58, 60).

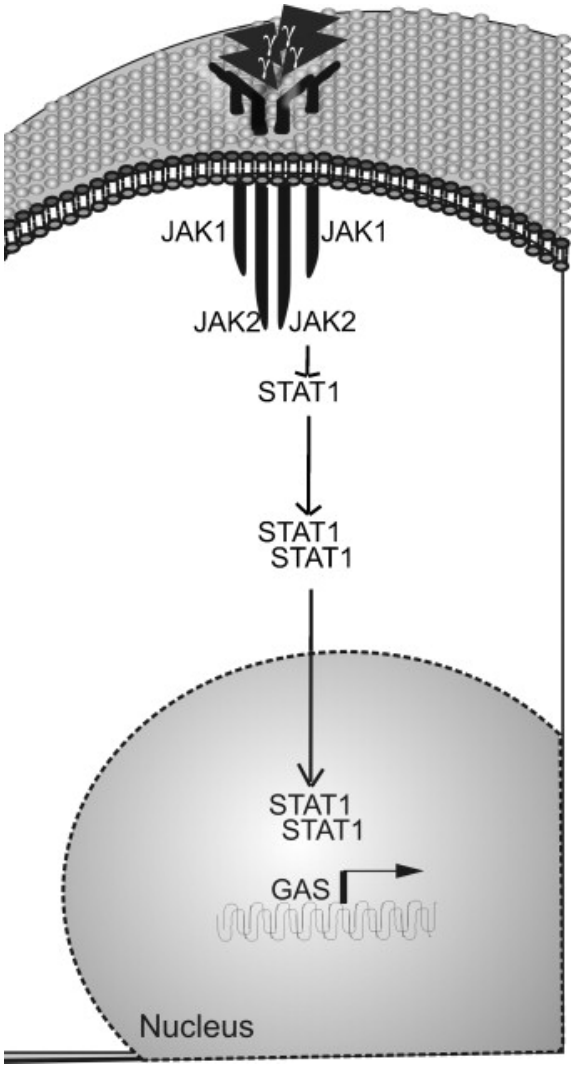


Figure 3. IFN-γ signaling.

IFN-γ binds to its receptor. This binding moves the IFN-γR associated JAKs closer to each other, causing their trans-phosphorylation. The JAKs then phosphorylate STAT1 in the cytosol, making it able to dimerize and translocate to the nucleus where it causes transcription of genes containing GAS or ISRE sequences (58).

1.3 Monocytic co-stimulatory and adhesion molecules

1.3.1 CD40, CD86 and CD54

Monocytes express various receptors and co-stimulatory molecules on their surface. When activated, expression of these co-stimulatory molecules increases and they play a role in signaling to other cells of the immune system. CD86, also called B7-2, is a surface molecule expressed by APCs that functions as a co-stimulatory molecule for CD4 T cell activation by binding to CD28, which activates CD4 T cells to express cytokines and surface molecules leading to their differentiation into effector T cells. These cytokines and surface molecules are also important for further activation of the APC and for promoting the CD8 T cell differentiation. Among these up-regulated proteins, is CD40, which is

mainly expressed by APCs and functions to increase their activation. It also functions as a co-stimulatory molecule for CD8 T cell proliferation when CD40L on CD4 T cells binds to CD40 on the APCs, causing up-regulation of CD86, among others (1, 61). This, in turn, provides the naive CD8 T cells with further co-stimulation and pushes them towards becoming fully differentiated CD8 cytotoxic T cells. CD54, also known as intercellular adhesion molecule 1 (ICAM-1), is yet another molecule important for the interaction between T cells and APCs. ICAM-1 is one of several molecules that facilitates the binding between APCs and naive T cells when the latter are sampling MHC molecules for antigens. ICAM-1 interacts with lymphocyte function associated antigen 1 (LFA-1) on the T cell surface (1).

CD40, CD86 and CD54 are up-regulated during immune response and inflammation and have been implicated in inflammatory diseases, such as RA and atherosclerosis (61-64).

1.4 Natural products

As mentioned in the introduction, people have for centuries turned to nature when they need to cure disease and promote health. Until very recently in human history, all that was available were crude extracts of plants or other natural components. Some of the oldest documentation of crude drug use dates back 4-5000 years, documenting the use of plant derived substances by Egyptians and the people of Mesopotamia (65). One can further assume that although records of earlier use of herbal medicines might not exist, ancient civilizations must indeed also have depended on nature for survival. It was not until 200 years ago that the first active ingredient from a medicinal plant was isolated when Friedrich Sertürner isolated morphine from opium and marked a new era in pharmacology history (66).

In today's modern society, a large proportion of the drugs we make use of are based on chemical leads from traditional medicine. Therefore, discovering novel compounds and understanding their effects is a pressing subject as we constantly race against new and developing pathogens (67, 68). A systematic screening of natural products has proven to be the most effective means to finding new drug leads. Almost half of the drugs approved from 1994 to 2007 were based on natural products (69). Of those, thirteen natural-product related drugs were approved from 2005 to 2007 and five of those represented the first members of new classes of drugs (70). Many natural compounds that have been isolated from plants, animal products such as milk, marine animals, algae, fungi, bacteria and more, have been shown to have immunoregulatory, anti-inflammatory and other biomedically relevant effects (71-77). Fewer than 15% of higher plant species are thought to have been examined for bioactivity (78) and hundreds of thousands of marine species and even millions of microbes are yet to be discovered (79). From the year 2007 to 2008 as many as 197 bioactive marine compounds were discovered (80). Therefore, one can assume there is much to gain in this field of research.

Screening of natural products focuses mainly on testing the effects of so-called secondary metabolites. Secondary metabolites are those that, in contrast to primary metabolites, are not directly involved in the basic metabolism of an organism. Secondary metabolites typically function to protect the organism against e.g. predators or parasites and thereby provide the organism with an evolutionary advantage (81). Adaptation to Iceland’s unique habitat may have encouraged plants and

other organisms to develop biological compounds with novel properties, as organisms evolve to survive under diverse conditions. However, this is a largely unexplored and exciting subject.

1.4.1 Lichens

Lichens have been used in folk medicine for centuries and in Iceland there is a rich tradition for the use of lichens such as *Cetraria islandica* to alleviate disease and as a dietary supplement. Lichens are composite organisms consisting of a mycobiont (a fungal component) and a photobiont (a photosynthetic partner) living in symbiosis. The mycobiont is typically of the phylum Ascomycota or Basidiomycota. The photobiont can be green algae or cyanobacteria, or both. The symbiosis is so intimate that if separated, the organisms can rarely survive on their own. Lichen growth is very slow, but their lifespan is long. They can survive in extreme conditions and can even be found living on rocks in the Antarctica (82). Evolution is thought to have provided lichens with an array of unique secondary metabolites, making them capable of surviving in extremely rough terrains.

Various lichen extracts and compounds have been shown to affect inflammatory responses in both murine models and human cell culture models (83-86). Because the lichen symbiosis is so close, the isolated extracts most likely contain material from both symbiotic partners and even from surrounding or other symbiotic microorganism as well. Therefore it is important to further purify these extracts in order to find the actual bioactive component and perhaps its origin.

1.4.2 Polysaccharides

Research of the bioactivity of polysaccharides from various sources has become an increasingly large field of study. With the isolation and detailed characterization of polysaccharides becoming faster and more convenient, as well as *in vitro* and *in vivo* models becoming more sophisticated, the number of studies has increased greatly (87). Many extracts of polysaccharides as well as pure polysaccharides isolated from nature have been shown to affect immune responses, where some have been shown to up-regulate inflammation (88) while others down-regulate it (89, 90).

Various polysaccharides isolated from lichens have immunomodulating effects (91-93).

1.4.3 Cyanobacteria

Cyanobacteria can fix atmospheric nitrogen and make it readily available to the organisms in their closest surroundings. They also perform photosynthesis and are ubiquitous in almost any type of terrain in the world. As mentioned previously, cyanobacteria are common in lichen symbiosis as the photobiont and most often lichen cyanobacteria are of the *Nostoc* genus (94).

The cyanobacterium *Nostoc commune* is known to produce complex extracellular polysaccharides that are important for the organisms tolerance of harsh environmental conditions. It can survive extreme temperatures, hydration and pH changes, and can grow in habitats where few other species are able to maintain themselves (95, 96). Edible cyanobacteria have long been used for dietary

supplementation and a subspecies of *Nostoc commune* have been shown to possess anti-inflammatory properties *in vitro* (97). Crude extracts of *Nostoc commune* are also biologically active against certain protozoa (98).

Figure 4. *Nostoc commune* (IS: Túnkrepja).

Image captured by Hörður Kristinsson, floraislands.is.

1.4.4 Nc-5-s

Nc-5-s is a complex heteroglycan that was isolated from the cyanobacterium *Nostoc commune*. The molecular weight of Nc-5-s was estimated to be about 1500 kDa and the monosaccharide composition was determined to be approximately Glc/GlcA/Xyl/Man/Ara/Rib/Gal in ratios of 24:24:15:13:13:7:4 (99).

2 Aims

For centuries, plants and other natural products have been used to promote health, yet little is known about how these products exert their effects. Today's research is focused on comprehending these complex effects and the current study is a contribution to that understanding, as well as the screening of novel natural products for bioactivity.

The main aim of the study was to determine the effects of the purified heteroglycan Nc-5-s from the cyanobacterium *Nostoc commune* on the inflammatory responses of THP-1 monocytes, and how the effects were mediated on an intracellular level.

Specific aims:

1. To confirm the effects of Nc-5-s on cytokine secretion by THP-1 monocytes
2. To determine the effects of Nc-5-s on:
    a. Cell viability
    b. IL-8 secretion
    c. Expression of surface molecules
    d. Intracellular signaling pathways downstream of the receptors TLR4 and IFN-γR

3 Materials and Methods

3.1 Isolation and characterization of Nc-5-s

The Nc-5-s heteroglycan was kindly provided by professor Elín Soffía Ólafsdóttir and associate professor Sesselja Ómarsdóttir at the Faculty of Pharmaceutical Sciences. The isolation and characterisation of the heteroglycan was performed by their students and was not a part of this project.

Nc-5-s was isolated from the cyanobacterium *Nostoc commune* and structurally characterized as previously described (99). In short, *Nostoc commune* was collected in Hvalfjörður, south-western Iceland, then dried and powdered, extracted through a series of steps using organic solvents and further purified with anion-exchange chromatography as shown in Figure 5. When used for experiments, the dry Nc-5-s was dissolved in culture medium.

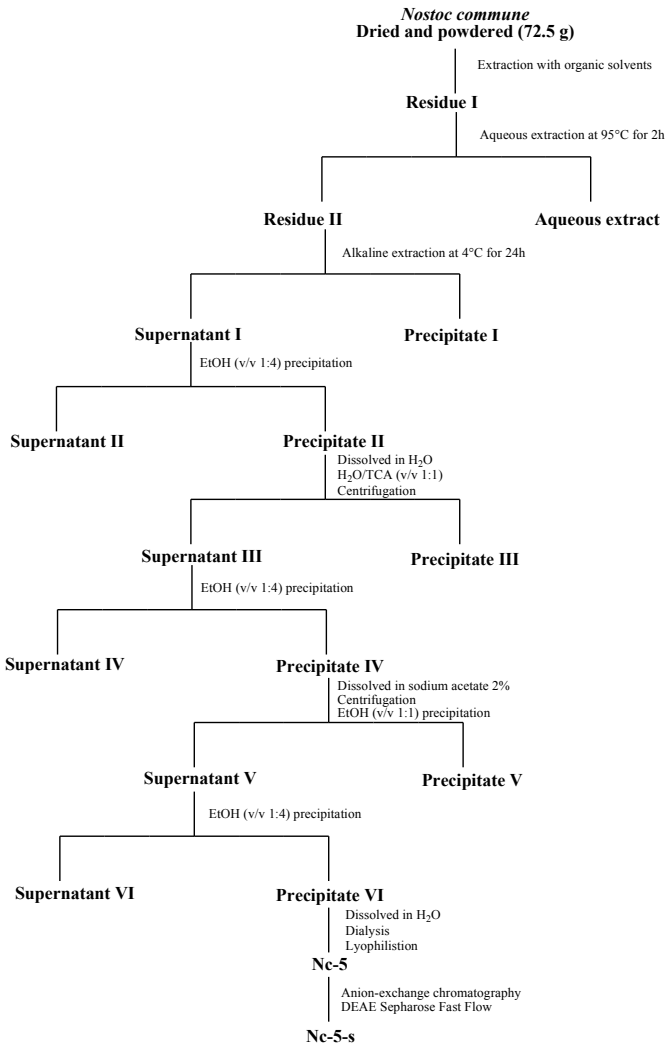


Figure 5. Extraction and fractionation of *Nostoc commune.*

(99).

3.2 Cell culture

3.2.1 THP-1 cells

The THP-1 cell line (DSMZ) was maintained in 75 cm^2 EasYFlasks™ plastic cell culture flasks (Nunc) in RPMI-1640 medium supplemented with 2.05 mM L-glutamine (Gibco, Invitrogen), 10% heat inactivated fetal calf serum (FCS), 100 U/mL penicillin and 100 μg/mL streptomycin. The cells were passaged twice a week. The passage was performed by centrifugation of the cell suspension at 300xg for 10 min and re-suspension in fresh medium at a concentration of 2.7-2.8x10^5 cells/mL. Cell count was determined using Trypan blue staining with a Countess® cell counter (Invitrogen). The cells were cultured in a 95% humidified incubator at 37°C and 5% CO_2. Each cell batch was passaged a maximum of 20 times.

The THP-1 cells were used as undifferentiated, activated monocytes following priming with IFN-γ at 100 u/mL (R&D Systems) for 3 h and stimulation with bacterial LPS at 0.75 μg/mL (Sigma-Aldrich). Nc-5-s at concentrations indicated for each experiment was added to the cell culture either along with the IFN-γ (-3 h) or along with the LPS (0 h). When the MEK1/2 inhibitor U0126 (Cell Signaling) was used, it was added to the cells along with the LPS at concentrations indicated (see Figure 6).

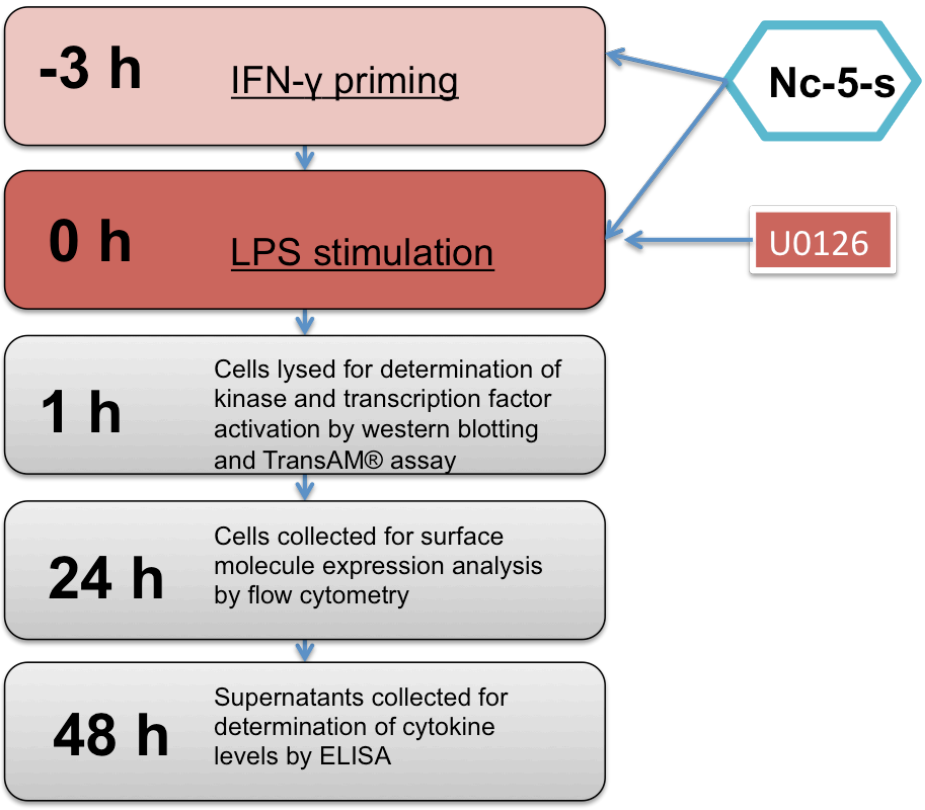


Figure 6. Timeline for experimental setups.

Cells were primed with IFN-γ for 3 h and stimulated with LPS. Nc-5-s was added at -3 h or at 0 h. The MEK1/2 inhibitor U0126 was added at 0 h. Samples for Western blotting, TransAM® DNA binding assay, flow cytometry and cytokine measurements were collected at indicated time points. Experiments for each application were carried out separately and the timeline is therefore not an overview of one single experiment, but many.

3.2.2 Assessment of cell viability using Trypan blue

Cells were seeded in 6 well plates at a density of $1x10^6$ cells/mL, 2 mL/well and incubated with Nc-5-s at the highest concentration tested (100 μg/mL) for 48 h, with or without IFN-γ and LPS. After incubation, 10 μL of each sample was collected and stained with an equal amount of Trypan blue stain (0.4%). Cell number was then determined using a Countess® cell counter.

3.2.3 Assessment of Nc-5-s cytotoxicity using Via-Probe

Cells were seeded and incubated in the same way as in chapter 3.2.2. After incubation, cells were washed with 2 mL staining buffer (Phosphate buffered saline (PBS) with 0.5% bovine serum albumin (BSA), 2 mM Ethylenediaminetetraacetic acid (EDTA) and 0.1% NaN_3), centrifuged at 1200 rpm for 5 min at 4°C and re-suspended in 0.5 mL staining buffer with 3 μL of Via-Probe™ cell viability solution (BD Biosciences). Cells were then incubated for 15 min on ice and in the dark followed by immediate collection of the cells on a flow cytometer (FACScan, BD Biosciences). Collected data was analyzed using Flowing Software, version 2.5.0 (Turku Centre for Biotechnology, University of Turku).

3.3 Determination of cytokine concentration using ELISA

Cells were seeded in 48 well plates at a density of $5x10^5$ cells/mL, 1 mL/well, primed with IFN-γ (-3 h) and stimulated with LPS (0 h). At the same time as the LPS was added, Nc-5-s was added at 1, 10 or 100 μg/mL and the cells cultured for 48 h (with or without inhibition with U0126 MEK1/2 inhibitor). Thereafter, cells were collected, centrifuged at 300xg for 10 min and supernatants collected and stored at -80°C.

Cytokine concentration in the collected supernatants was measured by sandwich Enzyme-linked immunosorbent assay (ELISA) DuoSets® from R&D Systems, according to manufacturers instructions. Flat bottomed 96-well ELISA maxisorp plates (Nunc) were coated with mouse anti-human capture antibodies against the cytokine of interest overnight at room temperature (RT). The following day, the antibody solution was discarded and the plates blocked with blocking buffer (PBS with 1% BSA, 5% sucrose and 0.05% NaN_3) for 1 hour at RT to avoid unspecific binding of proteins in the supernatants to the plates. Next, the plates were washed four times with washing buffer (PBS with 0.05% Tween), supernatants and standards added in appropriate dilutions and the plates incubated for 2 h at RT. Samples and standards were diluted in ELISA buffer (PBS with 1% BSA) or TRIS-ELISA buffer (Tris buffered saline (TBS) with 0.1% BSA and 0.05% Tween) (for IL-8). After incubation, the plates were washed three times and cytokine specific biotinylated goat anti-human detection antibodies added and the plates incubated for two hours at RT, followed by washing. Detection antibodies were diluted in ELISA buffer or Tris-ELISA buffer (for IL-8). Next, horseradish peroxidase (HRP)-conjugated streptavidin was added to the wells and the plates incubated for 20 min in the dark. After a final washing, TMB ONE substrate solution (Kem-En-Tec), was added to the wells. Plates were incubated in the dark until a dark blue color had developed in the highest standard and then the

reaction was stopped by adding 0.18 M sulphoric acid to each well. Finally, the absorbance was measured at 450 nm using a micro-plate reader.

Each sample or standard was run in duplicate. Dilutions of the supernatants varied from undiluted to 1/10 dilution, depending on the cytokine measured. Two-fold serial dilutions of standards for the respective cytokines were included in each plate and a standard curve constructed. Values in pg/mL were calculated based on the standard curve. The concentration of a cytokine in the supernatants of cells incubated with Nc-5-s was divided by the concentration of the cytokine in the supernatants of cells incubated without Nc-5-s to develop the secretion index (SI).

Previously, former M.Sc. student Guðný Ella Thorlacius, had started investigating the effects of the Nc-5-s heteroglycan on cytokine secretion by THP-1 monocytes (100). In chapter 4.2, results from her study as well as the present one, will be shown as pooled data.

3.4 Expression of surface molecules

Expression of the surface molecules TLR4, TLR2, CD14, CD40, CD54 and CD86 were determined using flow cytometry.

Cells were primed with IFN-γ (-3 h) and stimulated with LPS (0 h). At the same time as the LPS was added, Nc-5-s was added at 100 µg/mL and the cells cultured for 24 h. Thereafter, 100 µL of sample were pipetted into tubes and 5 µL of fluorescence-labeled monoclonal antibodies were added (Table 1). Appropriate isotype-specific antibodies were used as controls (Table 1). Cells were incubated with the antibodies for 20 min, on ice and in the dark and then washed with 2 mL staining buffer (PBS with 0.5% BSA, 2 mM EDTA and 0.1% NaN_3) and centrifuged at 1200 rpm for 5 min at 4°C. Finally, the cell pellet was resuspended and fixed with 300 µL of 1% paraformaldehyde in PBS and collected using FACScan. Analysis was performed using Flowing Software, version 2.5.0 (Turku Centre for Biotechnology, University of Turku) and results expressed as percentage positive cells and as mean fluorescence intensity (MFI).

Table 1. Antibodies and isotype controls for flow cytometry analysis.

Antigen	Cat#	Isotype	Conjugate	Dilution	Supplier
TLR4	12-9917-41	mouse IgG2a	PE	1:5	eBioscience
TLR2	558319	mouse IgG1	Alexa647	none	BD Pharmingen
CD14	mca596	mouse IgG2a	APC	none	AbD Serotec
CD54	mca1615	mouse IgG1	FITC	none	AbD Serotec
CD86	mca1118	mouse IgG1	FITC	none	AbD Serotec
CD40	mca1590	mouse IgG2a	PE	none	AbD Serotec
Isotype control	mca928	mouse IgG1	FITC	none	AbD Serotec
Isotype control	mca929	mouse IgG2a	PE	1:5 or none	AbD Serotec
Isotype control	mca929	mouse IgG2a	APC	none	AbD Serotec
Isotype control	557732	mouse IgG1	Alexa647	none	BD Pharmingen

3.5 Assessment of intracellular signaling pathway activation using Western blotting

To determine the activation/phosphorylation of kinases and transcription factors, cells were seeded in 6 well plates at a density of $1x10^6$ cells/mL, 2 mL/well, primed with IFN-γ (-3 h) and stimulated with LPS (0 h). Nc-5-s was added at 100 µg/mL either with IFN-γ or with LPS and the cells were cultured for 1 h after the addition of LPS. Finally, cells were collected and lysed using either whole cell lysis or by separating cytoplasmic and nuclear fractions.

3.5.1 Whole cell lysis

To determine phosphorylation of Akt and the MAP kinases (ERK1/2, p38 and SAPK/JNK) as well as the breakdown of the NF-κB inhibitor IκBα, whole cell lysates of treated cells were prepared as follows:

Cells were collected into pre-cooled 2 mL Eppendorf tubes, centrifuged at 450xg for 5 min at RT and supernatants discarded. Cells were then re-suspended, washed with 1 mL ice cold PBS, centrifuged again and the PBS carefully pipetted off. The cell pellets were then lysed in 200 µL Western Lysis Buffer (25 mL 1M Tris-HCl pH 7.4, 15 mL 5M NaCl, 5 mL NP-40, 334 mL dH_2O) with added aliquots of stock solutions of protease (MINI-Tab, Roche) and phosphatase (PhosStop, Roche) inhibitors. Next, samples were sonicated on ice in a Bioruptor® sonicator (Diagenode) for a total of 6 min, with the time divided in intervals of 30 sec sonication followed by 30 sec of rest. Cells were put on ice for 20 min and then centrifuged at 14000 rpm for 5 min at 4°C to spin down cell debris. Finally, the supernatant (whole cell lysate) was collected and stored at -20°C. If applicable, protein concentration was measured using Bradford Assay.

3.5.2 Nuclear extraction

To determine levels of expression and phosphorylation of transcription factors, nuclear lysates of treated cells were made. Cytoplasmic fractions were also obtained using this method and were used to determine phosphorylation of the SAPK/JNK MAP kinase in the cytoplasm.

To make separate nuclear and cytoplasmic lysates, a Nuclear Extract Kit from Active Motif was used according to the manufacturers instructions. Cells were collected into pre-chilled conical tubes and washed with PBS containing phosphatase inhibitors. Samples were centrifuged at 500 rpm for 5 min at 4°C and the supernatant discarded. The cell pellets were gently re-suspended in Hypotonic buffer, transferred to a 1.5 mL Eppendorf tube and incubated on ice for 15 min. Detergent was added to the samples and they were vortexed at highest speed for 10 sec and then centrifuged at 14000xg for 1 min at 4°C. Supernatants were then transferred to a new 1.5 mL Eppendorf tube and kept as the cytoplasmic fraction. The cell pellets were re-suspended in Complete Lysis Buffer and vortexed for 10 sec at highest speed. The suspensions were then incubated for 30 min on ice and on a rocking platform at 150 rpm. Finally, samples were vortexed for 30 sec at highest speed, centrifuged for 10

min at 14000xg at 4°C and the supernatants (nuclear fractions) transferred to a 500 μL microcentrifuge tube. Samples were stored at -80°C.

3.5.3 SDS-PAGE gel electrophoresis and iBlot transfer

Samples were electrophoresed on 8-12% SDS-PAGE gels using the Mini-PROTEAN Tetra Cell equipment from Bio-Rad©. For each sample, an equal amount of lysate and 2X Sample buffer (see Table 2) was pipetted into a PCR tube and the samples heated at 95°C for 5 min to denature the proteins. Samples were then loaded onto gels and electrophoresed for 2-3 hours at 100-110 volts.

Table 2. 2x sample buffer

2X Sample buffer
4 mL 1MTris-HCl pH 7.0 (.1M final)
8 mL Glycerol (20% final)
16 mL 10% SDS (4% final)
4 mL 14M 2-Beta Mercaptoethanol (1.4 M final)
2 mL saturated bromophenol blue (0.05% final)
6 mL dH_2O

After electrophoresis, proteins were blotted onto a nitrocellulose membrane, using iBlot® dry transfer system (Invitrogen). In short, the gels were placed on a bottom buffer matrix covered with a nitrocellulose membrane that was positioned in the iBlot® gel transfer device. The gels were covered with wet filter paper, the top buffer matrix and finally a sponge. Proteins were blotted using a 5:40 min transfer. The membranes were then blocked with 5% skimmed milk powder in TBS for 1 h at RT and thereafter incubated with primary antibodies (Table 3) diluted in 5% BSA (for anti-phospho antibodies) or non-fat dried milk (for all other antibodies) in 0.1% TBS-Tween on a rocker at 4°C overnight. In all cases possible, dual labeling with rabbit and mouse antibodies was performed.

The following day the membranes were washed with 0.1% TBS-Tween three times for 5 min and then incubated with Near-infrared (NIR) labeled secondary antibodies (Table 4) diluted in 0.1% TBS-Tween with 0.01% SDS for 30-60 min at RT. Finally, the membranes were washed as before in addition to a 5 min wash with TBS and scanned using the Odyssey Infrared Imaging System (Li-Cor Biosciences).

Table 3. Primary antibodies used for Western blotting.

Antibody against	Cat#	Isotype	Dilution	Supplier
β-Actin	4967	Rabbit	1:1000	Cell Signaling
β-Actin	3700	Mouse IgG2b	1:1000	Cell Signaling
IκBα	4814	Mouse IgG1	1:1000	Cell Signaling
p38	9212	Rabbit	1:1000	Cell Signaling
Phospho-p38	9216	Mouse IgG1	1:1000	Cell Signaling
ERK1/2 (p44/42)	4696	Mouse IgG1	1:1000	Cell Signaling
Phospho-ERK1/2 (p44/42)	4370	Rabbit IgG	1:1000	Cell Signaling
Akt	2920	Mouse IgG1	1:1000	Cell Signaling
Phospho-Akt	4060	Rabbit IgG	1:1000	Cell Signaling
SAPK/JNK	9258	Rabbit IgG	1:1000	Cell Signaling
phospho-SAPK/JNK	9255	Mouse IgG1	1:2000	Cell Signaling
LaminB1	33-2000	Mouse IgG1	1:1000	Invitrogen
Phospho-c-fos	sc-81485	Mouse IgG1	1:200	Santa Cruz Biotech.
NF-κB p65	sc-372	Rabbit IgG	1:500	Santa Cruz Biotech.
phospho-Stat1	9167	Rabbit IgG	1:1000	Cell Signaling

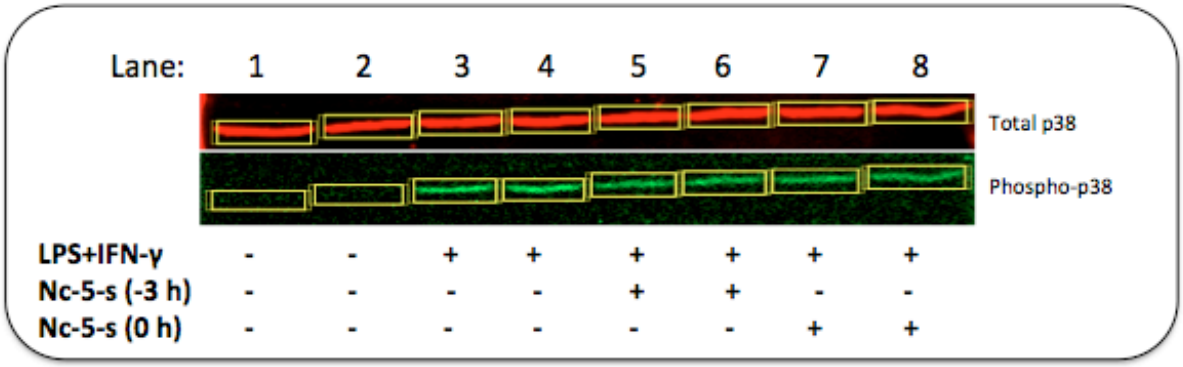
Table 4. Secondary antibodies used for Western blotting.

Antibody against	cat#	label	dilution	Supplier
Rabbit IgG	926-68021	IRDye 680LT	1:15000	Li-Cor Biosciences
Mouse IgG	926-32210	IRDye 800CW	1:15000	Li-Cor Biosciences

3.5.4 Analysis using Image Studio software

After scanning, the membranes were kept in TBS and re-probed if applicable. After final scanning, they were dried and stored.

Image Studio software from Li-Cor Biosciences was used to determine the intensity of bands for each of the proteins examined. Images of blots were digitally marked with lanes and rectangles drawn around the bands. The software then automatically quantified the intensity of the bands and compared it to the background around them. The numbers obtained were used to quantify the relative amount or relative activation of proteins in question. A ratio between the signal intensity of phosphorylated protein (or total protein in case of IκBα and NF-κB p65) and a loading control, being either the corresponding total protein or a housekeeping protein, was calculated. Results are shown as the ratio between the signal intensities, relative to a stimulated control in each experiment. An example of the analysis is shown in Figure 7.



Intensity values for each of the bands:

Channel	Lane	Signal	Channel	Lane	Signal	800/700
700	L01	*191000*	800	L01	*511*	0.00268
700	L02	*216000*	800	L02	*332*	0.00154
700	L03	*166000*	800	L03	*3810*	0.02295
700	L04	*157000*	800	L04	*4060*	0.02586
700	L05	*164000*	800	L05	*3510*	0.02140
700	L06	*157000*	800	L06	*3630*	0.02312
700	L07	*150000*	800	L07	*4100*	0.02733
700	L08	*147000*	800	L08	*2320*	0.01578

Figure 7. Processing and analysis of Western Blotting results.

The example shows how the activation of p38 kinase was quantified using Image Studio software. Red bands from the 700 nm scanning channel represent the total p38 protein and green bands from the 800 nm scanning channel represent the phosphorylated protein. Each sample was loaded onto the gel in duplicate. Bands were marked with a rectangle and the software quantified the intensity of the signal in each rectangle. The quantifications from the software were then moved into Microsoft Excel and the ratio of phosphorylated to total protein was calculated. Results are shown as the ratio of phosphorylated to total protein relative to the stimulated control.

3.6 Determination of c-fos DNA binding activity using TransAM® transcription factor ELISA-kit

For detection and quantification of c-fos/AP-1 DNA binding activity in nuclear extracts, a commercially available TransAm® kit from Active Motif was used according to manufacturer's instructions.

Microwell strips coated with an immobilized TPA response element (TRE (TGAGTCA)) were provided with the kit. Wells were incubated with 30 μL Complete binding buffer and 20 μL of sample (nuclear lysate) for 1 h at RT on a rocking platform set to 100 rpm. Next, wells were washed 3 times with Wash buffer. A diluted c-fos antibody, specific to an epitope on c-fos that is only accessible upon DNA binding, was then added to each well. Strips were incubated with the primary antibody for 1 h at RT. Again, wells were washed 3 times with Washing buffer and then a HRP-conjugated secondary antibody was added and the strips incubated for 1 h at RT. After one final wash, Developing solution was added to each well and incubated at RT away from light until a dark blue color had developed in the positive control wells. Then a Stop solution was added and the absorbance measured on a spectrophotometer at 450 nm.

The absorbance of each sample was compared to the absorbance of the IFN-γ and LPS stimulated control and results presented as arbitrary units based on these ratios.

3.7 Statistical analysis

Results are presented as means ± standard error of the means (SEM). A paired T-test was used when comparing two groups only. When more than two groups were compared, a repeated measures ANOVA, followed by Tukey's post hoc test, was used. A p-value of $p<0.05$ was considered statistically significant and results are labeled with * for $p<0.05$ or ** for $p<0.01$.

4 Results

4.1 Effects of Nc-5-s on viability and appearance of THP-1 monocytes

Possible cytotoxic effects of the heteroglycan Nc-5-s on THP-1 monocytes were tested using both Trypan blue staining and Via-Probe™ DNA stain. Trypan blue staining showed that Nc-5-s at the highest concentration tested (100 μg/mL) did not affect viability of THP-1 monocytes, neither when added to unstimulated nor when added to stimulated cells (Figure 8).

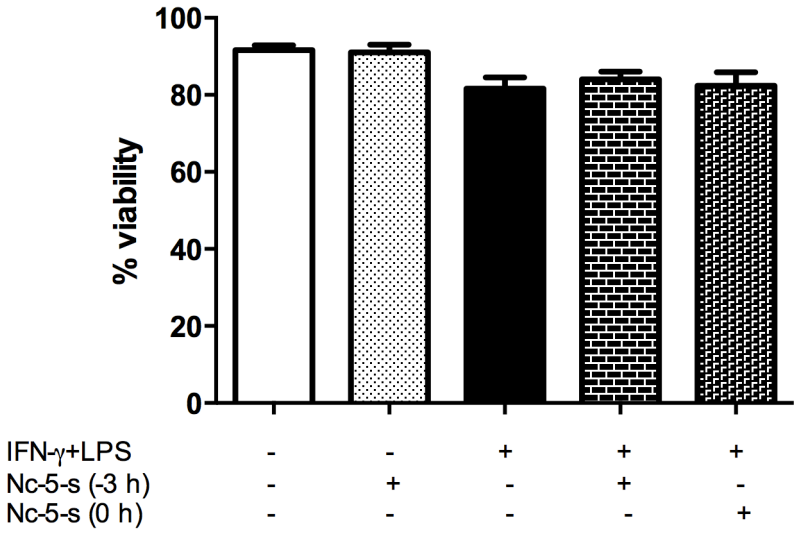


Figure 8. Effects of Nc-5-s on viability of THP-1 monocytes measured with Trypan blue staining.

Cells were treated with or without IFN-γ for 3 h and LPS for 48 h. Nc-5-s was added either simultaneously with IFN-γ (-3 h) or along with LPS (0 h) at a concentration of 100 μg/mL. Results are shown as a mean percentage of viable cells ± SEM. n=3.

Staining with Via-Probe also showed that Nc-5-s at a concentration of 100 μg/mL did not have cytotoxic effects on unstimulated cells (Figure 9). Stimulation with IFN-γ for 3 h and LPS for 48 h decreased cell viability, whereas addition of Nc-5-s to stimulated cells attenuated the cytotoxic effects of IFN-γ + LPS stimulation (Figure 9).

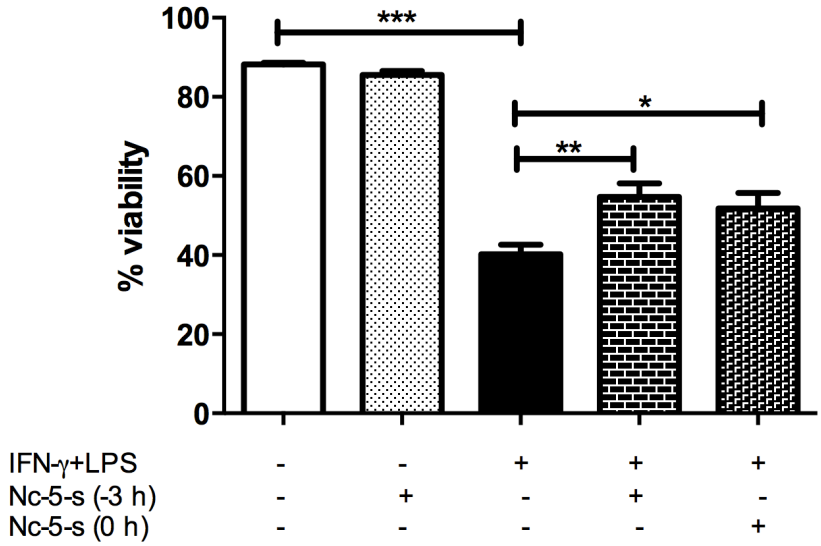


Figure 9. Effects of Nc-5-s on viability of THP-1 monocytes measured with Via-Probe DNA staining.

Cells were treated with or without IFN-γ for 3 h and LPS for 48 h. Nc-5-s was added either simultaneously with IFN-γ (-3 h) or along with LPS (0 h) at a concentration of 100 μg/mL Results are shown as a mean percentage of viable cells ± SEM. n=3. Statistical significance is indicated with * for $p<0.05$, ** for $p<0.01$ or *** for $p<0.001$.

The appearance of the THP-1 cells under a light microscope reflected the effects of stimulation and Nc-5-s on viability measured with Via-Probe DNA staining. Unstimulated THP-1 cells were present in the medium as single cells and did not adhere to each other (Figure 10, A). Cells that had been primed with IFN-γ and stimulated with LPS clumped, this being one of the characteristics of THP-1 cells being on a path towards terminal differentiation and ultimately, cell death (Figure 10, B). Cells that were stimulated in the presence of Nc-5-s, clumped less than those that were stimulated without Nc-5-s (Figure 10, C).

Figure 10. Effects of Nc-5-s on appearance of THP-1 monocytes.

Unstimulated cells (A), cells primed with IFN-γ and stimulated with LPS for 48 h (B) and cells primed with IFN-γ and stimulated with LPS for 48 h with Nc-5-s being added along with LPS (C). Photographic images of cells, taken using a light microscope at 5x magnification and a digital camera.

4.2 Effects of Nc-5-s on cytokine secretion by stimulated THP-1 monocytes

THP-1 monocytes treated with Nc-5-s secreted less IL-6 than cells incubated without Nc-5-s (Figure 11). The effects of Nc-5-s on IL-6 secretion were dose dependent, with the highest dose causing more than 50% decrease in IL-6 secretion. In contrast, Nc-5-s at the highest concentration tested increased TNF-α and IL-8 secretion by THP-1 monocytes with the effect on IL-8 being more than threefold (Figure 11). Nc-5-s had no effect on LPS-induced IL-10 or IL-12p40 secretion by the THP-1 cells (Figure 11).

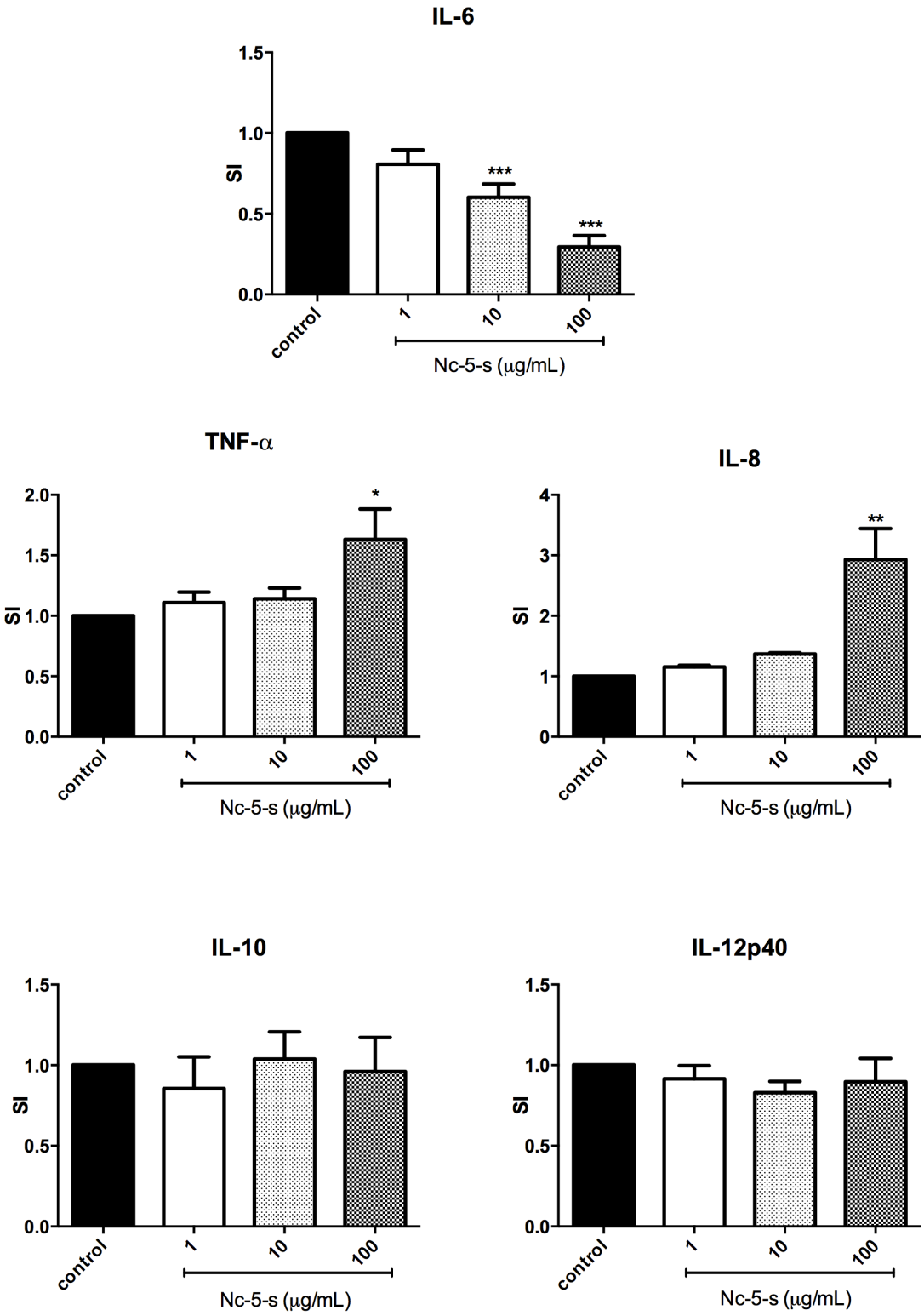


Figure 11. Effects of Nc-5-s on cytokine secretion by stimulated THP-1 monocytes.

THP-1 monocytes were primed with IFN-γ for 3 h and then stimulated with LPS for 48 h. Nc-5-s was added to the cells along with LPS at the indicated concentrations. Results are shown as the mean of the secretion index (the concentration of a cytokine in the supernatants of cells incubated with Nc-5-s divided by the concentration of the cytokine in the supernatants of cells incubated without Nc-5-s) ± SEM. For IL-6 and IL-12p40 n=7, for IL-10 n=6, for TNF-α n=5 and for IL-8 n=3. Statistical significance is indicated with * for $p<0.05$, ** for $p<0.01$ or *** for $p<0.001$.

4.3 Effects of Nc-5-s on expression of surface markers on THP-1 monocytes

4.3.1 Effects of Nc-5-s on TLR4, CD14 and TLR2 expression by THP-1 monocytes

Nc-5-s did not affect expression of TLR4 or CD14, neither the percentage of cells expressing the molecules nor the mean expression level (MFI), when compared with stimulated control (Figure 12). There was no change in the percentage of cells expressing TLR4 upon stimulation of the cells, whereas the MFI of TLR4 was increased. Although the percentage of cells expressing CD14 increased upon stimulation, less than 10% of the cells expressed CD14. Stimulation of the cells also increased mean expression levels of CD14 on the cells.

Because the LPS used to stimulate the cells was not 100% pure, there was also a slight possibility of it stimulating the cells through TLR2 (101). Therefore the effects of Nc-5-s on TLR2 expression were also assessed. Nc-5-s did not affect the mean percentage of cells expressing TLR2 or the mean expression levels of TLR2 (Figure 12). Stimulation of the cells had little effect on the expression of TLR2.

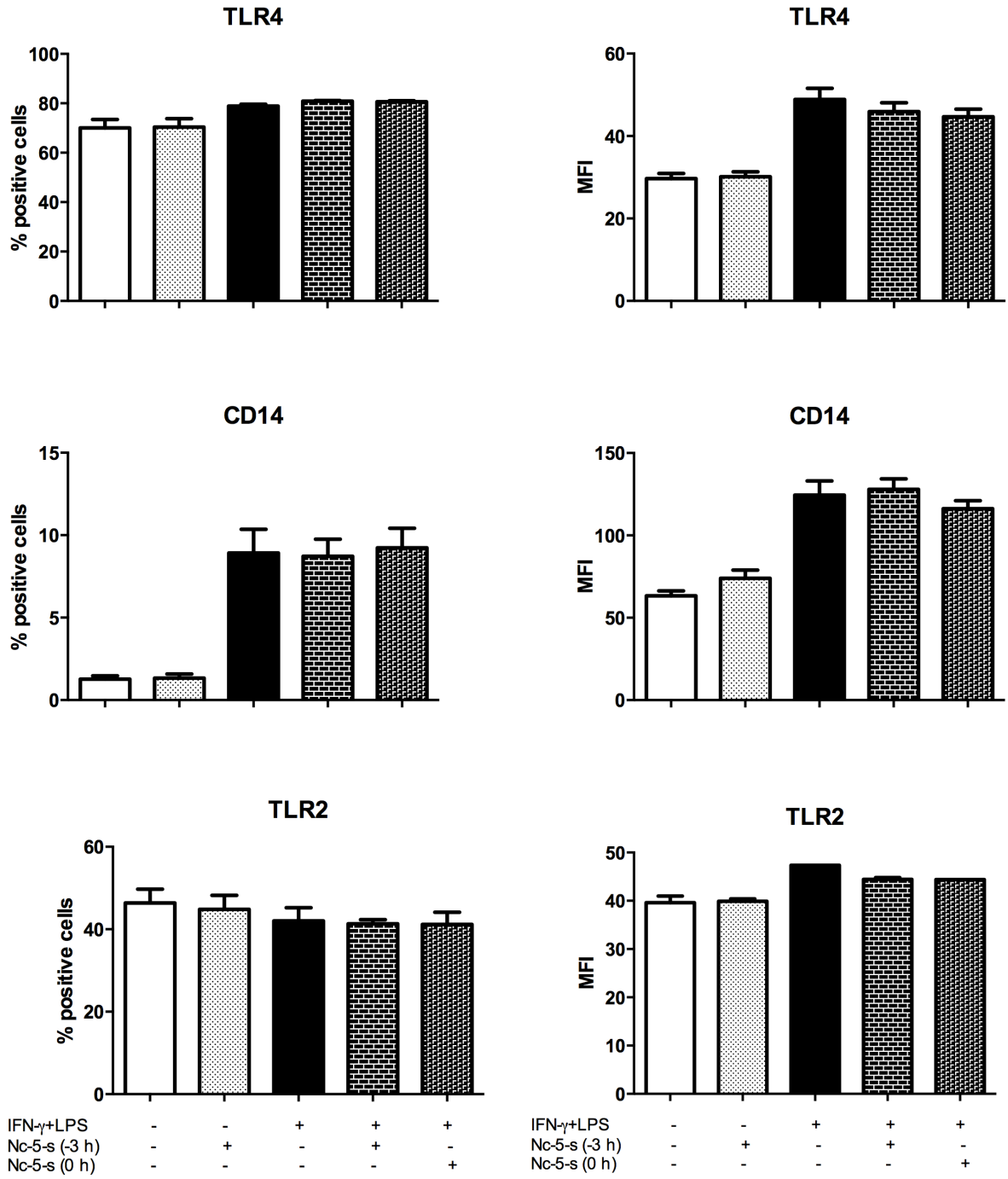


Figure 12. Effects of Nc-5-s on TLR4, CD14 and TLR2 expression by stimulated THP-1 monocytes.

THP-1 monocytes were primed with IFN-γ for 3 h and then stimulated with LPS for 24 h. Nc-5-s was added along with IFN-γ (-3 h) or LPS (0 h) at a concentration of 100 μg/mL. Results are shown as mean percentage of positive cells or mean fluorescence intensity (MFI) ± SEM. For TLR4 n=3 and for CD14 and TLR2 n=2.

4.3.2 Effects of Nc-5-s on CD40, CD54 and CD86 expression by THP-1 monocytes

Nc-5-s did not affect the expression (percentage of positive cells or MFI) of the co-stimulatory molecules CD40, CD54 and CD86, compared with the stimulated control (Figure 13). Stimulation had no effect on the percentage of CD40 positive cells, but did increase the MFI. However, stimulation increased both the percentage of CD54 positive cells as well as the MFI for CD54. Finally, stimulating the cells caused an up-regulation in the expression of CD86 but did not cause a change in the MFI.

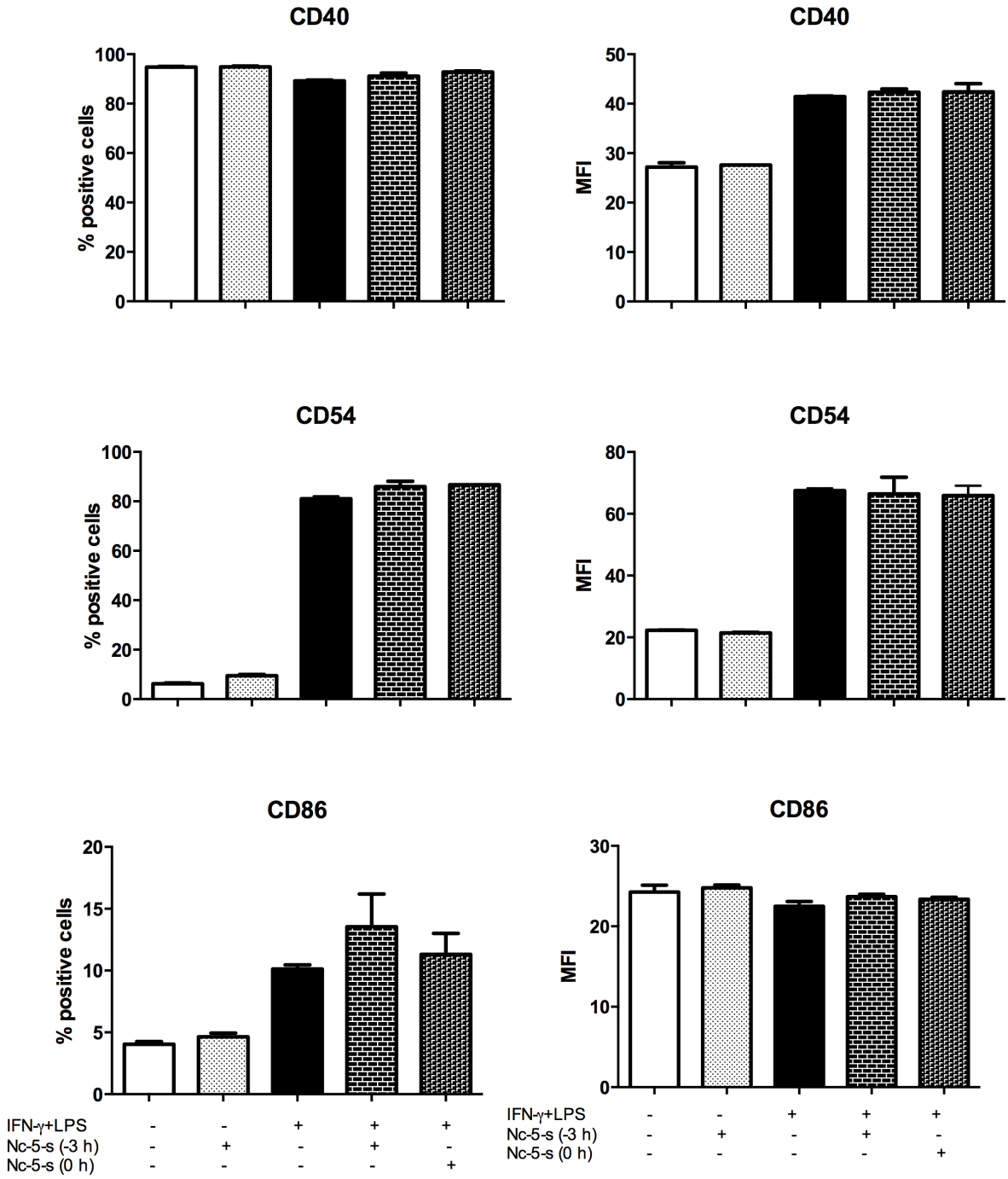


Figure 13. Effects of Nc-5-s on CD40, CD54 and CD86 expression by stimulated THP-1 monocytes.

THP-1 monocytes were primed with IFN-γ for 3 h and then stimulated with LPS for 24 h. Nc-5-s was added to the cells along with LPS, at a concentration of 100 μg/mL. Results are shown as mean percentage of positive cells or mean fluorescence intensity (MFI) ± SEM. For CD40 and CD54 n=2 and for CD86 n=3.

4.4 Effects of Nc-5-s on activation of intracellular signaling pathways

Whether Nc-5-s mediated its effects on cytokine secretion by THP-1 monocytes by affecting intracellular signaling pathways associated with inflammatory responses was examined by measuring activation of several intracellular signaling pathways.

Time curves for activation of kinases and transcription factors as well as the breakdown of the IκBα inhibitor following LPS stimulation are shown in Appendix 1. Maximal activation of the kinases and transcription factors and maximal breakdown of IκBα occurred after 60 min of LPS stimulation (Figures A1-A3). Therefore the 60 min time point was used for LPS stimulation in the following experiments.

4.4.1 Effects of Nc-5-s on activation of MAP kinases in THP-1 monocytes

Nc-5-s decreased phosphorylation of ERK1/2 by more than 50% regardless of whether it was added to the cells along with IFN-γ or along with LPS (Figure 14). Nc-5-s had no effect on phosphorylation of p38 kinase (Figure 14).

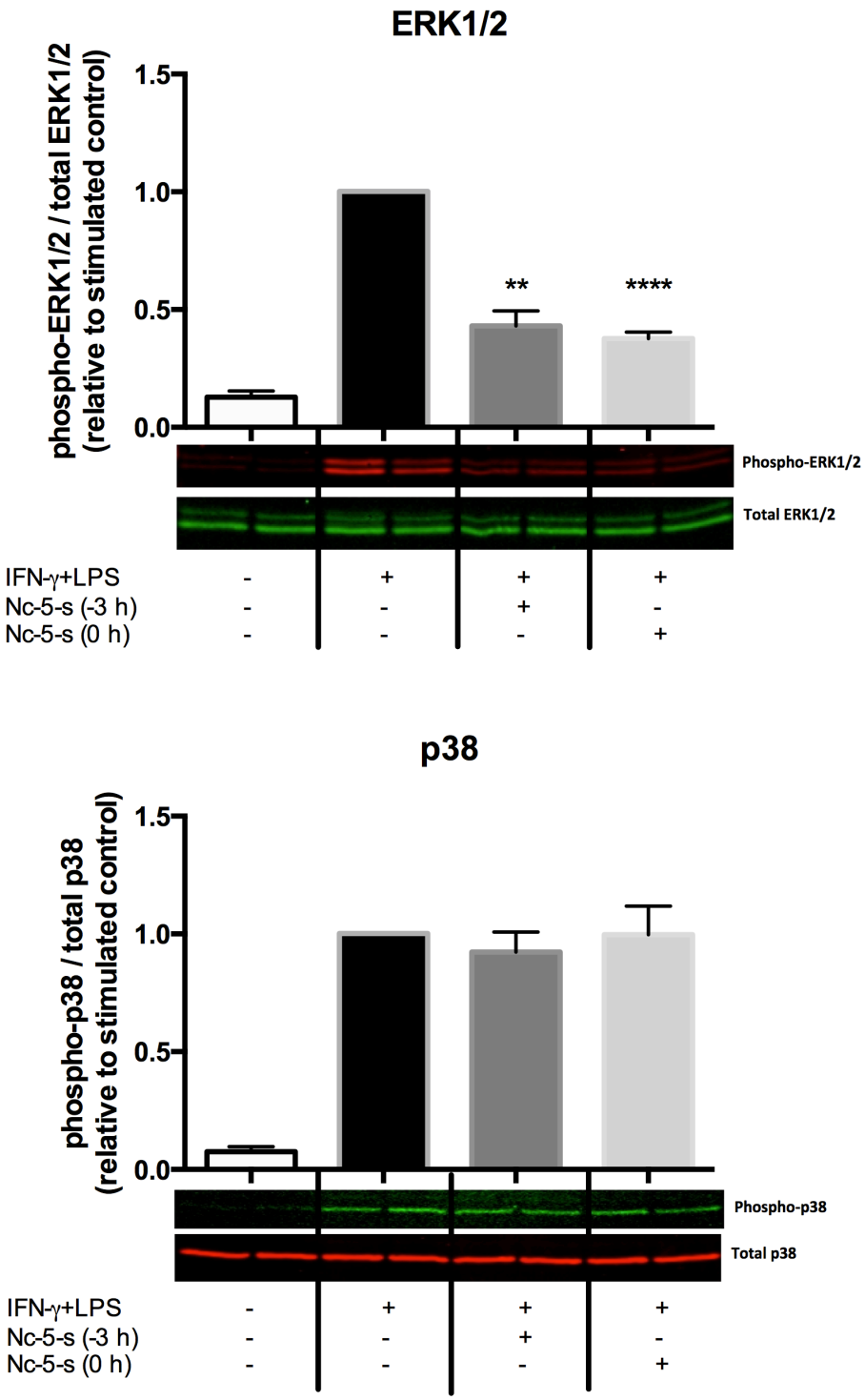


Figure 14. Effects of Nc-5-s on phosphorylation of ERK1/2 and p38 in THP-1 monocytes.

THP-1 monocytes were primed with IFN-γ for 3 h and then stimulated with LPS for 1 h. Nc-5-s was either added to the cells along with IFN-γ (-3 h) or along with LPS (0 h), at a concentration of 100 μg/mL. Bars represent the ratio of the mean signal intensity for phosphorylated protein versus total protein, relative to the same ratio for the stimulated control (black bar) ± SEM with n=3. **Different from stimulated control $p<0.01$ or **** $p<0.0001$. The blots are representative of three independent experiments with a duplicate of each sample.

Whether Nc-5-s affected activation of the SAPK/JNK kinase could not be determined as there was almost no activation of this kinase with IFN-γ and LPS treatment of the THP-1 monocytes. This was confirmed both in whole cell and cytosolic lysates, using Western blotting (data not shown).

4.4.2 Effects of Nc-5-s on activation of the PI3K/Akt pathway in THP-1 monocytes

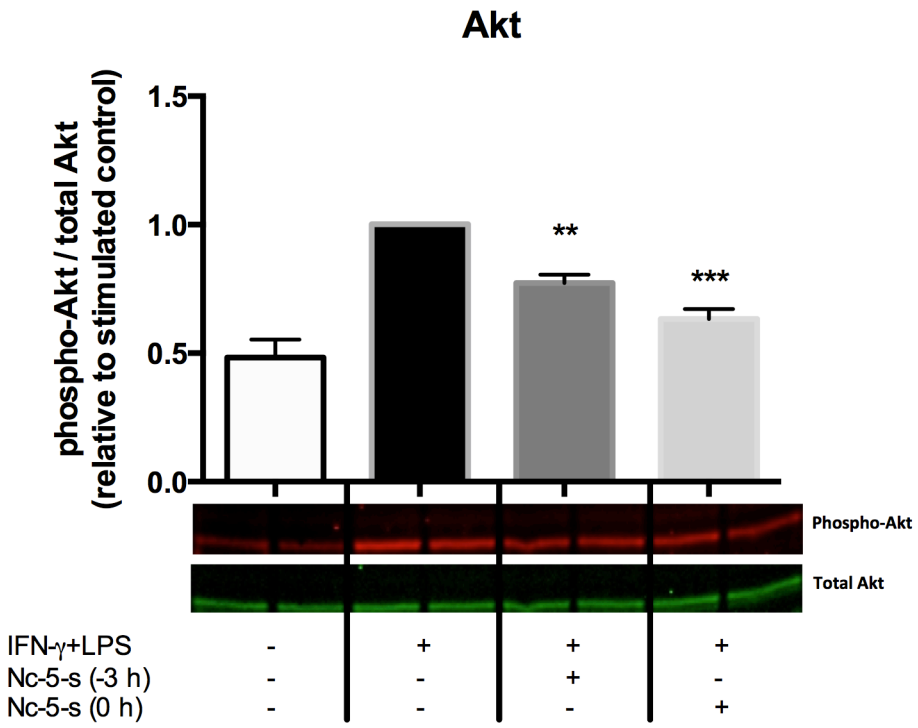


Figure 15. Effects of Nc-5-s on phosphorylation of the Akt kinase in THP-1 monocytes.

THP-1 monocytes were primed with IFN-γ for 3 h and then stimulated with LPS for 1 h. Nc-5-s was either added to the cells along with IFN-γ (-3 h) or along with LPS (0 h), at a concentration of 100 μg/mL. Bars represent the ratio of the mean signal intensity for phosphorylated protein versus total protein, relative to the same ratio for the stimulated control (black bar) ± SEM with n=3. **Different from stimulated control P<0.01 or ***p<.001. The blots are representative of three independent experiments with a duplicate of each sample.

Nc-5-s attenuated LPS-induced activation of the Akt kinase, both when it was added to the cells along with IFN-γ and when it was added along with LPS (Figure 15). With Akt being a downstream kinase of PI3K, these results indicate that PI3K activation may also be decreased.

4.4.3 Effects of Nc-5-s on activation of the NF-κB pathway in THP-1 monocytes

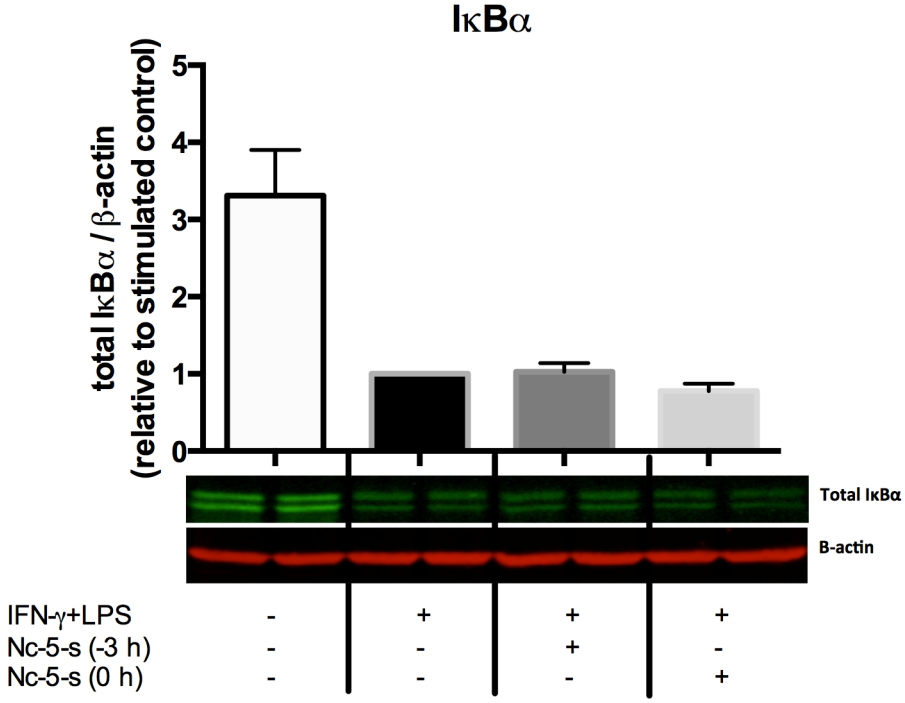


Figure 16. Effects of Nc-5-s on the breakdown of IκBα in THP-1 monocytes.

THP-1 monocytes were primed with IFN-γ for 3 h and then stimulated with LPS for 1 h. Nc-5-s was either added to the cells along with IFN-γ (-3 h) or along with LPS (0 h), at a concentration of 100 μg/mL. Bars represent mean signal intensity for the ratio of total protein versus β-actin control, relative to the same ratio for the stimulated control (black bar) ± SEM with n=3. The blots are representative of three independent experiments with a duplicate of each sample.

Nc-5-s did not affect the breakdown of the NF-κB inhibitor IκBα in LPS stimulated THP-1 monocytes (Figure 16), indicating that the NF-κB pathway is not involved in the effects of Nc-5-s on cytokine secretion by THP-1 monocytes. However, when examining the nuclear localization of NF-κB, there was a tendency towards less NF-κB being translocated to the nucleus when the cells were stimulated in the presence of Nc-5-s (Figure 17).

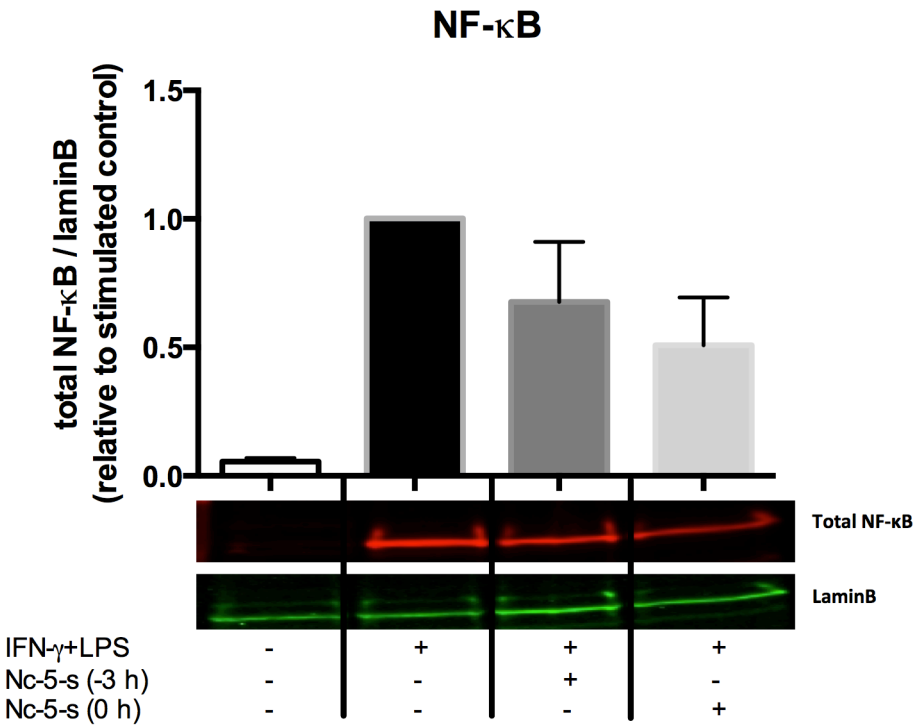


Figure 17. Effects of Nc-5-s on nuclear localization of NF-κB in THP-1 monocytes.

THP-1 monocytes were primed with IFN-γ for 3 h and then stimulated with LPS for 1 h. Nc-5-s was either added to the cells along with IFN-γ (-3 h) or along with LPS (0 h), at a concentration of 100 μg/mL. Bars represent mean signal intensity for the ratio of total protein versus a LaminB nuclear control, relative to the same ratio for the stimulated control (black bar) ± SEM with n=2. The blots are representative of two independent experiments using nuclear lysates.

4.4.4 Effects of Nc-5-s on AP-1 and STAT1 transcription factor activity in THP-1 monocytes

4.4.4.1 Effects of Nc-5-s on phosphorylation of AP-1 and STAT1 in THP-1 monocytes

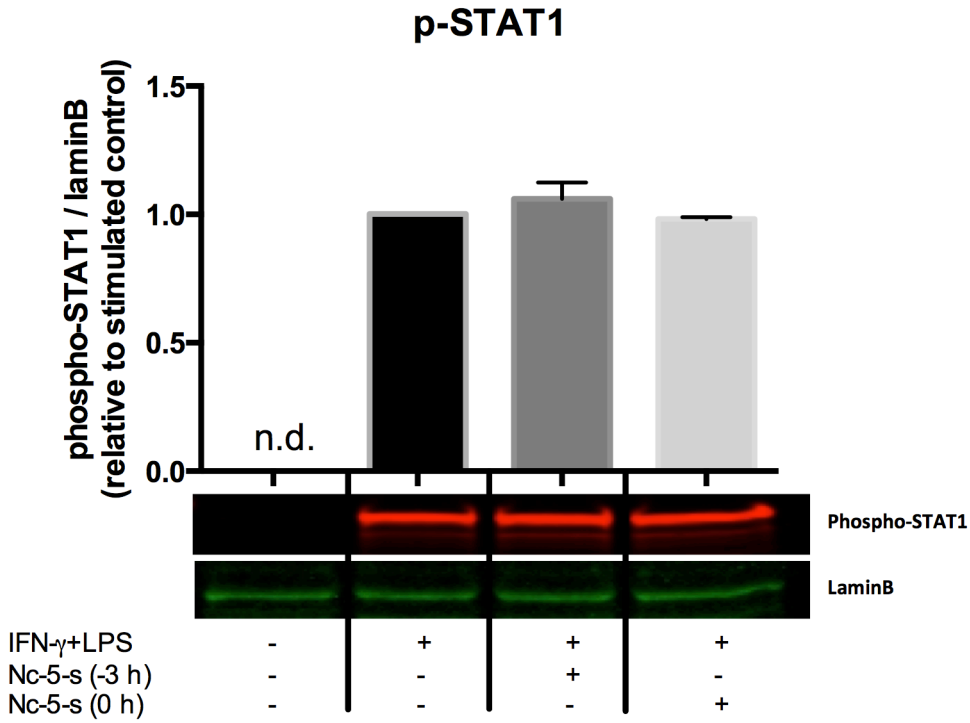


Figure 18. Effects of Nc-5-s on the phosphorylation of STAT1 in THP-1 monocytes.

THP-1 monocytes were primed with IFN-γ for 3 h and then stimulated with LPS for 1 h. Nc-5-s was either added to the cells along with IFN-γ (-3 h) or along with LPS (0 h), at a concentration of 100 μg/mL. Bars represent mean signal intensity for the ratio of phosphorylated protein versus a LaminB nuclear control, relative to the same ratio for the stimulated control (black bar) ± SEM with n=2. The blots are representative of two independent experiments using nuclear lysates.

Nc-5-s did not affect phosphorylation of the STAT1 transcription factor, based on the two experiments performed (Figure 18).

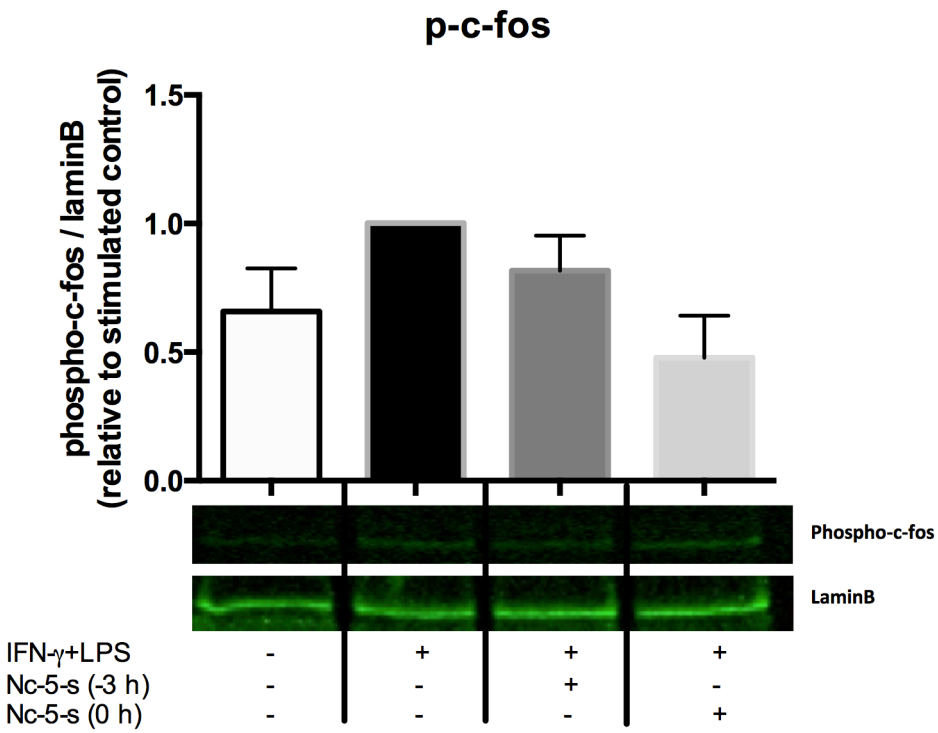


Figure 19. Effects of Nc-5-s on the phosphorylation of c-fos in THP-1 monocytes.

THP-1 monocytes were primed with IFN-γ for 3 h and then stimulated with LPS for 1 h. Nc-5-s was either added to the cells along with IFN-γ (-3 h) or along with LPS (0 h), at a concentration of 100 μg/mL. Bars represent mean signal intensity for the ratio of phosphorylated protein versus a LaminB nuclear control, relative to the same ratio for the stimulated control (black bar) ± SEM with n=3. The blots are representative of three independent experiments using nuclear lysates.

There was little expression of c-fos in stimulated THP-1 monocytes and therefore it was difficult to quantify. Nc-5-s did not affect phosphorylation of the c-fos subunit of the AP-1 transcription factor (Figure 19). Whilst there was a tendency towards lower c-fos phosphorylation in cells cultured with Nc-5-s, the difference was not statistically significant and most likely an artifact due to weak signals.

4.4.4.2 Effects of Nc-5-s on AP-1 DNA binding in THP-1 monocytes

Because the c-fos protein was scarce in simulated THP-1 monocytes and difficult to detect using Western blot, a c-fos ELISA based DNA binding assay, a more sensitive method for the assessment of c-fos activation, was also used.

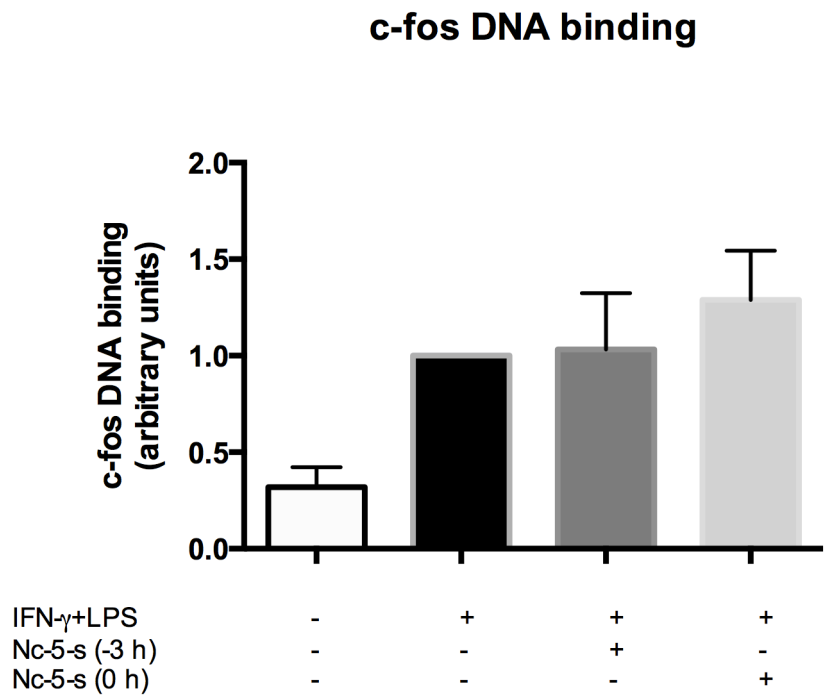


Figure 20. Effects of Nc-5-s on c-fos DNA binding ability in THP-1 monocytes.

THP-1 monocytes were primed with IFN-γ for 3 h and then stimulated with LPS for 1 h. Nc-5-s was either added to the cells along with IFN-γ (-3 h) or along with LPS (0 h), at a concentration of 100 μg/mL. Bars represent mean absorbance for each sample as a ratio of the absorbance of the stimulated control (black bar) ± SEM with n=4. Nuclear lysates were used.

Nc-5-s did not affect binding of c-fos to its regulatory TRE DNA element compared with a stimulated control (Figure 20).

4.5 Effects of the MEK1/2 inhibitor U0126 on cytokine secretion by THP-1 monocytes

In order to investigate whether Nc-5-s had similar effects on the THP-1 monocytes as a known ERK1/2 inhibitor, the effects of the commercially available inhibitor U0126 on the cells were tested.

The MEK1/2 inhibitor U0126 attenuated LPS-induced secretion of all cytokines tested (Figure 21). The inhibition was dose dependent and resulted in more than 60% inhibition in secretion of all the cytokines at the highest concentration used (Figure 21).

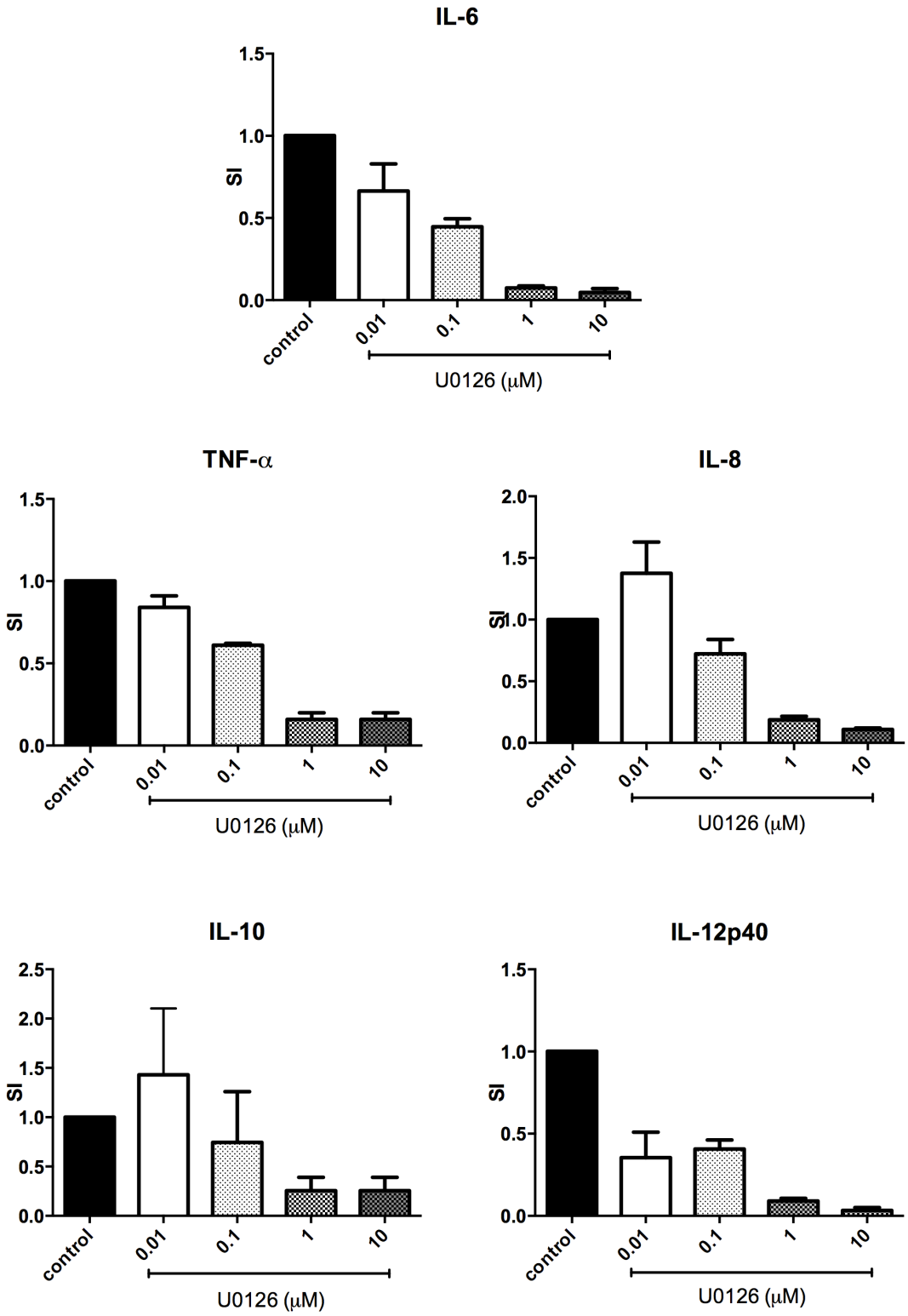


Figure 21. Effects of the MEK1/2 inhibitor U0126 on cytokine secretion by THP-1 monocytes.

THP-1 monocytes were primed with IFN-γ for 3 h and then stimulated with LPS for 48 h. U0126 at the indicated concentrations was added with the LPS. Results are shown as the mean of the secretion index ± SEM. For IL-6, TNF-α, IL-8 and IL-12p40 n=3, except for 0.01 μM U0126 where n=2. For IL-10, n=2.

5 Discussion

The heteroglycan Nc-5-s, isolated from the cyanobacterium *Nostoc commune*, affects LPS-induced cytokine secretion and activation of intracellular signaling pathways in THP-1 monocytes. The effects on cytokine secretion can neither be considered as anti- nor pro-inflammatory since Nc-5-s reduced secretion of the pro-inflammatory cytokine IL-6 while increasing secretion of TNF-α and IL-8, which are also considered pro-inflammatory. Nc-5-s did not affect secretion of the pro-inflammatory cytokine IL-12p40, or the anti-inflammatory cytokine IL-10. Intracellular signaling pathways downstream of TLR4, the receptor for LPS, were also affected by Nc-5-s. Of the three MAP kinases studied, ERK1/2, p38 and SAPK/JNK, only ERK1/2 was affected, with its phosphorylation being decreased by Nc-5-s. Phosphorylation of Akt kinase of the PI3K/Akt pathway was also decreased by Nc-5-s. Although there was no effect of Nc-5-s on breakdown of the NF-κB inhibitor IκBα there was a tendency towards decreased nuclear localization of the NF-κB transcription factor. The present study, combined with a prior M.Sc. project (100), is the first study to examine the effects of Nc-5-s on immune responses.

In the present study, LPS stimulation of THP-1 monocytes resulted in an increase in the secretion of IL-6, TNF-α and IL-8. Addition of Nc-5-s to the cells lowered LPS-induced IL-6 secretion, but increased TNF-α and IL-8 secretion. These results were surprising as all these cytokines are pro-inflammatory and IL-6 and TNF-α are often affected in similar ways during activation in the presence of natural products (102). This is e.g. seen during acute inflammation and has been extensively studied in the pathogenesis of RA, where both IL-6 and TNF-α are typically elevated (103) and inhibition of TNF-α reduces IL-6 levels (104). TNF-α is an early phase inflammatory cytokine that can up-regulate IL-6 expression (105) and *in vitro* IL-6 levels rise shortly after a rise in TNF-α levels (10, 106). In contrast to studies showing IL-6 and TNF-α to be regulated in a similar way, there are also studies showing that plant extracts and isolated compounds are capable of decreasing LPS-induced IL-6 secretion by THP-1 macrophages, without affecting TNF-α secretion (107, 108). Furthermore, in a recent study, quince peel polyphenols increased LPS-induced IL-6 secretion by PMA differentiated THP-1 macrophages while attenuating secretion of IL-8 and TNF-α (109). These effects are opposite to that found with Nc-5-s in the present study, but show that natural products can have opposite effects on IL-6 and TNF-α secretion by THP-1 cells. In the study with the quince peel polyphenols there was a link between the IL-6 increase and the TNF-α decrease, as adding IL-6 to the cells led to a dose-dependent decrease in TNF-α secretion. It is therefore possible that in the present study the decreased IL-6 secretion caused by Nc-5-s may have resulted in the increase in the TNF-α concentrations. As IL-8 secretion has been shown to be induced by TNF-α (110) the increase in IL-8 secretion by the THP-1 cells stimulated in the presence of Nc-5-s may have resulted from the increase in TNF-α.

In this thesis, results for the effects of Nc-5-s on IL-6, TNF-α, IL-12p40 and IL-10 from the present study and a former M.Sc. project (100) were combined. Combining the results for IL-6, TNF-α and IL-10 was not a problem since the effects of Nc-5-s were similar in both projects. However, the effects of Nc-5-s on IL-12p40 in the two projects were opposite, with around 40% reduction in IL-12p40 being found in four separate studies in the previous project, whilst in the present study, three repeated experiments showed a tendency towards about 30% increase in IL-12p40 secretion by THP-1 monocytes stimulated in the presence of Nc-5-s. When combined, the results show that Nc-5-s has no effect on IL-12p40 secretion by the stimulated THP-1 monocytes. The reason for the different results obtained in the two projects is not clear. All experimental setups were the same and the effects of Nc-5-s on other cytokines tested were repeatable. However, human error is a factor that can never be excluded. As the IL-12p40 subunit was measured for assessment of IL-12 secretion and this subunit is shared with the cytokine IL-23 (43) it is not likely that Nc-5-s affected IL-12 or IL-23 secretion by the THP-1 monocytes in the present study. Nc-5-s did not affect secretion of IL-10 with the concentrations of IL-10 in the supernatants of stimulated cells being low and often just barely above the detection limit of the ELISA. The low concentrations of IL-10 in the supernatants may possibly be explained by the fact that IL-10 expression can be inhibited by priming of the monocytes with IFN-γ (111, 112).

Taken together, the results on the effects of Nc-5-s on inflammatory cytokine secretion show that the heteroglycan specifically affects certain cytokines but not others. Consistent with the dogma that TNF-α is the first pro-inflammatory cytokine to be secreted in acute inflammatory response (1) it may be assumed that Nc-5-s is first affecting TNF-α, which then could be further affecting IL-6 and IL-8 secretion. However, as mentioned earlier, it is also possible in this study that IL-6 is affecting TNF-α secretion. In order to determine whether TNF-α affects IL-6 secretion or vice versa, thorough time curves for secretion of these cytokines by the stimulated THP-1 monocytes need to be established. Also, the use of antibodies against IL-6 or TNF-α would provide information about their role in the secretion of the other cytokines. In a previous study, mouse macrophages treated with anti-IL-6 or anti-TNFα antibodies secreted more TNF-α or IL-6, respectively than untreated cells, indicating that production of TNF-α and IL-6 can be negatively regulated by each other (113). While IL-6 and TNF-α have many functions in common, such as inducing synthesis of acute phase proteins and fever, one of the most important functions of IL-6 is to regulate T cell differentiation and activation (1, 45). IL-6 induces the development of Th17 cells or, to a lesser extent, development of Th2 cells from naive T cells, while, at least in mice, suppressing the generation of Tregs (45). IL-6 has been implicated in the pathogenesis of RA as well as other inflammatory diseases (45). Blocking TNF-α with monoclonal antibody drugs, such as Infliximab, is the most commonly used antibody treatment for RA. However, not all patients respond well to TNF-α blockers and, therefore, blocking other inflammatory cytokines has been attempted (105). A blocker of the IL-6R (Tocilizumab) is available for treatment of RA and systemic juvenile idiopathic arthritis (114) and currently 19 patients in Iceland are being treated with an anti-IL-6 agent (B. Guðbjörnsson, personal communication, 24.07.13). However, Tocilizumab can cause unwanted toxicity and side effects and, therefore, alternative IL-6 blockers are being searched

for. Decreasing IL-6 secretion by cells of the innate immune system is an interesting property of the heteroglycan Nc-5-s that should be further tested in *in vivo* models of inflammation.

The current study clearly shows the importance of testing the effects of natural products on many cytokines rather than focusing on one or two, as is often the case in similar studies. If using only a single cytokine for screening, one might miss valuable and interesting effects that are exerted on other cytokines in the model system. Although it will never be possible to examine the effects of natural products on all cytokines, it is important to elucidate their effects on more than one cytokine, since a beneficial effect of a natural product or substance on one cytokine can be counteracted by a detrimental effect on another one or supplemented by a beneficial effect on another cytokine. To date, many different assays are available for analyzing a number of cytokines in one sample. However, these assays are very expensive and, therefore, not always possible to use.

Several studies have shown that natural products affect intracellular inflammatory pathways (8, 109, 115-118) and blocking the transcription of inflammatory cytokines might become a feasible way to treat inflammatory diseases in the future. Most inflammatory cytokine genes contain regulatory binding sites for NF-κB (44, 48, 51) and although Nc-5-s did not affect breakdown of the NF-κB inhibitor IκBα there were indications that it may have resulted in fewer NF-κB molecules translocating into the nucleus, which is an obligatory step prior to its facilitation of the transcription of target genes. These results were, however, not statistically significant and need to be repeated. For NF-κB to translocate to the nucleus and be able to cause transcription of genes, two events are needed. First, its inhibitor IκBα needs to be degraded and second, phosphorylation of its subunits, such as p65, is required (119). Although Nc-5-s did not affect degradation of IκBα it may have affected phosphorylation of p65 and thereby affected translocation of NF-κB into the nucleus.

Another set of pathways that are activated downstream of TLR4 are the MAP kinases. In the THP-1 monocytes only two of the three MAP kinases studied were activated to a point where the activation was quantifiable using Western blotting. LPS stimulation of the THP-1 monocytes had little effect on activation of the SAPK/JNK kinase, which is consistent with a previous report (120) and personal communication with the technical service at Cell Signaling Technology saying that in order to properly activate SAPK/JNK, THP-1 cells have to be differentiated using PMA (Cell Signaling Technical Support, personal communication, 08.04.13). Activating SAPK/JNK in undifferentiated THP-1 cells is, however, not impossible as stimulation with compounds such as ovalbumin effectively activates SAPK/JNK and ERK1/2 while leaving p38 unaffected (121). In the present study Nc-5-s specifically attenuated LPS-induced activation of ERK1/2 without affecting p38. This suggests that even though most often more than one MAP kinase is affected by external substances (117, 122), indicating that they are closely regulated, they can indeed be independently affected. Previous studies have shown that regulating the ERK1/2 pathway plays a role in regulating the production of TNF-α (123), IL-6 (124, 125) and IL-8 (115, 122). In the current study, it is therefore possible that the reduced activation of

ERK1/2 caused the reduced secretion of IL-6. However, the increased TNF-α and IL-8 production by the THP-1 monocytes treated with Nc-5-s cannot be explained by its effects on ERK1/2 activation, as decreased activation of ERK1/2 would be expected to decrease secretion of TNF-α and IL-8 (122). That inhibition of ERK1/2 leads to less production of TNF-α and IL-8 was confirmed using the MEK1/2 (the kinase upstream of ERK1/2) inhibitor, U0126, showing that it reduced secretion of all five cytokines tested.

It is known that the ERK1/2 kinase can activate the c-fos subunit of the AP-1 transcription factor, which is commonly involved in regulation of inflammatory gene transcription (24-26). Several studies show that various natural products can inhibit activation of the c-fos and/or the c-jun subunits of AP-1 and thereby have anti-inflammatory effects (8, 76, 126). However, in the present study the effects of Nc-5-s on cytokine secretion by the THP-1 cells were probably not mediated through modulation of c-fos as Nc-5-s did not affect phosphorylation of c-fos in the THP-1 monocytes. There are other downstream effectors of ERK1/2, such as Egr1 (34, 123), Lin1 and Sap1 (24), but only Egr1 has been directly implicated as an effector in inflammatory responses. Further studies on the effects of Nc-5-s on other downstream effectors of ERK1/2 are needed to elucidate how this MAP kinase plays a role in the altered cytokine secretion by THP-1 monocytes.

In addition to decreasing activation of ERK1/2, Nc-5-s also decreased activation of Akt, one of the kinases of the PI3K/Akt pathway. Less is known about this pathway in the context of inflammation than the NF-κB and the MAP kinase pathways and there are data in the literature showing that it can be both pro- (37, 38) and anti-inflammatory (31, 33-36). However, the most recent literature suggests that it may be serving as a negative regulator of inflammation (31). Thus, decreased Akt phosphorylation would lead to more inflammation (34) and this could be the underlying cause for the increased TNF-α secretion by THP-1 monocytes stimulated in the presence of Nc-5-s.

Direct comparison of the results of this study to other studies is not possible since the immunomodulatory effects of Nc-5-s have not been tested before. However, the results can be compared with results from studies examining the effects of other lichen polysaccharides on inflammatory responses. In a study conducted at the Faculty of Pharmaceutical Sciences at the University of Iceland, 11 lichen-derived polysaccharides were screened for immunomodulatory effects on monocyte-derived DCs (91). Four of those polysaccharides, including one heteroglycan, had potential anti-inflammatory effects by increasing secretion of IL-10 by the DCs. Other heteroglycans in the same study increased IL-12 secretion by the DCs (91). In the present study Nc-5-s affected neither IL-10 nor IL-12 secretion by THP-1 monocytes, indicating that each heteroglycan may have a unique effect on immune responses.

How the heteroglycan mediates its effects outside the THP-1 cells remains to be answered. It may bind to a receptor on the cells or compete with other molecules and sterically hinder their binding to

the cells. Cells of the myeloid lineage express many receptors on their surface that bind carbohydrates, such as the C-type lectin receptors (CLRs). CLRs can bind to carbohydrates expressed on the surface of various pathogens and thus participate in pathogen recognition. Over sixty CLRs have been identified in humans. Among them are DC-SIGN, DEC-205, Dectin-1 and the mannose receptor (127). Whether Nc-5-s binds to any of the CLRs is not known but it is possible that oligosaccharides on the surface of the intact heteroglycan or oligosaccharides produced by breakdown of the heteroglycan can bind to one or more of the CLRs on the surface of the THP-1 cells. Although binding of carbohydrates to the CLRs most often leads to inflammatory responses (128), they can, in some instances, lead to immunotolerance and anti-inflammatory responses (127). To elucidate how Nc-5-s is mediating its effects, the heteroglycan could be labeled and its location and/or binding monitored using microscopy or flow cytometry. However, the process of such labeling could be complex and perhaps not even possible. Also, if the heteroglycan is broken down in the cell culture, unlabeled parts might be the ones causing the effects, and those would then not be detectable. Deliberately breaking down Nc-5-s and repeating the experiments presented in this thesis with individual parts of the heteroclycan could shed a light on whether it is the heteroglycan as a whole or merely parts of it that are causing the observed effects. Finally, it is unlikely that Nc-5-s competes with LPS for binding to TLR4, as that would be expected to decrease secretion of all cytokines that are induced by LPS as well as inhibit activation of all the pathways downstream of LPS.

The THP-1 monocytic cell line used as a model in the present study is commonly used for testing the effects of natural products on immune responses (6-8). Using cell lines for such testing is less time consuming and less expensive than using animal models. However, an *in vitro* model has many limitations and is still very far from imitating how such products would affect inflammatory responses in living organisms. In order to determine whether the overall effects of Nc-5-s are anti-or pro-inflammatory, an *in vivo* experimental animal model of inflammation must be used.

6 Conclusion

The heteroglycan Nc-5-s, from the cyanobacterium *Nostoc commune*, attenuated LPS-induced secretion of IL-6 but increased TNF-α and IL-8 secretion by THP-1 monocytes. These effects of Nc-5-s may be mediated through the ERK1/2 MAP kinase pathway, the PI3K/Akt pathway and/or the NF-κB pathway. Nc-5-s may, therefore, be an interesting new candidate in the search for novel anti-IL-6 agents to be used in inflammatory related disorders. Decreasing IL-6 secretion may lead to inhibition of development of Th17 cells that are involved in the pathogenesis of many chronic inflammatory diseases. In the long run, these effects could be beneficial in treatment of autoimmune disease.

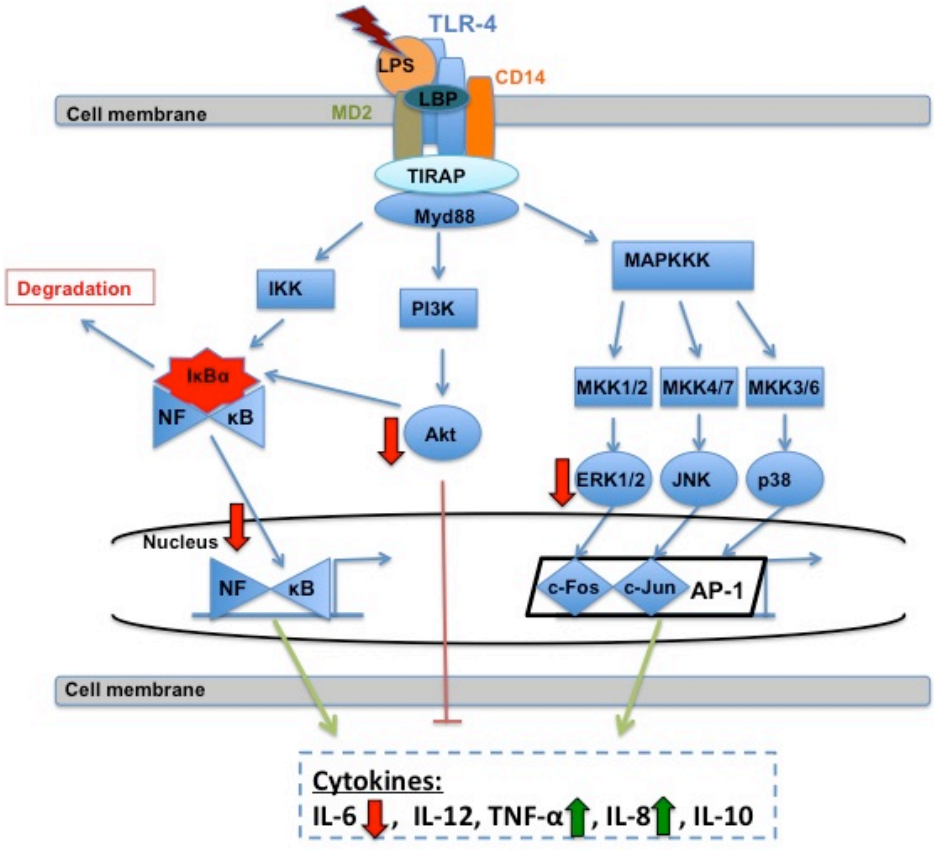


Figure 22. An overview of the effects of Nc-5-s on LPS stimulated TLR4 signaling in THP-1 monocytes.

Red arrows indicate that Nc-5-s causes a decrease in activation/cytokine secretion and green arrows that it causes an increase in activation/cytokine secretion.

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Appendix 1

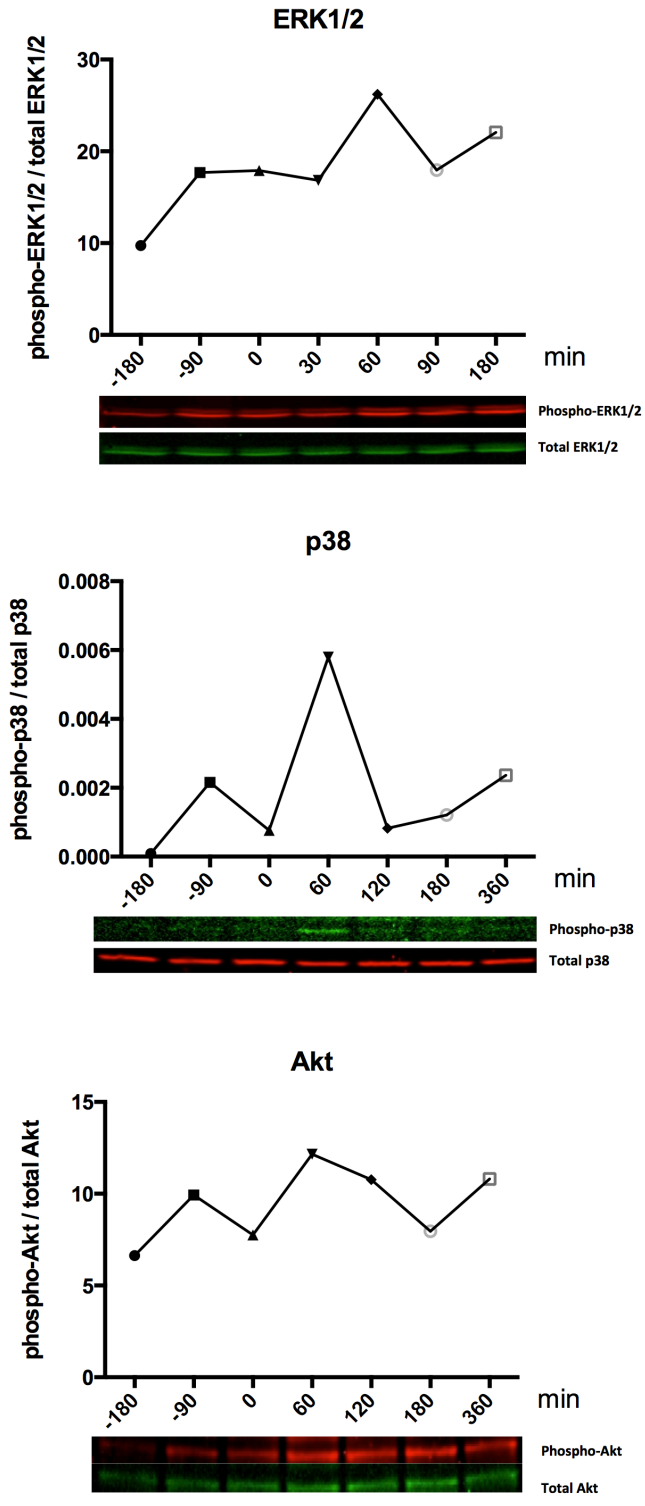

Figure A1. Time curves for phosphorylation of MAP kinases and Akt kinase.

THP-1 monocytes were primed with IFN-γ for 3 h (-180 min) and then stimulated with LPS (0 min) for the indicated time. Samples were collected and lysed for Western blotting at each of the indicated time points. Each graph represents results from one experiment that has been repeated more than once.

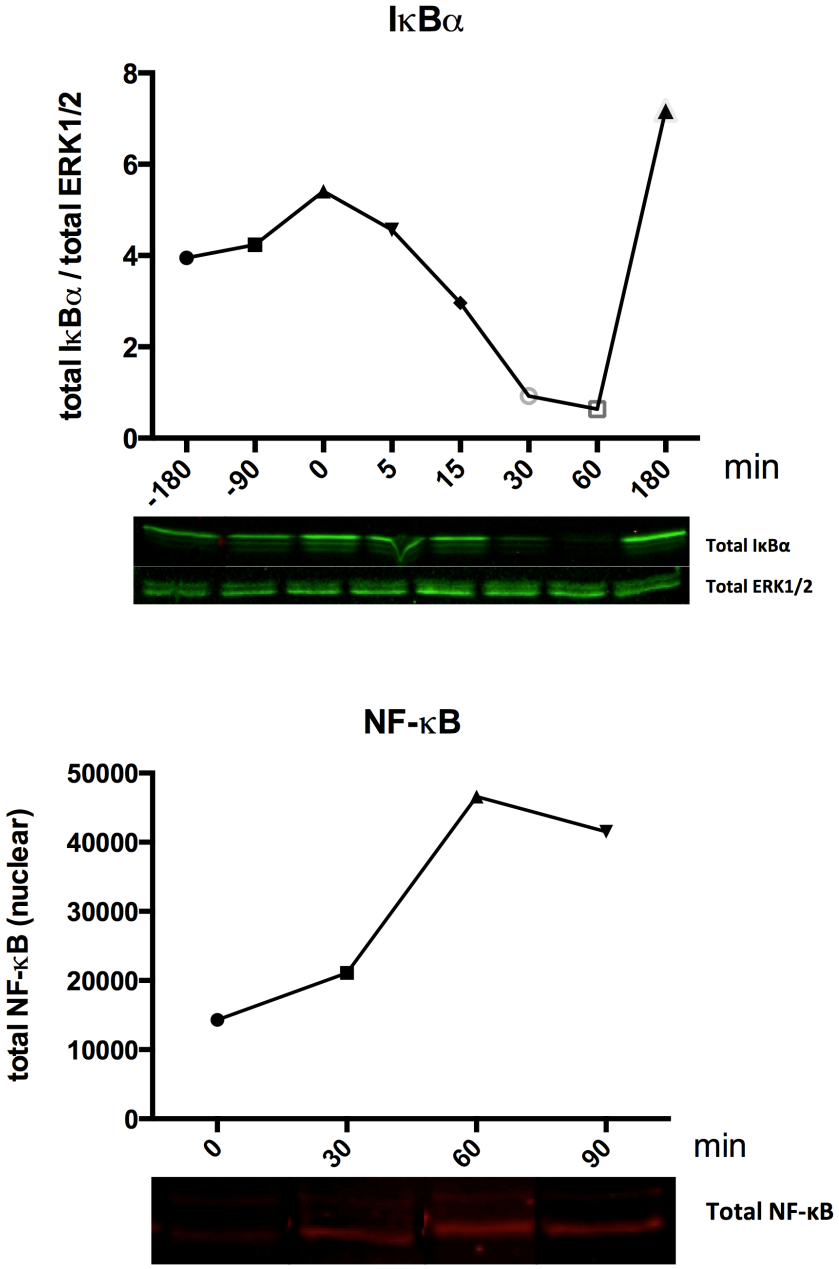

Figure A2. Time curves for breakdown of IκBα and nuclear localization of NF-κB.

THP-1 monocytes were primed with IFN-γ for 3 h (-180 min) and then stimulated with LPS (0 min) for the indicated time. Samples were collected and lysed for Western blotting at each of the indicated time points. Each graph represents results from one experiment that has been repeated more than once.

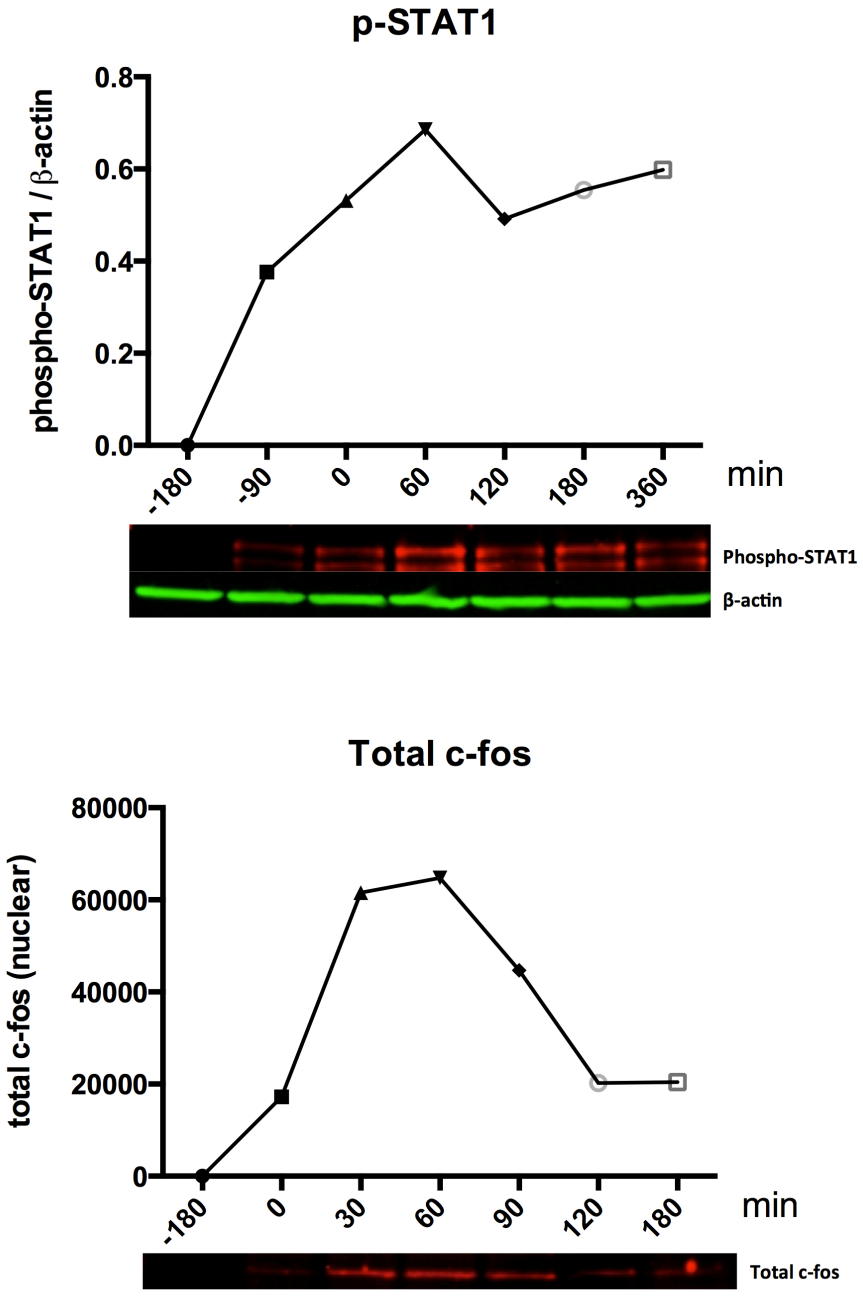

Figure A3. Time curves for phosphorylation of STAT1 and nuclear c-fos accumulation.

THP-1 monocytes were primed with IFN-γ for 3 h (-180 min) and then stimulated with LPS (0 min) for the indicated time. Samples were collected and lysed for Western blotting at each of the indicated time points. Each graph represents results from one experiment that has been repeated more than once.

Manuscript in preparation

A heteroglycan from the cyanobacterium *Nostoc commune* modulates LPS-induced inflammatory cytokine secretion by THP-1 monocytes through phosphorylation of ERK1/2 and Akt

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Abstract

Icelandic lichens have been used in folk medicine to alleviate inflammatory diseases. The cyanobacterium *Nostoc commune* is common in lichen symbiosis and is known to produce complex exopolysaccharides. A heteroglycan (Nc-5-s) has been isolated from *Nostoc commune*. The aim of this study was to determine the effects of Nc-5-s on the inflammatory response of LPS-stimulated human THP-1 monocytes and how the effects are mediated. THP-1 monocytes primed with IFN-γ and stimulated with LPS in the presence of Nc-5-s secreted less of the pro-inflammatory cytokine IL-6 than THP-1 monocytes stimulated without Nc-5-s. In contrast, Nc-5-s increased LPS-induced secretion of the pro-inflammatory cytokines TNF-α and IL-8. Nc-5-s decreased LPS-induced phosphorylation of the extracellular regulated kinase (ERK) 1/2 and Akt kinases, but did not affect phosphorylation of the p38 kinase or breakdown of the nuclear factor kappa B (NF-κB) inhibitor, IκBα. These results show that Nc-5-s differently affects secretion of the pro-inflammatory cytokines IL-6, TNF-α and IL-8 by THP-1 monocytes and that these effects may be mediated through the ERK1/2 pathway and/or the Akt/phosphoinositide 3-kinase pathway and their downstream effectors. The potential of Nc-5-s to decrease IL-6 secretion may be of importance as IL-6 induces the development of Th17 cells that have been implicated in autoimmune diseases. However, the concomitant increase in the pro-inflammatory cytokines TNF-α and IL-8 may hinder its use as an anti-inflammatory agent.

Keywords

THP-1, IL-6, IL-8, TNF-α, ERK1/2, Akt

Abbreviations

ELISA, enzyme-linked immunosorbent assay; ERK1/2, extracellular regulated kinase 1/2; FACS, fluorescence-activated cell sorting; FCS, fetal calf serum; IFN, interferon; IL, interleukin; TNF, tumor necrosis factor; MAP, mitogen-activated protein; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; RA, rheumatoid arthritis; SDS, sodium dodecyl sulfate; SI, secretion index; TBS, tris-buffered saline; TLR, toll-like receptor.

Introduction

A broad spectrum of plants and lichens has traditionally been used for promoting health or for prevention and treatment of different disorders, like infectious illness and autoimmune diseases. Lichens consist of a mycobiont and a photobiont growing in symbiosis. Cyanobacteria are common in lichen symbiosis and are most often of the *Nostoc* genus (Rikkinen et al., 2002). The cyanobacterium *Nostoc commune* is known to produce complex extracellular polysaccharides that are important for the organism's tolerance of harsh environmental conditions. A complex heteroglycan, called Nc-5-s, has been isolated from *Nostoc commune* and its structure determined (Jensen et al., 2013). Its effects on inflammatory responses have not been examined, but a previous study has shown that several polysaccharides from lichens have immunomodulating effects on dendritic cells (Omarsdottir et al., 2006). Although there are no reports of immunomodulating properties of polysaccharides from *Nostoc commune*, lipid extracts from *Nostoc commune* have been shown to have anti-inflammatory effects on macrophages (Ku et al., 2013) and crude extracts of *Nostoc commune* have been shown to be biologically active against certain protozoa (Broniatowska et al., 2011).

Inflammatory responses are essential but need to be tightly regulated. If regulation fails, it can lead to chronic inflammation and to diseases, such as rheumatoid arthritis (RA). In RA and other autoimmune diseases overexpression of inflammatory cytokines, such as IL-6 and TNF-α, has been implicated in progression of the disease (Brennan and McInnes, 2008). Therefore, inhibiting cytokines production or its effects has become one focus of treating these diseases.

Stimulation of human monocytes with lipopolysaccharide (LPS) is initiated when LPS binds to toll-like receptor 4 (TLR4) and the receptor associated proteins, CD14 and myeloid differentiation protein (MD2). Through this complex, several intracellular signaling pathways are activated. The nuclear factor kappa B (NF-κB) and Akt/phosphoinositide 3-kinase (PI3K) pathways as well as the mitogen activated protein (MAP) kinases become activated, leading to the production of pro-inflammatory (IL-6, IL-12 and TNF-α) and anti-inflammatory (IL-10) cytokines (Huang et al., 2012; Tamai et al., 2003) (reviewed in (Rossol et al., 2011)). ERK1/2 activates the activator protein (AP)-1 transcription factor through phosphorylating c-fos (Gilley et al., 2009) which can then dimerize with c-jun and bind 25 times more efficiently to the AP-1 DNA site than the c-jun dimer (Halazonetis et al., 1988).

The present study was formulated based on knowledge about biological activities of Icelandic lichens, cytokine overproduction in inflammatory diseases and regulation of cellular signaling. The aim was to examine the effects of a purified heteroglycan (Nc-5-s) from *Nostoc commune,* a common lichen photobiont*,* on inflammatory responses by human THP-1 monocytes and to elucidate how the effects are mediated on an intracellular level.

Materials and methods

Reagents

The Nc-5-s heteroglycan from *Nostoc commune* was isolated as previously described (Jensen et al., 2013) and dissolved in culture medium prior to use. Monoclonal antibodies for Western blotting were all purchased from Cell Signaling Technology (Danvers, MA, USA) except for antibodies against phospho-c-Fos and NF-κB p65 that were obtained from Santa Cruz Biotechnology (Dallas, TX, USA) and LaminB which was obtained from Invitrogen (Paislay, UK). The ERK1/2 inhibitor, U0126, was obtained from Cell Signaling. Antibodies for flow cytometric analysis were obtained from AbD Serotec (Kidlington, UK), except for the TLR2 antibody that was from BD Biosciences (San Jose, CA, USA) and the TLR4 antibody that was from eBioscience (San Diego, CA, USA).

Cell culture

Human THP-1 monocytes were obtained from The German Collection of Microorganisms and Cell cultures (Braunschweig, Germany) and maintained in RPMI 1640 culture medium supplemented with 2.05 mM L-glutamine, 10% heat inactivated fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin (all from Invitrogen). The cells were passaged twice per week to maintain logarithmic growth and cultured in a humidified incubator at 37°C and 5% CO_2. Cells were passaged a maximum of 20 times.

Viability assessment

Possible cytotoxic effects of Nc-5-s were tested using trypan blue staining and Via-Probe (BD Biosciences) after culturing THP-1 monocytes with Nc-5-s for 48 h. For viability analysis using Via-Probe, cells were rinsed with staining buffer (phosphate-buffered saline with 0.5% FCS and 20 mM EDTA) and resuspended in 0.5 ml staining buffer with 3 µl Via-Probe cell viability solution (BD Biosciences). Cells were incubated on ice in the dark for 15 min and analyzed on a FACScan (BD Biosciences) within 1 h. Cell viability was also assessed using trypan blue staining and counting cells using Countess automated cell counter (Invitrogen).

Cytokine concentration determination

Cells were seeded in 48 well culture plates, $5x10^5$ cells/ml in culture medium and primed with 100 U/ml IFN-γ (R&D Systems Inc., Minneapolis, MN, USA) for 3 h before addition of Nc-5-s and stimulation with 0.75 µg/ml LPS (*E. coli* 055:B*5;* Sigma-Aldrich, St. Louis, MO, USA) for 48 h. When

indomethacin (Sigma-Aldrich) was used it was added immediately prior to the LPS. Concentrations of IL-6, IL-8, IL-10, IL-12p40 and TNF-α in supernatants were measured by DuoSet ELISA kits (R&D Systems) according to the manufacturer's instructions. Results are presented as secretion index (SI), which is the concentration of the cytokine in medium from cells cultured with Nc-5-s divided by the concentration of the cytokine in medium from cells cultured without Nc-5-s.

Prostaglandin E_2 measurements

Cells were treated as described for determination of cytokine concentration and PGE_2 levels measured in culture supernatants using an EIA kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions.

Western blot analysis

THP-1 monocytes were seeded in 6 well plates, $1x10^6$ cells/ml in culture medium and primed with IFN-γ for 3 h, followed by stimulation with LPS for 1 h. Nc-5-s (100 µg/ml) was either added with IFN-γ or with LPS. Unstimulated cells and stimulated cells without Nc-5-s were used as controls. After treatment, cells were washed with ice cold PBS and then lysed with with either a mild NP-40 lysis buffer (25 ml 1M Tris-HCl pH 7.4, 15 ml 5M NaCl, 5 mL NP-40, 334 ml dH_2O) and sonicated shortly to produce a whole cell lysate or a Nuclear Extract kit from Active Motif (Carlsbad, CA, USA) to yield a nuclear lysate. Whole cell or nuclear lysates were then separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes using iBlot (Invitrogen). The membranes were blocked with 5% non-fat milk powder in Tris-buffered saline (TBS) for 1 h and then incubated with primary antibodies in a solution of TBS, bovine serum albumin and Tween-20 at 4°C overnight. Finally, the membranes were incubated with NIR-labeled anti-rabbit and/or anti-mouse secondary antibodies (Li-Cor Biosciences, Lincoln, NE, USA) in TBS with 0.1% Tween-20 and blots scanned using the Odyssey Infrared Imaging System (Li-Cor Biosciences) and analyzed using the accompanying Image Studio software.

Expression of surface molecules

THP-1 monocytes were seeded in 6 well plates, $1x10^6$ cells/ml in culture medium and primed with IFN-γ for 3 h, followed by stimulation with LPS for 24 h. Nc-5-s (100 µg/ml) was either added with IFN-γ or with LPS. Unstimulated cells and cells stimulated without Nc-5-s were used as controls. After treatment, approximately $1x10^5$ cells were harvested into FACS tubes and appropriate monoclonal antibodies or isotype controls added. The tubes were incubated on ice for 20 min, cells washed with staining buffer, resuspended and fixed in 1% paraformaldehyde. Finally, samples were collected on

FACScalibur (BD Biosciences) and data analyzed using Flowing Software (Cell Imaging Core, Turku Centre for Biotechnology, Turku, Finland).

AP-1 activation analysis

C-fos/AP-1 DNA binding activity in nuclear extracts was performed with a TransAm® kit (Active Motif, Carlsbad, CA, USA).according to the manufacturer's instructions.

Statistical analysis

Results are presented as mean ± standard error of the mean (SEM). A paired T-test was used when comparing two groups. When more than two groups were compared, a repeated measures ANOVA, followed by Tukey's post hoc test, was used. A p-value less than 0.05 was considered statistically significant. Results are labeled with * for $p<0.05$ or ** for $p<0.01$.

Results

Effects of Nc-5-s on cell viability and appearance

Incubating THP-1 monocytes with 100 µg/ml of Nc-5-s for 48 h did not affect viability of the cells (data not shown). Nc-5-s did not affect the appearance of unstimulated cells , which were present in the medium as single cells. Following priming and stimulation of the cells they clumped but Nc-5-s seemed to attenuate that clumping (figures to be produced before submitting the manuscript).

Effects of Nc-5-s on LPS-induced secretion of cytokines

THP-1 monocytes stimulated in the presence of the heteroglycan Nc-5-s secreted less of the pro-inflammatory cytokine IL-6 than monocytes stimulated without the heteroglycan (Fig. 1A). The inhibitory effect of Nc-5-s on secretion of IL-6 was dose dependent. In contrast, the highest dose of Nc-5-s (100 µg/ml) increased LPS-induced secretion of the pro-inflammatory cytokines TNF-α and IL-8, with the effect on IL-8 being more than three-fold (Figs. 1B and C). Nc-5-s did not affect LPS-induced secretion of IL-10 or IL-12p40 by the THP-1 monocytes (Figs. 1D and E).

To test if the effects of Nc-5-s on secretion of IL-6 could be mediated by prostaglandins, indomethacin, a cyclooxygenase inhibitor, was added to the cells prior to LPS stimulation. Indomethacin did not prevent Nc-5-s mediated down-regulation of IL-6 secretion by the THP-1 monocytes (data not shown). Neither did Nc-5-s affect LPS-induced PGE_2 secretion by the THP-1

cells (data not shown), further supporting the concept that prostaglandins are not involved in the effects of Nc-5-s on cytokine secretion.

Effects of Nc-5-s on activation of MAP kinases and the Akt/PI3K pathway

In order to investigate how Nc-5-s mediates its effects on cytokine secretion by THP-1 monocytes we investigated whether it affected activation of various intracellular pathways associated with inflammatory responses. Nc-5-s decreased LPS-induced phosphorylation of ERK1/2 more than 50% (Fig. 2A). The effect was seen both when Nc-5-s was added along with IFN-γ or along with LPS. Nc-5-s did not affect LPS-induced phosphorylation of the p38 MAP kinase (Fig. 2B). The SAPK/JNK kinase was not activated by the priming and stimulation (IFN-γ and LPS) used (data not shown).

Nc-5-s attenuated LPS-induced activation of the Akt kinase, both when it was added to the cells along with IFN-γ and when it was added along with LPS (Fig. 2C). With Akt being a downstream kinase of PI3K, these results indicate that PI3K activation may also be decreased.

Effects of Nc-5-s on activation of the transcription factors NF-κB, AP-1 and STAT1

Nc-5-s did not affect LPS-induced breakdown of the NF-κB inhibitor, IκBα (Fig. 3A), indicating that the NF-κB pathway is not involved in the effects of Nc-5-s on cytokine secretion by THP-1 monocytes. However, when examining nuclear localization of the NF-κB, there was a tendency towards less NF-κB being translocated to the nucleus when the cells were stimulated in the presence of Nc-5-s than when they were stimulated without Nc-5-s (Fig. 3B). Nc-5-s did not affect phosphorylation of the c-fos subunit of the AP-1 transcription factor nor did it affect phosphorylation of the STAT1 transcription factor (data not shown). Neither did Nc-5-s affect binding of c-fos to its regulatory TRE DNA element (data not shown).

Effects of the U0126 on cytokine production by THP-1 monocytes

As the effects of Nc-5-s on LPS-induced cytokine secretion by THP-1 cells may, at least in part, be mediated through the ERK1/2 kinase, we investigated whether a known MEK1/2 inhibitor affected LPS-induced cytokine secretion by THP-1 cells in the same way as Nc-5-s. The MEK1/2 inhibitor, U0126, decreased LPS-induced secretion of IL-6 as did Nc-5-s (Fig. 1S A). However, in contrast to the effects of Nc-5-s on IL-8 and TNF-α secretion, U0126 also inhibited secretion of these two cytokines (Figs. 1S B and C). U0126 also inhibited secretion of IL-10 and IL-12p40 (Figs. 1S D and E). Therefore, while Nc-5-s decreased LPS-induced activation of ERK1/2 in THP-1 cells, does not cause the same effects on secretion of all cytokines as a known inhibitor of the ERK1/2.

Effects of Nc-5-s on expression of surface molecules involved in signal transduction following LPS stimulation

Nc-5-s did not affect expression levels of TLR4, TLR2, CD14, CD40, CD54 or CD86 on unstimulated or stimulated THP-1 cells (data not shown). Neither did Nc-5-s affect the proportion of unstimulated and stimulated cells expressing these surface markers (data not shown).

Discussion

The heteroglycan Nc-5-s, isolated from the cyanobacterium *Nostoc commune*, attenuated LPS-induced secretion of the pro-inflammatory cytokine IL-6 while increasing secretion of TNF-α and IL-8, which are also considered pro-inflammatory. Therefore, the overall effect of Nc-5-s on the immune response by the THP-1 monocytes cannot be considered either anti- or pro-inflammatory, but rather a specific effect on these cytokines.

Decreased IL-6 secretion by THP-1 monocytes stimulated in the presence of Nc-5-s may have been caused by a decrease in activation of the MAP kinase, ERK1/2, the only MAP kinase of the three studied, that was affected by Nc-5s, and/or by a decrease in activation of the Akt kinase of the PI3K/Akt pathway. U0126 a known inhibitor of ERK1/2 reduced IL-6 secretion by the THP-1 cells similarly to Nc-5-s, although more effectively. Although attenuated activation of ERK1/2 by Nc-5-s did not result in reduced activation of the c-fos subunit of the AP-1 transcription factor, that is most commonly implicated in regulation of inflammatory gene transcription (Bakiri et al., 2011; Gilley et al., 2009; Roskoski, 2012), there are other downstream effectors of ERK1/2, such as Egr1 (Guha and Mackman, 2002; Shi et al., 2002) that have also been implicated in inflammatory responses and need to be further studied.

The increase in TNF-α and IL-8 secretion by THP-1 cells treated with Nc-5-s is puzzling as activation of ERK1/2, that is down-regulated by Nc-5-s in the present study, has been shown to be critical for TNF-α and IL-8 production (Scherle et al., 1998) and in the present study the ERK1/2 inhibitor, U0126, effectively decreased secretion of both these cytokines. Other natural products have also been shown to affect TNF-α and IL-8 secretion by THP-1 monocytes in the same direction. The antifungal drug, terbinafine, increased secretion of TNF-α and IL-8 by THP-1 monocytes as well as activation of ERK1/2 (Mizuno et al., 2010) and the fluoroquinolone, garenoxacin, inhibited IL-8 production through a negative regulation of the ERK1/2 pathway (Hara et al., 2011). We could find no reports showing opposite effects of natural products on TNF-α and IL-8 production and ERK1/2 activation.

In contrast to the decreased IL-6 and the increased TNF-α and IL-8 secretion by THP-1 cells stimulated in the presence of Nc-5-s in the present study, there is one report showing just the opposite, i.e. decreased secretion of TNF-α and IL-8 and increased secretion of IL-6 by THP-1 derived macrophages stimulated in the presence of a polyphenolic extract from the Tunisian quince Cydonia oblonga Miller (Essafi-Benkhadir et al., 2012). In that study it was shown that the increased IL-6

production could be responsible for the decrease in TNF-α and IL-8 production as incubation of the cells with IL-6 decreased TNF-α secretion. In addition, mouse macrophages treated with anti-IL-6 antibodies secreted more TNF-α than untreated cells, indicating that production of TNF-α can be negatively regulated by IL-6 (Yimin and Kohanawa, 2006). Therefore, whether the decrease in IL-6 secretion caused by Nc-5-s in the present study may have led to the increase in TNF-α (and perhaps also the IL-8) secretion by the THP-1 cells remains to be established, but is a possibility. However, IL-6 levels have been shown to rise shortly after the rise in TNF-α levels in several *in vitro* studies (Huang et al., 2012; Schildberger et al., 2013) and therefore it may be assumed that Nc-5-s is first affecting TNF-α, which then could be further affecting IL-6 and IL-8 secretion. As IL-8 secretion has been shown to be induced by TNF-α (Leonard and Yoshimura, 1990) the increase in IL-8 secretion by the THP-1 cells stimulated in the presence of Nc-5-s may have resulted from the increase in TNF-α.

The considerable reduction in IL-6 secretion by THP-1 cells stimulated in the presence of Nc-5-s may be of importance as IL-6 induces the development of Th17 cells and blockade of IL-6 signaling is effective in treating experimental models of autoimmune and chronic inflammatory diseases, such as inflammatory bowel diseases, diabetes, multiple sclerosis, asthma and RA (Neurath and Finotto, 2011) and a blocker of the IL-6R (Tocilizumab) is available for treatment of RA and systemic juvenile idiopathic arthritis (Md Yusof and Emery, 2013). However, the concomitant increase in TNF-α and IL-8 secretion may hinder the potential use/development of Nc-5-s as an IL-6 blocker, as TNF-α and IL-8 are involved in neutrophil activation and recruitment to the inflamed tissue (Huber et al., 1991) and modulation of the secretion of TNF-α and IL-8 is thought to be important to counteract pathologic inflammation.

Acknowledgements

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Figure legends

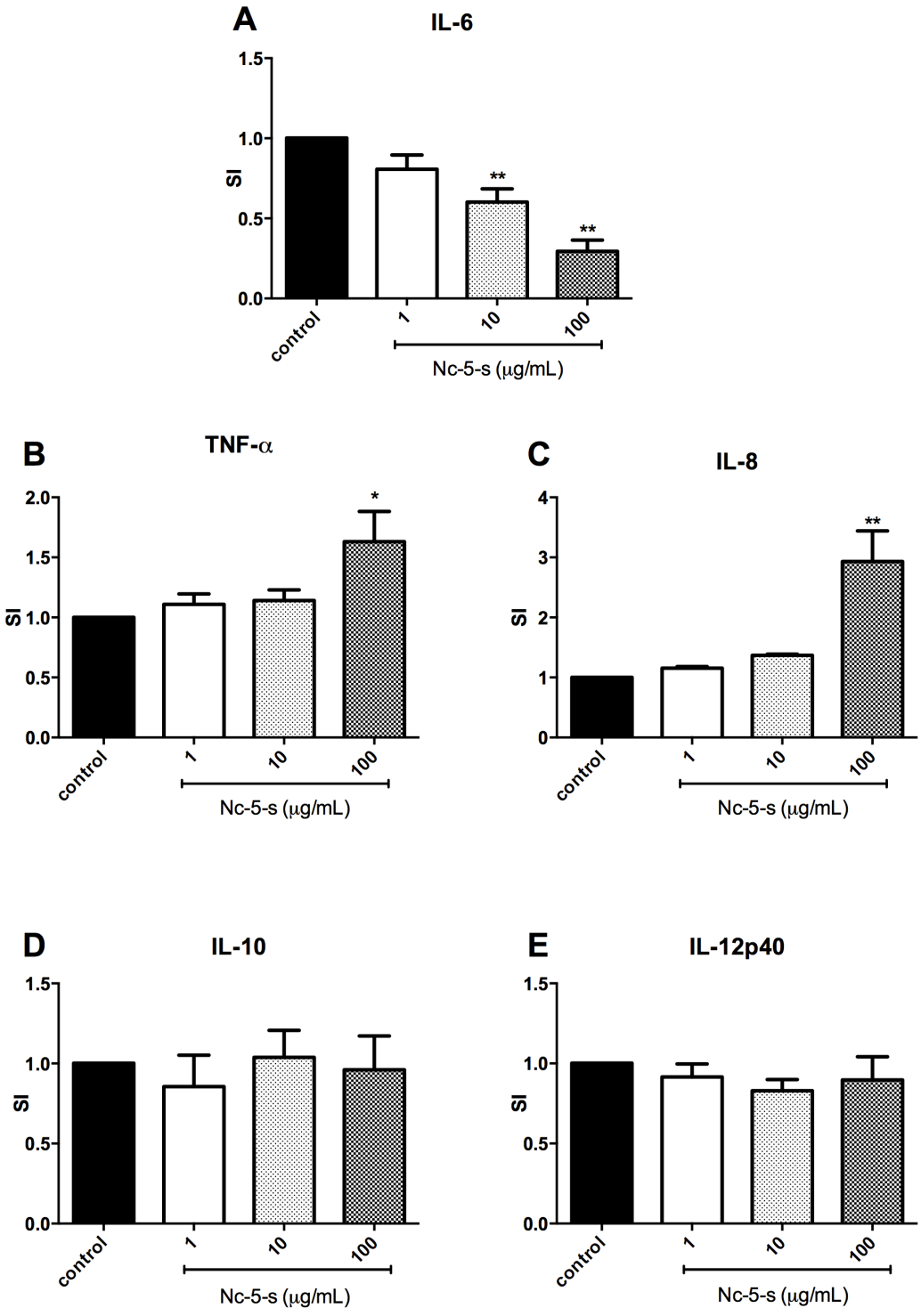
Figure 1. Effects of Nc-5-s on LPS-induced secretion of IL-6 (A), TNF-α (B), IL-8 (C), IL-10 (D) and IL-12p40 (E) by THP-1 monocytes. THP-1 monocytes were primed with IFN-γ for 3 h and then stimulated with LPS for 48 h. Nc-5-s was added to the cells along with LPS at the indicated concentrations. Results are shown as the mean of the secretion index (the concentration of the cytokine in the supernatants of cells incubated with Nc-5-s divided by the concentration of the cytokine in the supernatants of cells incubated without Nc-5-s) ± SEM. For IL-6 and IL-12 n=7, for IL-10 n=6, for TNF-α n=5 and for IL-8 n=3. *Different from stimulated control $p<0.05$ or ** $p<0.01$.

Figure 2. Effects of Nc-5-s on LPS-induced phosphorylation of ERK1/2 (A), p38 (B) and Akt (C) kinases. THP-1 monocytes were primed with IFN-γ for 3 h and then stimulated with LPS for 1 h. Nc-5-s was either added to the cells along with IFN-γ (-3 h) or along with LPS (0 h), at a concentration of 100 µg/mL. Whole cell protein lysates were analyzed using Western blotting. Bars represent the ratio of the mean signal intensity for phosphorylated protein versus total protein, relative to the same ratio for the stimulated control (black bar) ± SEM. n=3. **Different from stimulated control $p<0.01$. The blots are representative of three independent experiments.

Figure 3. Effects of Nc-5-s on LPS-induced breakdown of IκBα (A) and nuclear localization of NF-κB (B). THP-1 monocytes were primed with IFN-γ for 3 h and then stimulated with LPS for 1 h. Nc-5-s was either added to the cells along with IFN-γ (-3 h) or along with LPS (0 h), at a concentration of 100 µg/mL. Bars represent mean signal intensity for the ratio of total protein versus β-actin control, relative to the same ratio for the stimulated control (black bar) ± SEM with n=3. The blots are representative of three independent experiments using whole cell lysates (IκBα) or nuclear lysates (NF-κB).

Figure 1S. Effects of the MEK1/2 inhibitor U0126 on LPS-induced secretion of IL-6 (A), TNF-α (B), IL-8 (C), IL-10 (D) and IL-12p40 (E) by THP-1 monocytes.THP-1 monocytes were primed with IFN-γ for 3 h and then stimulated with LPS for 48 h. U0126 at the indicated concentrations was added with the LPS. Results are shown as the mean of the secretion index ± SEM. For IL-6, TNF-α, IL-8 and IL-12 n=3, except for the 0.01 µM concentration of U0126 where n=2. For IL-10, n=2.

Figure 1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IL-6
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0.5
0.0
**
**
control
1
10
100
Nc-5-s (μg/mL)
B
TNF-α
SI
2.0
1.5
1.0
0.5
0.0
*
control
1
10
100
Nc-5-s (μg/mL)
C
IL-8
SI
4
3
2
1
0
**
control
1
10
100
Nc-5-s (μg/mL)
D
IL-10
SI
1.5
1.0
0.5
0.0
control
1
10
100
Nc-5-s (μg/mL)
E
IL-12p40
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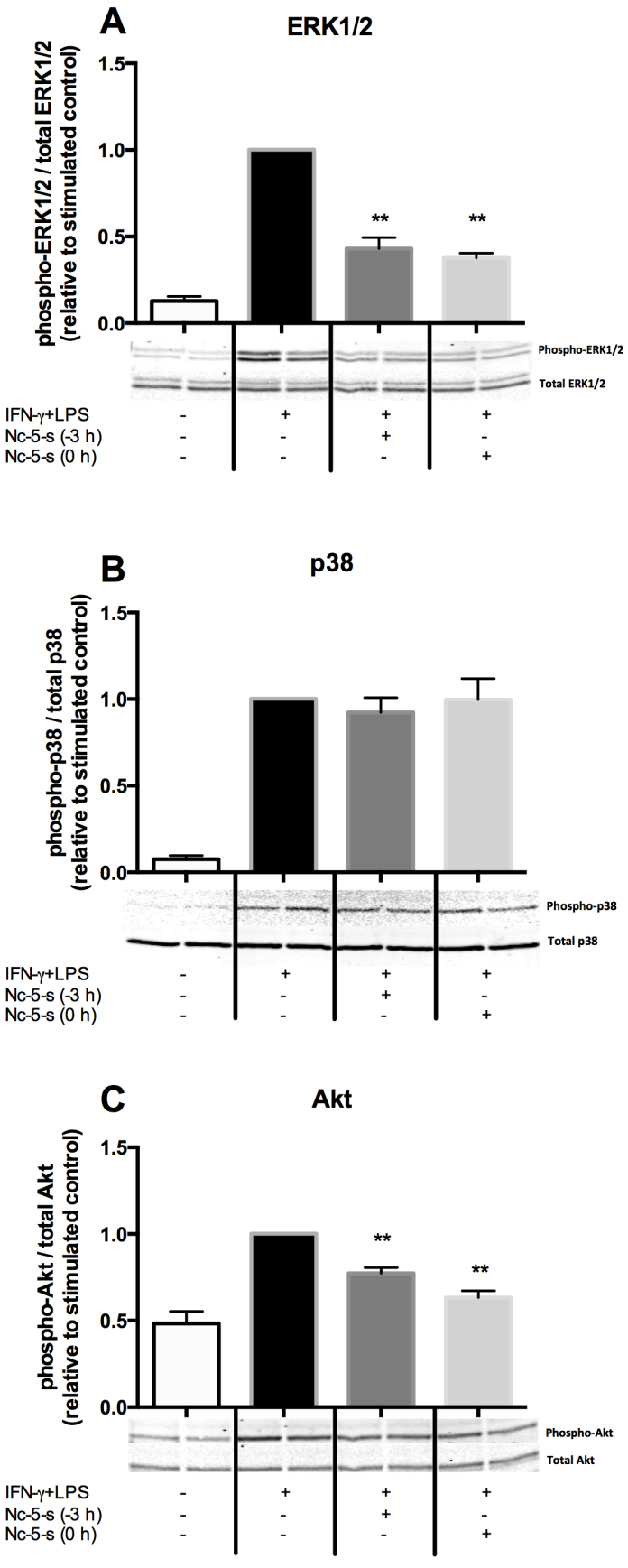
1.0
0.5
0.0
control
1
10
100
Nc-5-s (μg/mL)

Figure 2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ERK1/2
phospho-ERK1/2 / total ERK1/2
(relative to stimulated control)
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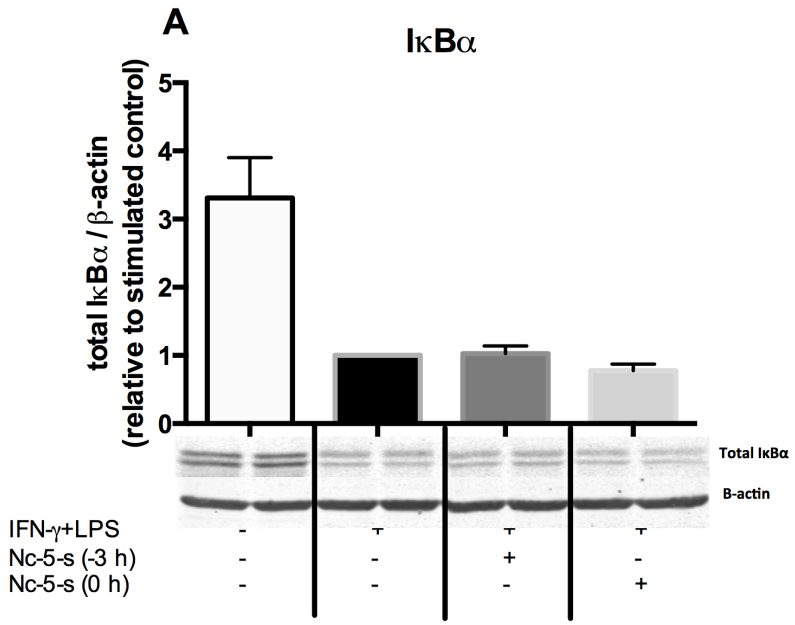
1.0
0.5
0.0
**
**
Phospho-ERK1/2
Total ERK1/2
IFN-γ+LPS - + + +
Nc-5-s (-3 h) - - + -
Nc-5-s (0 h) - - - +
B
p38
phospho-p38 / total p38
(relative to stimulated control)
1.5
1.0
0.5
0.0
Phospho-p38
Total p38
IFN-γ+LPS - + + +
Nc-5-s (-3 h) - - + -
Nc-5-s (0 h) - - - +
C
Akt
phospho-Akt / total Akt
(relative to stimulated control)
1.5
1.0
0.5
0.0
**
**
Phospho-Akt
Total Akt
IFN-γ+LPS - + + +
Nc-5-s (-3 h) - - + -
Nc-5-s (0 h) - - - +

Figure 3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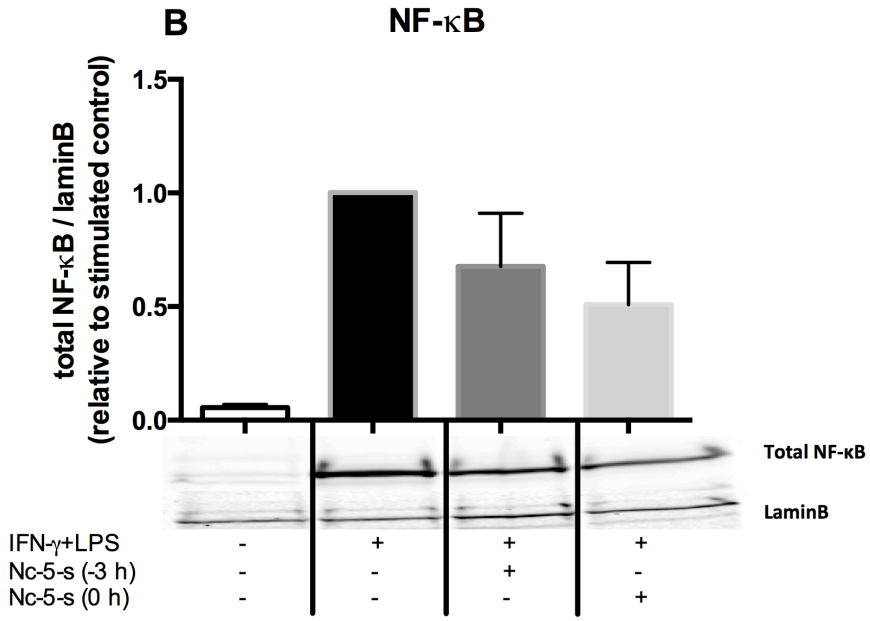
IκBα
total IκBα / β-actin
(relative to stimulated control)
0
1
2
3
4
5
Total IκBα
B-actin
IFN-γ+LPS
Nc-5-s (-3 h)
Nc-5-s (0 h)
- + + +
- - + -
- - - +


B
NF-κB
total NF-κB / laminB
(relative to stimulated control)
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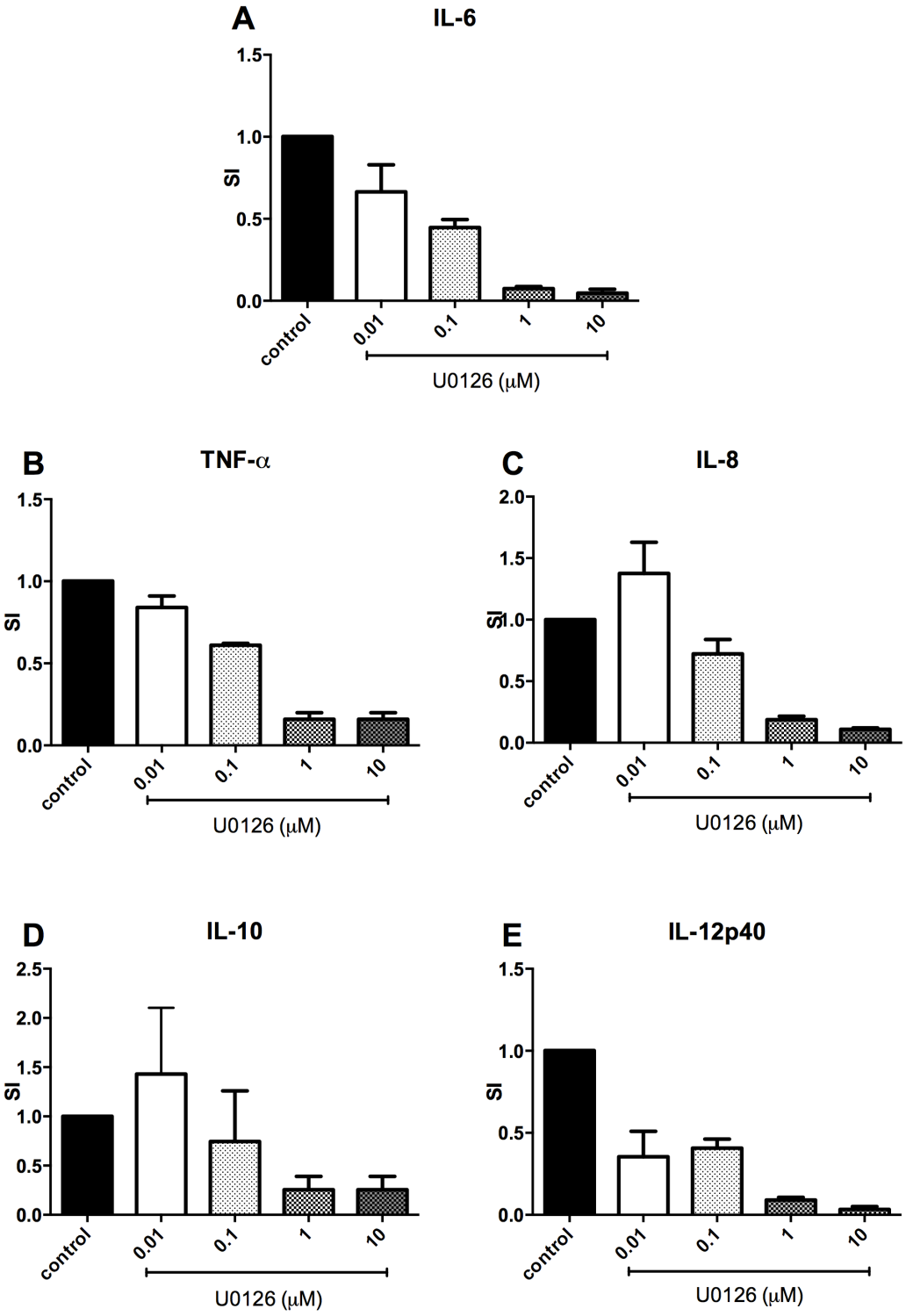
0.5
1.0
1.5
Total NF-κB
LaminB
IFN-γ+LPS
Nc-5-s (-3 h)
Nc-5-s (0 h)
- + + +
- - + -
- - - +

Figure 1S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IL-6
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1.0
0.5
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control
0.01
0.1
1
10
U0126 (μM)
B
TNF-α
C
IL-8
2.0
D
IL-10
2.5
E
IL-12p40