



Isolation of a polysaccharide from *Achillea millefolium* and its effects on inflammatory responses in THP-1 monocytes

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**Thesis for the degree of Master of Science
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School of Health Sciences**



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Thesis for the degree of Master of Science

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Einangrun fjölsykru úr vallhumli og áhrif hennar á ónæmisviðbrögð í THP-1 einkjörnungum

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Abstract

Isolation of a polysaccharide from *Achillea millefolium* and its effects on inflammatory responses in THP-1 monocytes

The use of drug substances derived from natural sources has a long tradition in medicine. Investigations of *Achillea* species have shown that many components from plants of this genus are highly bioactive. *Achillea millefolium* has been used in traditional medicine to heal inflamed cuts and alleviate rheumatism. Aqueous extract from *A. millefolium* has been shown to have anti-inflammatory effects on dendritic cells *in vitro*, which was attributed to polysaccharides in the extract. Several compounds have been isolated from *A. millefolium* but only a few have been tested for their immunological effects.

The aim of this study was to use bioguided fractionation to isolate a polysaccharide from *A. millefolium* and determine its effects on cytokine secretion and activation of intracellular pathways in THP-1 monocytes.

A water extract from dry *A. millefolium* was prepared and dialyzed. Ion exchange chromatography was used to fractionate the extract and PD-10 column to desalinate. Molecular weight determination was performed using HP-GPC analysis. THP-1 monocytes were primed with IFN- γ for 3 h and then stimulated with LPS for one (activation of intracellular pathways), 24 (expression of surface molecules) or 48 h (cytokine secretion). Am-25-d was added along with the LPS. Concentrations of IL-1 β , IL-6, IL-8, IL-10, IL-12p40, IL-23, IL-27 and TNF α in the culture medium were determined by ELISA. Expression of the surface markers TLR4, CD14, CD40, CD54, CD80, CD86 and HLA-DR was determined using flow cytometry. Activation of MAP kinases, PI3K/Akt and NF- κ B was investigated using Western blotting.

Am-25 had the most pronouncing effects on cytokine secretion by THP-1 monocytes of the four fractions from the ion exchange column. Following desalting, the molecular weight of Am-25-d was determined to be around 270 kDa.

THP-1 monocytes stimulated in the presence of Am-25-d secreted more of the anti-inflammatory cytokine IL-10 than monocytes stimulated in the absence of the polysaccharide. Furthermore, secretion of the pro-inflammatory cytokines IL-1 β , IL-8, IL-12p40, IL-23 and TNF- α was also increased in THP-1 monocytes stimulated in the presence of Am-25-d. The ratio between IL-12p40 and IL-10 was decreased in THP-1 monocytes stimulated in the presence of Am-25-d compared with that in THP-1 monocytes stimulated without it. Am-25-d did not affect expression of the surface molecules examined. Am-25-d decreased LPS-induced phosphorylation of the ERK1/2 MAP kinase but had no effect on p38 kinase. Am-25-d also decreased phosphorylation of the Akt kinase, suggesting inhibition

of the PI3K/Akt pathway. Am-25-d did not affect breakdown of the NF- κ B inhibitor, I κ B α , although translocation of NF- κ B to the nucleus had small tendency towards being decreased when THP-1 monocytes were stimulated in the presence of Am-25-d.

Am-25-d enhanced secretion of both pro- and anti-inflammatory cytokines. Therefore, whether Am-25-d is having a pro- or anti-inflammatory effect on THP-1 monocytes is not clear. The pro-inflammatory effects may be mediated through the PI3K/Akt pathway, but how the anti-inflammatory effects are regulated is not conclusive.

Ágrip

Einangrun fjölsykru úr vallhumli og áhrif hennar á ónæmisviðbrögð í THP-1 einkjörnungum

Notkun náttúruafur til heilsubóta á sér langa hefð í lækningum. Rannsóknir á plöntum af *Achillea* ættinni hafa sýnt að mörg innihaldsefni þeirra hafa mikla lífvirkni. *Achillea millefolium*, eða vallhumall, hefur verið notaður í alþýðulækningum í aldaðir til að lina bólgur og minnka einkenni gigtar. Sýnt hefur verið fram á bólguhemjandi áhrif í angafrumum meðhöndluðum með vatnsútdrætti úr vallhumli sem rakin eru til fjölsykra í útdrættinum. Nokkur efnasambönd hafa verið einangruð úr vallhumli en aðeins fáar rannsóknir hafa verið gerðar til að skoða áhrif þeirra á ónæmiskerfið. Rannsóknir á fjölsykrum úr plöntum hafa sýnt fram á ónæmisstýrandi áhrif þeirra, bæði *in vitro* og *in vivo*.

Markmið þessarar rannsóknar var að notast við lífvirknileidda einangrun á fjölsykru úr vallhumli og skoða áhrif hennar á boðefnamyndun og virkjun innanfrumuboðleiða í THP-1 einkjörnungum.

Vatnsútdráttur var útbúinn úr þurrkuðum vallhumli og hann svo dýlisaður. Jónskiptaskiljun var notuð til úrhlutunar á útdrættinum og PD-10 súla til að afsalta. Til að áætla mólþyngd var notast við HP-GPC greiningu. THP-1 einkjörnungar voru næmdir með IFN- γ í 3 klst og svo örvaðir með LPS í eina (virkjun innanfrumuboðleiða), 24 (tjáning yfirborðssameinda) eða 48 klst (boðefnamyndun). Am-25-d var sett út í frumuræktina á sama tíma og LPS. Styrkur boðefnanna IL-1 β , IL-6, IL-8, IL-10, IL-12p40, IL-23, IL-27 og TNF- α úr floti frumnanna var mældur með ELISA aðferð. Tjáning yfirborðssameindanna TLR4, CD14, CD40, CD54, CD80, CD86 og HLA-DR var metin með frumflæðisjá. Virkjun á MAP kínösum, PI3K/Akt boðleiðinni og NF- κ B var athuguð með Western blot aðferð.

Af þeim fjórum úrhlutunum sem fengust af jónskiptasúlunni, hafði Am-25 mestu áhrifin á seytun boðefna í THP-1 einkjörnungum. Eftir afsöltun, var mólþyngd Am-25-d áætluð um 270 kDa.

THP-1 einkjörnungar sem örvaðir voru í návist Am-25-d seyttu meira af bólguhemjandi boðefninu IL-10 heldur en THP-1 einkjörnungar örvaðir án sykrunnar. Þar að auki, var seytun á bólguhvetjandi boðefnunum IL-1 β , IL-8, IL-12p40, IL-23 og TNF- α einnig aukið í THP-1 einkjörnungum örvuðum í návist fjölsykrunnar. Hlutfallið á milli IL-12p40 og IL-10 minnkaði í THP-1 einkjörnungum örvuðum í návist Am-25-d borið saman við þá sem voru örvaðir án sykrunnar. Am-25-d hafði engin áhrif á tjáningu mældra yfirborðssameinda. Am-25-d dró úr LPS-aukinni fosfæringu á ERK1/2 MAP kinasanum en hafði engin áhrif á virkjun p38 kinasans. Am-25-d minnkaði einnig fosfæringu á Akt kinasanum, sem gefur til kynna hindrun á PI3K/Akt boðleiðinni. Am-25-d hafði ekki áhrif á niðurbrot á NF- κ B hindranum I κ B α , þó að tilfærsla NF- κ B inn í kjarnann hafi haft tilhneigingu til að minnka þegar THP-1 einkjörnungar voru örvaðir í návist Am-25-d.

Am-25-d jók seytun bæði bólguhvetjandi og bólguhemjandi boðefna. Þess vegna er ekki ljóst hvort Am-25-d hafi bólguhvetjandi eða bólguhemjandi áhrif á THP-1 einkjörnunga. Bólguhvetjandi áhrifunum gæti verið miðlað í gegnum PI3K/Akt boðleiðina, en hvernig bólguhemjandi áhrifunum er miðlað er ekki ljóst.

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Declaration of contribution

Oddný Þóra Logadóttir performed all experimental work included in this thesis, except for collection of the Yarrow and isolation and differentiation of DCs.

Plants collected by a former M.Sc. student Guðbjörg Jónsdóttir was used in this project. She collected the Yarrow at Hvanneyri in early July 2008.

The DC culture was performed by Hildur Sigurgrímsdóttir, at the Department of Immunology.

List of abbreviations

AP-1	Activator protein 1
APCs	Antigen presenting cells
BSA	Bovine serum albumin
DCs	Dendritic cells
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal regulated kinases
FCS	Fetal calf serum
GM-CSF	Granulocyte macrophage-colony-stimulating factor
GTPase	guanosine triphosphatase
HLA-DR	Human leukocyte antigen-DR
HP-GLC	High-performance gel permeation chromatography
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
ICAM	Intercellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IKK	I κ B kinase
IL	Interleukin
imDCs	Immature dendritic cells
IRF	Interferon regulatory factor
JNK	c-Jun N-terminal kinases
LBP	Lipopolysaccharide binding protein
LFA	Lymphocyte function associated antigen
LPS	Lipopolysaccharide
MAPKs	Mitogen-activated protein kinases
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
NF- κ B	Nuclear factor-kappa B
NK cell	Natural killer cell
NO	Nitric oxide
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate buffered saline

PBMCs	Peripheral blood mononuclear cells
PI3K	phosphatidylinositol 3-kinase
PRRs	pathogen recognition receptors
RT	Room temperature
SI	Secretion index
Th	T helper
TLRs	Toll like receptors
TNF	Tumor necrosis factor
Treg	Regulatory T cell
TRIF	TIR domain-containing adaptor inducing interferon- β

1 Introduction

For centuries humans have relied on nature for medicines to treat of a wide spectrum of diseases. Extracts from plants and organisms were the first, and for a long time, the only medicines available to humans. Although in the Western world, they are largely supplanted by active pharmaceutical ingredients, crude extracts remain the primary treatment of many diseases for the majority of the world's population. However, more studies are needed to examine most of these natural products, if they acutely do have an effect and then how these effects are regulated in the body.

Inflammation is a crucial part of normal immune response that plays a role in protecting the body from harmful factors such as pathogens. An inactive regulation of inflammation can lead to chronic inflammation that has been linked to many diseases in the Western society. Screening and testing the effects of natural product can provide valuable information on fighting inflammation and diseases.

1.1 The immune system

The body is protected from infectious agents and the damage they can cause, by a variety of effector cells and molecules that together make up the immune system. Microorganisms that we encounter daily in our life cause disease only occasionally. Most pathogens are detected and destroyed within minutes or hours of their appearance in the body by defense mechanisms of the innate immune system. However, when our immune system is overpowered or too slow to tackle an infection or inflammation we turn to antibiotics, antivirals and anti-inflammatory drugs for assistance.

Most of the pathogens we encounter in our daily life do not get past our first line of defense, i.e. the skin and the mucosa. If the first line of defense fails, we have two distinct yet intricately connected systems to tackle the invading pathogens; the innate and the adaptive immune systems (1).

Innate immune responses occur rapidly upon exposure to an infectious organism. Both the innate and the adaptive immune systems can distinguish between self and non-self. Innate immunity relies on secreted proteins and pathogen recognition receptors (PRRs), such as toll-like receptors (TLRs). These receptors sense the presence of pathogens by recognizing molecules called pathogen associated molecular patterns (PAMPs) (reviewed in (1)). PAMPs, like lipopolysaccharide (LPS), play a pivotal role in the initiation of a variety of host responses caused by infection with Gram-negative bacteria. Such action leads to systemic inflammatory response, for instance up-regulation of pro-and anti-inflammatory cytokine genes, resulting in secretion of cytokines into the blood stream (2).

All cells of the immune system arise from the same pluripotent hematopoietic stem cells in the bone marrow, which give rise to two kinds of progenitor cells; myeloid cells and lymphoid cells. The myeloid progenitor cells develop into monocytes, dendritic cells (DCs) and granulocytes, i.e. neutrophils, eosinophils and basophils. The monocytes are the precursors of the long-lived tissue residing macrophages. These cells comprise most of the cells that are classified within the innate immune system. The induced innate response can either succeed in clearing the infection or will contain it while an adaptive response develops (1).

The adaptive response will harness many of the same effector mechanisms used by the innate immune system, such as complement-mediated phagocytosis, but will target it with much greater precision. Responses by the adaptive immune system take days rather than hours to develop, contrary to the innate immune system. However, the adaptive system is capable of eliminating infections more efficiently because of the specific recognition function of lymphocytes. These cells can recognize and then respond to individual antigens by means of highly specialized antigen receptors on their surface (1).

The lymphoid progenitor cells give rise to natural killer (NK) cells, B lymphocytes, T lymphocytes and DCs (1). The wide spectrum of pathogens requires different responses for both their recognition and their destruction. B lymphocytes and T lymphocytes have different roles in the immune system and different types of antigen receptors. B cells recognize native antigens from the extracellular environment and, when activated, they proliferate and differentiate into plasma cells that produce antibodies. These antibody molecules have an identical specificity to the receptor and are known as immunoglobulins (Ig). T lymphocytes detect peptides that have been constructed inside the body's cell, whether from extracellular antigens that have been ingested or from locally produced proteins. This is reflected in the effector actions of T cells, some directly kill cells infected with intracellular pathogens, such as viruses, while others mediate help to other cells, such as macrophages and B cells.

Some of the activated B cells and T cells turn into memory cells that are responsible for the long-lasting immunity, ready to differentiate into effector cells when re-exposed to their specific antigen (1).

1.1.1 Inflammation

Inflammation can be induced by variety of different stimulating factors, such as physical damage, microbial invasion and immune responses. Generally, controlled inflammation is considered to be a beneficial response that can protect the body from harmful factors, such as pathogens. Proteins and cells from the blood are recruited into infected tissues and help to directly destroy the pathogen. In addition, inflammation increases the flow of lymph carrying microbes and antigen-presenting cells (APCs) from the infected tissue to nearby lymphoid tissues where they activate lymphocytes and initiate the adaptive immune response. However, if the body's regulation of inflammation is dysfunctional, the inflammation may have an adverse effect on the body and can lead to chronic inflammation (1, 3).

1.1.2 Monocytes

Monocytes are cells of the innate arm of the immune system that play multiple roles in immune function. Monocytes circulate in the blood and migrate into tissues where they differentiate into macrophages, which are resident in nearly all tissues in the body. Under inflammatory and/or infectious conditions, monocytes can also directly differentiate into so-called inflammatory DCs (1, 4). Monocytes are also a pool of scavenger and potential effector cells in the blood and are therefore

important players in defense against pathogens as well as in the pathogenesis of inflammatory diseases (5).

Monocytes and macrophages are involved in inflammatory processes (Figure 1), and they have a profound capacity to synthesize and secrete pro- and anti-inflammatory cytokines (1).

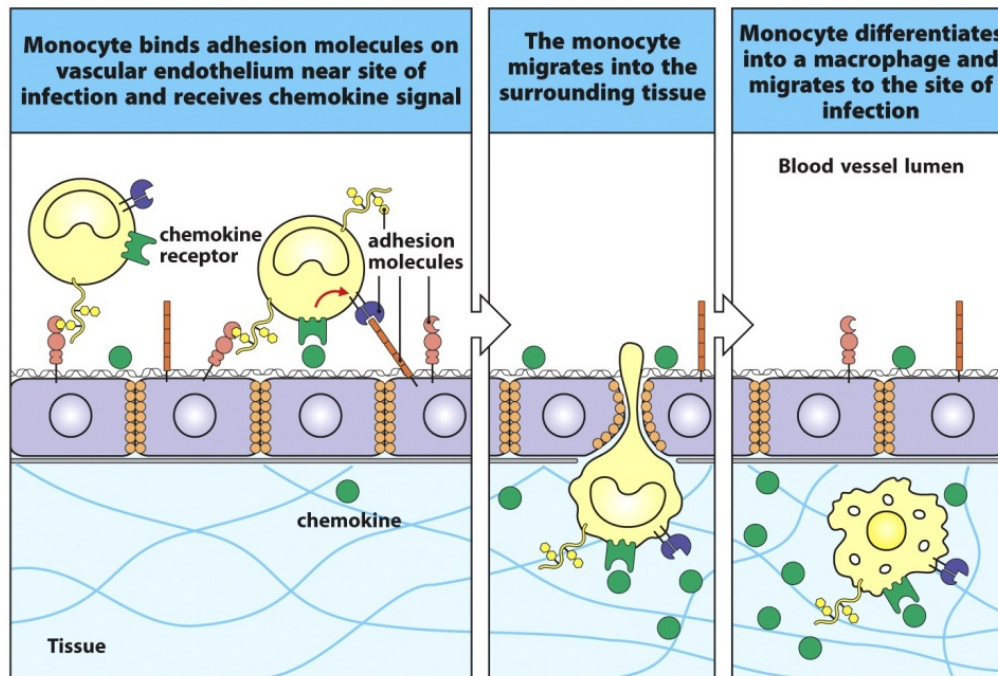


Figure 3.7 Janeway's Immunobiology, 8ed. (© Garland Science 2012)

Figure 1. Monocytes are involved in inflammatory responses

Monocyte becomes attached to the endothelium at the site of infection and receives cytokine and chemokine signal. Then the monocyte migrates into the infected tissue and differentiates into a macrophage. Figure from Murphy, *et al.* (1).

1.1.2.1 THP-1 monocytes

THP-1 cells are a human monocytic cell line derived from cells, first isolated in 1980 from a boy with acute monocytic leukemia. THP-1 cells are round in suspension and have distinct monocytic markers (6). They can either be used as undifferentiated monocytes or differentiated into macrophages by treating them with phorbol 12-myristate 13-acetate (PMA). These cells have been widely used as a model to study immune responses, because of similarities in their responses when compared with the monocyte fraction present in peripheral blood mononuclear cells (PBMCs). The THP-1 cells are a popular tool for studying modulation of inflammation and the effects of various compounds on the innate immune system (7, 8). Using cell lines rather than primary cells saves time and reduces cost. Cell lines have also some other advantages over human primary monocytes-macrophages, including their homogeneous genetic background that lowers the degree of variability in the cell phenotype (9).

Undifferentiated THP-1 monocytes do not respond sufficiently to LPS alone because of their low CD14 expression, a surface bound co-receptor for bacterial LPS detection. Therefore they need to be primed with interferon (IFN)- γ (10, 11).

1.1.3 Dendritic cells

DCs mature along both myeloid and lymphoid lineages in the bone marrow to become myeloid and plasmacytoid DCs, respectively. The myeloid DCs leave the bone marrow and travel with the bloodstream and enter tissues where they become immature DCs. Their main role in the immune system is to activate T-lymphocytes following pathogenic stimulation, rather than eliminating the microorganisms. Mature DCs display antigens from a pathogen to the antigen receptors on T lymphocytes along with other necessary signals to activate the T lymphocytes. Therefore, DCs are also APCs and form an important link between the innate- and the adaptive immune responses (1, 12).

APCs express major histocompatibility complex (MHC) class II molecules that deliver peptides, originating in the vesicular system, to the cell surface. MHC I molecules, which are expressed on all nucleated cells in humans, deliver peptides originating in the cytosol to the cell surface. DCs are the most potent APCs but macrophages and B cells can also act as APCs (1).

It has become clear in the past decades that DCs are important for the immune system and are important in studies of the immune system and its role in various diseases (13).

1.1.3.1 *In vitro* dendritic cell model

Three types of cells have been used to culture DCs *in vitro*, myeloid progenitor cells (CD34⁺ stem cells) isolated from bone marrow or umbilical cord blood, blood plasmacytoid cells or CD14⁺ monocytes from blood. To generate DCs from CD14⁺ monocytes, the monocytes are isolated from peripheral blood and incubated with granulocyte macrophage-colony stimulating factor (GM-CSF) and interleukin (IL)-4 to let them differentiated into immature DCs. The DC maturation is completed by stimulating the cells with tumor necrosis factor (TNF)- α and IL-1 β , with or without extra stimulation using, for example, LPS (14). DCs derived from CD14⁺ monocytes and CD34⁺ stem cells are morphologically, phenotypically and functionally similar, but differences can be seen in expression levels of some cell surface molecules, such as CD86 and HLA-DR. Both of these cell types are considered to be equally proficient for *in vitro* DC studies (15).

1.2 Cell signaling

Essential to the survival of every cell is to monitor the environment and to respond to external stimuli. The ability of cells to perceive and respond properly to their environment is the basis of development, tissue repair and immunity as well as normal tissue homeostasis. Failures in cell signaling are responsible for diseases, such as cancer and autoimmunity.

Cells use a large number of well-defined signaling pathways to manage their activity. These pathways fall into two main categories depending on how they are activated. Most of them are activated by external stimuli and lead to transfer of information from the cell surface to internal effector systems. The other group responds to information generated from within the cell, usually in the form of metabolic messengers.

During the process of development, specific cell types select out those signaling systems that are suitable to control their distinct functions (16).

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. By understanding cell signaling, diseases may be treated more effectively.

1.2.1 IFN- γ signaling

IFN- γ is a cytokine that is crucial for innate and adaptive immunity against viral and bacterial infections and tumor growth. It is mainly produced by NK cells and T cells (1).

When stimulated with IFN- γ , macrophages induce direct antimicrobial and antitumor mechanisms as well as up-regulate antigen processing pathways (17).

IFN- γ primarily signals through the JAK-STAT pathway. IFN- γ signals through the interferon- γ receptor (IFN- γ R), a heterodimeric receptor. When IFN- γ binds to the IFN- γ R, the JAKs transphosphorylate each other. Activated JAK then leads to phosphorylation of the transcription factor STAT1 in the cytosol, which enables STAT1 to translocate to the nucleus and cause transcription of inflammatory genes (18).

1.2.2 Toll-like receptors

TLR was first identified in *Drosophila* and found to play an important role in immune response against fungal infections (19). Later, a mammalian homologue of the *Drosophila* TLR was identified and shown to induce inflammatory gene expression (20). Today, 13 members of the mammalian TLR family have been identified. TLR1-10 are expressed in humans, but TLR11-13 are not (21). TLRs can either be extra- or intracellular, TLR3 and TLR7-9 are found inside the cell, in endosomal and lysosomal membranes, whereas TLR1-2, TLR5-6 and TLR10 are found in the plasma membrane (21-23).

TLRs are transmembrane signal receptor proteins that recognize various PAMPs. Each of them is devoted to recognizing a distinct set of molecular patterns that are not found in healthy cells. For

instance, TLR4 binds bacterial LPS, while TLR5 binds flagellin. DNA and RNA are ligands of the intracellular TLRs (23).

Signaling by TLRs in various cell types induces a diverse range of intracellular responses that result in production of inflammatory cytokines, chemotactic factors, antimicrobial peptides, and the antiviral cytokines IFN- α and IFN- β , the type I interferons. TLR signaling achieves this by being able to activate several different signaling pathways. Each of these pathways activates different transcription factors that bind to specific DNA sequences and thereby control the transcription of genetic information. Examples of these transcription factors are nuclear transcription factor (NF)- κ B, members of the interferon regulatory factor (IRF) transcription factor family and the activator protein 1 (AP-1) family. Members of the AP-1 family, such as c-jun go through yet another signaling pathway involving mitogen-activated protein kinases (MAPKs). NF- κ B and AP-1 act primarily to induce expression of pro-inflammatory cytokines and chemotactic factors, whereas IRF factors are important for inducing antiviral type I interferons (1). Dysfunction in TLR signaling has been linked to diseases, such as inflammatory disorders and cancer. These receptors and their downstream pathways, are therefore, of interest for understanding diseases and finding new drugs (24).

1.2.2.1 Toll-like receptor 4 signaling

As mentioned before, TLR4 binds bacterial LPS and induces innate immunity. LPS is a component of the outer membrane of Gram negative bacteria and stimulation with LPS has been extremely well defined. The composition of LPS can be slightly different between bacteria, but typically it consists of three parts; LipidA, core oligosaccharide and polysaccharide named O-antigen. LipidA is the part that is recognized by TLR4. To initiate response to LPS, TLR4 needs three accessory molecules; myeloid differentiation protein (MD)-2, LPS-binding protein (LBP) and CD14 (Figure 2).

LPS interacts with circulating LBP, which transfers it to CD14, a surface bound protein on monocytes, macrophages and DCs. CD14 then transfers the LPS to MD-2. Intracellular signal is initiated when MD-2 recognizes LPS. TLR4 then carries the signal inside the cell through Toll-interleukin-1 receptor (TIR), activating myeloid differentiation factor (MyD)88-dependent and MyD88-independent TLR4 signaling via different adaptor proteins (Figure 2).

The MyD88-dependent pathway signals through activation of I κ B kinase (IKK) and MAPK, also called MKK pathway (Figure 2). These pathways then activate NF- κ B and AP-1 and control the expression of pro-inflammatory cytokines and other immune related genes. In addition, phosphatidylinositol 3-kinase (PI3K) and AKT are also important factors downstream from MyD88 and mediate NF- κ B activation. The MyD88-independent pathway is mediated by the TIR domain-containing adaptor inducing interferon- β (TRIF), which activates IFR3 and induces expression of IFN- β and IFN-responsive genes (Figure 2) (25).

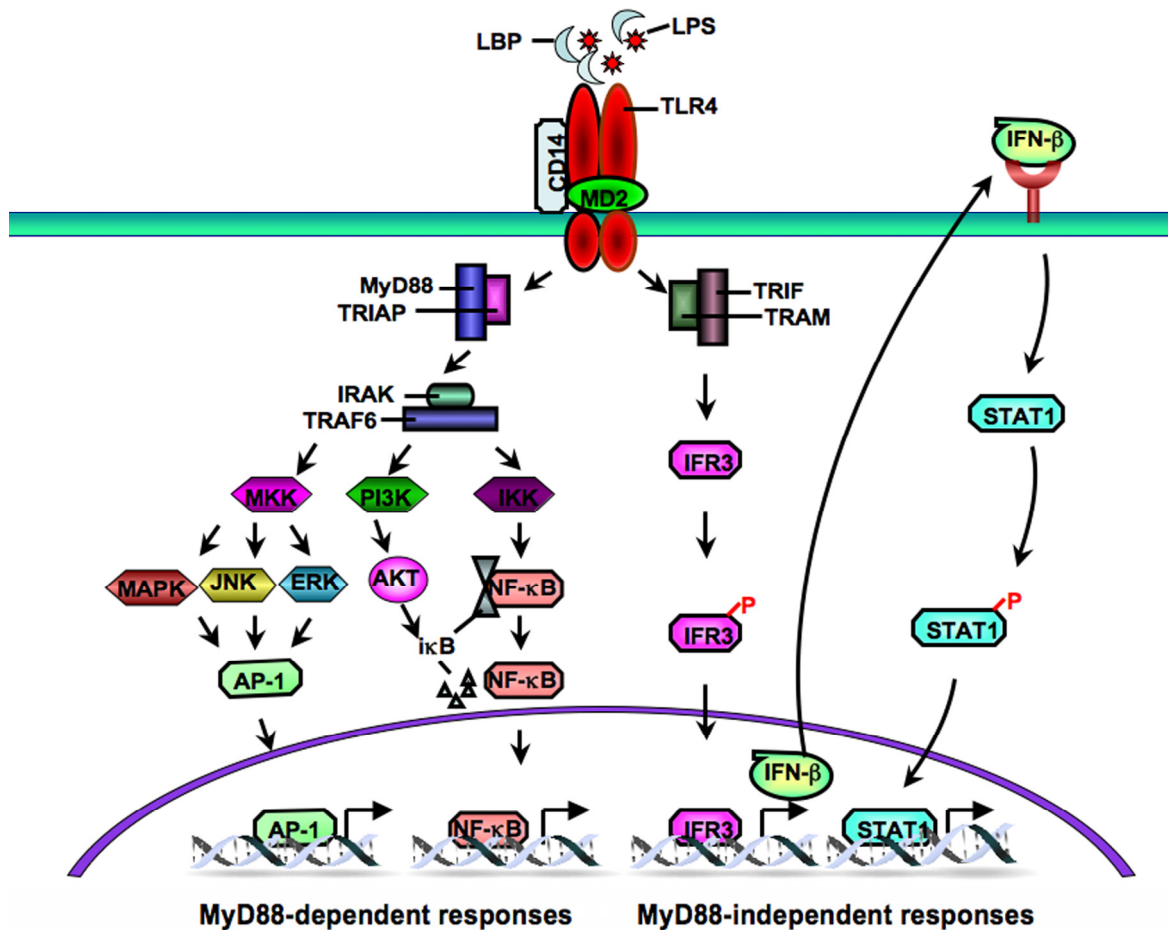


Figure 2. Overview of TLR4 signaling pathway.

When activated, TLR4 (red) carries the signal inside the cell and activates MyD88 dependent (left) and MyD88 independent (right) TLR4 signaling. The MyD88-dependent pathway signals through activation of MKK (pink), PI3K (green) and IKK (purple), which in turn leads to activation of NF-κB and AP-1, and regulates the expression of pro-inflammatory cytokines. The MyD88-independent pathway activates IFR3, which induces IFN-β expression. Figure from Guo and Friedman (25).

1.2.3 Mitogen activated protein kinase

The multifunctional MAPK is one of the most important and intensively studied signaling pathway. This signaling system consists of separate pathways that function to control a number of different cellular processes, such as gene transcription, metabolism, motility, cell proliferation and apoptosis. Dysfunction in MAPK regulation can cause various diseases, ranging from cancer to immunological, inflammatory and degenerative syndromes.

The MAPK pathway refers to a unit of three kinases, which together make up the MAPK cascade. These kinases are activated in response to a various stimuli, such as LPS, cytokines, growth factors, neurotransmitters, cellular stress and cell adherence by phosphorylating the next downstream kinase in the MAPK cascade. There are three kinase/phosphatase cycles consisting in the MAPK cascade; MAPK is activated via phosphorylation by MAPK kinase (MAPKK), which in turn is phosphorylated by MAPKKK kinase (MAPKKK). Once activated, MAPKs activate appropriate transcription factor, such as AP-1, via phosphorylation (Figure 3). Different downstream effectors are activated by the final MAPK components associated with the three main signaling pathways; extracellular-signal-regulated kinase (ERK) pathway, c-jun N-terminal kinase (JNK) pathway and p38 pathway (26, 27).

1.2.3.1 Extracellular signal regulated kinases

The ERK kinases are members of the MAPK family. The ERK kinases transmit signals from various extracellular agents to regulate a broad spectrum of cellular functions, such as proliferation, apoptosis, transcription, cytokinesis and differentiation. ERK1 and ERK2 are 84% identical and share many functions, thus they are normally referred as ERK1/2 (29, 30).

It is well known that LPS leads to activation of ERK1/2 in monocytes and macrophages (31). In response to stimuli, a member of the guanosine triphosphatase (GTPase) family, called Ras activates c-Raf, which is a MAPKKK, followed by phosphorylation of MEK, a MAPKK, which in turn activates the MAPK ERK1/2. ERK1/2 then causes phosphorylation and activation of c-fos, which is a part of the AP-1 transcription factor (32).

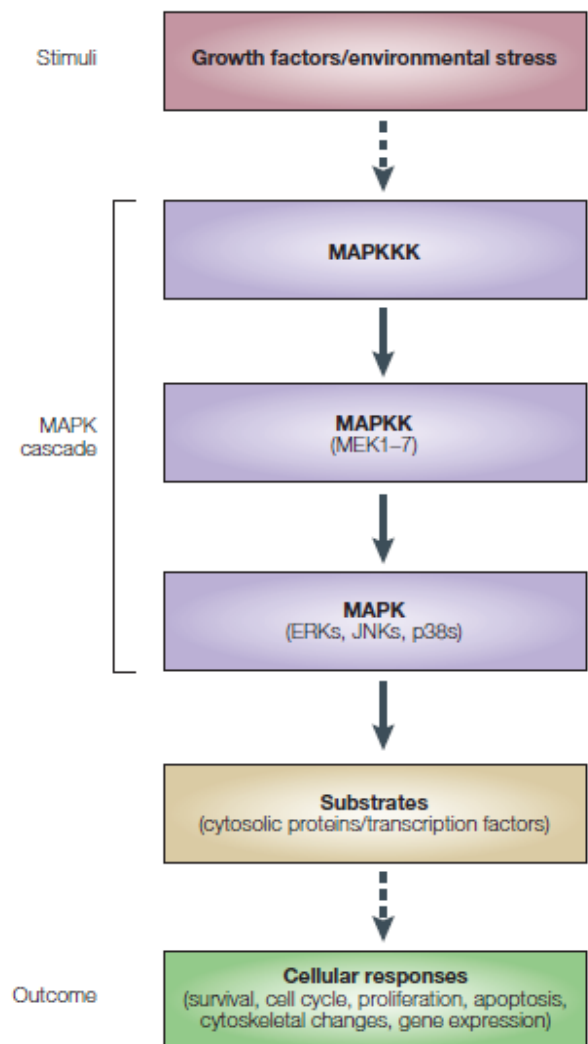


Figure 3. MAP kinase pathway.

The MAPK cascade consists of a series of three protein kinases – a MAPK and two upstream components, MAPKK and MAPKKK. Figure from Kumar *et al.* (28).

Dysregulation in ERK1/2 signaling are known for a wide range of pathologies, such as many types of cancers, diabetes, viral infection and cardiovascular disease (30).

1.2.3.2 p38

The p38 MAP kinases are a family of serine/threonine protein kinases that play a crucial role in cellular responses to external stress signals. p38 functions in the control of cell functions, such as apoptosis and the release of cytokines by macrophages and neutrophils (26).

It is activated by dual phosphorylation by upstream MAPKKs, such as MAP2K6, which is activated by several MAPKKKs. P38 kinase can phosphorylate many downstream kinases and transcription factors, including AP-1 (28).

1.2.4 PI3K / Akt pathway

Activation of the PI3K/Akt pathway is commonly detected after stimulation with TLR ligands. The resultant activation leads to phosphorylation of glycogen synthase kinase (GSK)-3 β , which is active in resting cells but is in an inactive state after phosphorylation. GSK-3 β then regulates various transcription factors, such as NF- κ B and AP-1, either negatively or positively (33).

Not much is known about the PI3K/Akt pathway compared with the other inflammatory signaling pathways. Some studies indicate that activation of PI3K results in anti-inflammatory events, such as inhibition of IL-12 and TNF- α expression (33-35), while other studies demonstrate pro-inflammatory effects, such as increase in NF- κ B phosphorylation and transcriptional activity (36, 37).

PI3K/Akt signaling pathway has been shown to be frequently disturbed in diseases, such as diabetes and many types of cancers (38, 39).

1.2.5 Nuclear transcription factor kappa-B

NF- κ B has often been termed a 'central mediator of the human immune response' because of the large variety of bacteria and viruses that activates its transcriptional activity (40). NF- κ B regulates inflammatory and immune responses as well as cellular growth by increasing expression of specific genes (41). These include genes encoding at least 27 different cytokines and chemokines, receptors involved in immune recognition, such as members of the MHC, and proteins involved in antigen presentation (40).

NF- κ B is activated by a large number of external stimuli such as the TNFs, IL-1 and the PAMPs, which are responsible for controlling processes such as inflammation, cell proliferation and apoptosis (42).

The IKK complex is the master kinase for NF- κ B activation. It contains two subunits, IKK α and IKK β . IKK phosphorylates the I κ B protein during inflammatory and immune responses. In the absence of specific extracellular signals, NF- κ B inhibitors, such as I κ B, tie NF- κ B down in the cytoplasm to prevent gene transcription mediated by NF- κ B. When the cells receive an appropriate stimuli, including TNF- α or PAMPs, the IKK complex induces I κ B phosphorylation, leading to required release

of NF- κ B that can translocate in to the nucleus where it binds to specific promoter elements to activate gene expression (43).

1.2.6 Monocytic co-stimulatory and adhesion molecules

Monocytes express various receptors and co-stimulatory molecules on their surface and the expression of these molecules increases when they are activated. The primary role of co-stimulatory and adhesion molecules is to participate in signaling to other cells of the immune system (1).

1.2.6.1 CD40

CD40 is a transmembrane receptor of the TNF gene superfamily. It is expressed on many cell types like APCs, endothelial cells and fibroblasts. Interactions between CD40 and CD40 ligand (CD40L) induce expression of cytokines, chemokines, growth factors, adhesion molecules and other molecules (1, 44).

In monocytes and macrophages, CD40 functions to increase their activation. It induces the production of pro-inflammatory cytokines and chemokines, such as IL-1 α / β , IL-6, IL-8, IL-10, IL-12, and TNF- α . Signaling through CD40 in monocytes and macrophages also results in up-regulation of other co-stimulatory molecules and nitric oxide (NO) generation (45).

1.2.6.2 CD80 and CD86

The surface molecules CD80 and CD86, also called B7-1 and B7-2 are expressed on APCs and are required for the induction of T cell responses. CD80 and CD86 function as co-stimulatory molecules. Upon binding of CD80 and CD86 to CD28 on T cells, the T cells become activated and express cytokines and surface molecules leading to their differentiation into effector T cells.

1.2.6.3 CD54

CD54, also known as intercellular adhesion molecule 1 (ICAM-1), is a cell adhesion molecule that plays an important role in the interaction between APCs and T cells. CD54 interacts with lymphocyte function associated antigen 1 (LFA-1) on the T cell surface to enable the binding between an APC and a naïve T cell, when the T cell is sampling antigens with MHC molecules (1, 46).

1.2.6.4 HLA-DR

The MHC gene family in humans is termed human leukocyte antigen (HLA) locus. HLA and related genes play many important roles in the immune system. HLA genes and proteins are associated with autoimmunity, immunity to infections, tumor development and more (47). HLA-DR are MHC class II proteins expressed on APCs and present peptides to CD4 T cells.

1.2.7 Cytokines

The development of an effective immune response involves lymphoid cells, inflammatory cells, and hematopoietic cells. The complex interactions among these cells are mediated by a group of proteins, collectively designated cytokines, which denote their role in cell-to-cell communication. Cytokines are

low-molecular weight regulatory proteins or glycoproteins secreted by white blood cells and various other cells in the body in response to a number of stimuli. These proteins assist in regulating the development of immune effector cells and some cytokines possess direct effector functions of their own (48). Cytokines can act in an autocrine manner, affecting the behavior of the cell that releases it, in a paracrine manner, affecting the behavior of adjacent cells and some cytokines are stable enough to act in an endocrine manner, affecting the behavior of distant cells (1).

Cytokines bind to specific receptors on the membrane of target cells, triggering signal transduction that ultimately alters gene expression in the target cells. The susceptibility of the target cell to a particular cytokine is determined by the presence of specific membrane receptors. Frequently, the cytokines and their receptors exhibit very high affinity for each other. Cytokines regulate the intensity and duration of the immune response by stimulating or inhibiting activation, proliferation, and/or differentiation of various cells and by regulating secretion of antibodies or other cytokines. Binding of a given cytokine to responsive target cells generally stimulates increased expression of cytokine receptors and secretion of other cytokines, which in turn affect other target cells. Thereby, the cytokines secreted by even a small number of lymphocytes activated by an antigen can influence the activity of numerous cells involved in the immune response.

Although a variety of cells can secrete cytokines, the two primary producers are T-helper (Th) cells and macrophages. Cytokines released from these cells activate an entire network of interacting cells. Among the numerous physiologic responses that require cytokine involvement are development of cellular and humoral immune responses, induction of the inflammatory response, control of cellular proliferation and differentiation and healing of wounds (48).

1.2.7.1 Interleukin-12

IL-12 is a pro-inflammatory cytokine produced in response to intracellular parasites, bacteria and bacterial products, such as LPS (1, 49). It plays an important role in the interaction between the innate and the adaptive immune systems by regulating inflammatory responses (50). IL-12 is composed of two covalently linked subunits, IL-12p35 and IL-12p40. These subunits are coded by different genes on separate chromosomes in humans, and therefore regulated independently (51).

IL-12 is produced mainly by monocytes, macrophages, DCs and B cells. The major function of IL-12 is induction of IFN- γ production, enhancement of cytotoxicity of NK and cytotoxic T cells, and differentiation of naïve T cells into Th1 effector cells (52).

As mentioned before, inflammation is essential for normal immune response against infections and IL-12 plays a crucial role in that fight. However, unregulated secretion of IL-12 has been linked to Th 1 mediated autoimmune diseases (51).

1.2.7.2 Interleukin-10

IL-10 is an anti-inflammatory cytokine that acts in an inhibitory manner to suppress immune responses and inflammation. IL-10 is a homodimer and is encoded by the *IL-10* gene. It is produced by activated immune cells, in particular monocytes, macrophages and T-cells. Moreover, B-cells, cytotoxic T cells, $\gamma\delta$ T cells, NK cells, mast cells, as well as neutrophilic and eosinophilic granulocytes synthesize IL-10.

Which of these cells are mainly responsible for the presence of IL-10 depends on the stimulus, type of affected tissue and time point in the immune process.

IL-10 affects the differentiation of DCs by inhibiting their secretion of IL-12 and thus impairing their ability to promote T-cell activation and Th1 differentiation. It suppresses T cell production of IL-2, TNF- α and IL-5 and inhibits APCs by reducing their expression of MHC molecules and co-stimulatory molecules (1).

Monocytes and macrophages secrete IL-10 after activation with various endogenous and exogenous mediators, such as bacterial LPS. These cells also secrete IL-10 during clearance of apoptotic cell (53).

Not all the effects of IL-10 are immunosuppressive; it can enhance B-cell survival and maturation into plasma cells and increase the activity of CD8 T cells. However, the dominant effects *in vivo* are immunosuppressive (1).

1.2.7.3 Interleukin-1 β

IL-1 is a critical mediator of the inflammatory response that plays an important role in the development of pathologic conditions leading to chronic inflammation. The IL-1 family comprises eleven members, of which IL-1 α and IL-1 β are the most studied ones. IL-1 β is a pro-inflammatory cytokine that is mainly secreted by monocytes and macrophages (54). IL-1 β has numerous biological effects, such as, activation of many inflammatory processes, induction of expression of acute-phase proteins, role in neuroimmune responses and more (55, 56).

1.2.7.4 Interleukin-6

The IL-6 family is composed of IL-6, leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), oncostatin M (OSM), IL-11 and cardiotrophin 1 (CT-1).

IL-6 is mostly regarded as a pro-inflammatory cytokine, although it has also been shown to have anti-inflammatory effects (57). IL-6 is produced by many different cell types, such as monocytes, macrophages, B cells and T cells. It plays a role in series of responses, such as immune responses, acute phase reaction and hematopoiesis.

IL-6 production is regulated by various stimuli, such as LPS, which enhances IL-6 production in monocytes and fibroblasts, whereas glucocorticoids inhibit its production. IL-6 stimulates target cells via a membrane bound IL-6 receptor. T cells, monocytes, macrophages, and neutrophils, all express the IL-6 receptor, and when this receptor is activated, it can have an array of different effects (57, 58).

1.2.7.5 Interleukin-8

IL-8 is a pro-inflammatory chemokine that belongs to the CXC chemokine subfamily. It is produced by various cell types, such as monocytes, lymphocytes, granulocytes, fibroblasts and endothelial cells. IL-8 has a chemotactic activity and plays an important role in acute inflammation by recruiting and activating neutrophils.

High levels of IL-8 have been detected in inflamed tissues and biofluids in many inflammatory diseases. LPS and inflammatory cytokines induce production of IL-8 by activating NF- κ B and AP-1

transcription factors. In contrast, IL-8 expression is inhibited by IL-4, IL-10, transforming growth factor (TGF) β , some interferons and immunosuppressive drugs (59).

1.2.7.6 Interleukin -23

IL-23 was identified only recently and is a heterodimeric pro-inflammatory cytokine secreted by activated macrophages and DCs (60, 61). It is comprised of two subunits, IL-23p19, an IL-12p35 related polypeptide, and IL-12p40, which it shares with IL-12 and belongs to the IL-6/IL-12 family. Both the p35 and the p19 subunits have sequence homology to IL-6 and G-CSF, making them members of the gp130-class of long chain cytokines (62). IL-23 is an important part of the inflammatory response against extracellular infections, mainly by inducing proliferation and IL-17 production by Th17 cells (63).

1.2.7.7 Interleukin-27

IL-27 is a recently identified cytokine, bridging innate and adaptive immunity (64). It is a heterodimeric cytokine composed of a p28 subunit, an IL-12p35-related polypeptide, and Epstein-Barr virus-induced gene 3 (EB13) subunit, an IL-12p40-related protein (65). IL-27 can both promote anti- and pro-inflammatory immune responses (66) and plays a role in initiation of T cell responses. Recent studies suggest that IL-27 also plays a key role in the regulation of monocyte cell function. The IL-27 receptor chains are expressed by primary human monocytes and they respond to IL-27 by inducing STAT1 and STAT3 phosphorylation (64). In addition, IL-27 can affect macrophage activation in a similar manner as IL-10 and IL-4 by inhibiting IL-12p40 expression (67).

1.2.7.8 Tumor necrosis factor alpha

TNF- α is a pro-inflammatory cytokine secreted mostly by monocytes, macrophages and T cells in response to various inflammatory stimuli, such as bacterial and viral pathogens. TNF- α is involved in many different cellular responses, such as inflammation, cell growth and differentiation (51). The pro-inflammatory functions of TNF- α include up-regulation of endothelial cells adhesion molecule expression, stimulation of cytokine expression, activation of neutrophils, antiviral effects and more (68). Although TNF- α is required for protection against bacterial infection, it is also involved in autoimmune diseases, rheumatoid arthritis, chronic inflammation and diabetes. Agents that block TNF- α action, such as thalidomide, soluble TNF receptors and anti-TNF antibodies, have been approved for diseases like rheumatoid arthritis and inflammatory bowel disease (69).

1.3 Natural products

Various natural compounds have been used for centuries to cure human diseases and to promote health. Excluding the last few decades, all there was available were crude extracts from plants and other organisms. Some of the oldest documentation of such use dates back 4-5000 years to Egyptians and the people of Mesopotamia (70).

Despite the enormous opportunities in medical chemistry these days, natural products have been a valuable source of many active substances of Western medicine. A large numbers of critical drugs in use today are of natural origin (71, 72). Indeed, it is estimated that more than 40% of all pharmacologic agents in use today are either natural products or their semi-synthetic derivatives (Figure 4) (73-76), thereof about 60-70% of antibacterial and anticancer drugs are derived from nature (77). The first active compound from a medicinal plant was not isolated until 200 years ago, when Friedrich Sertümer isolated morphine from opium and subsequently marked a new era in history of pharmacology (78).

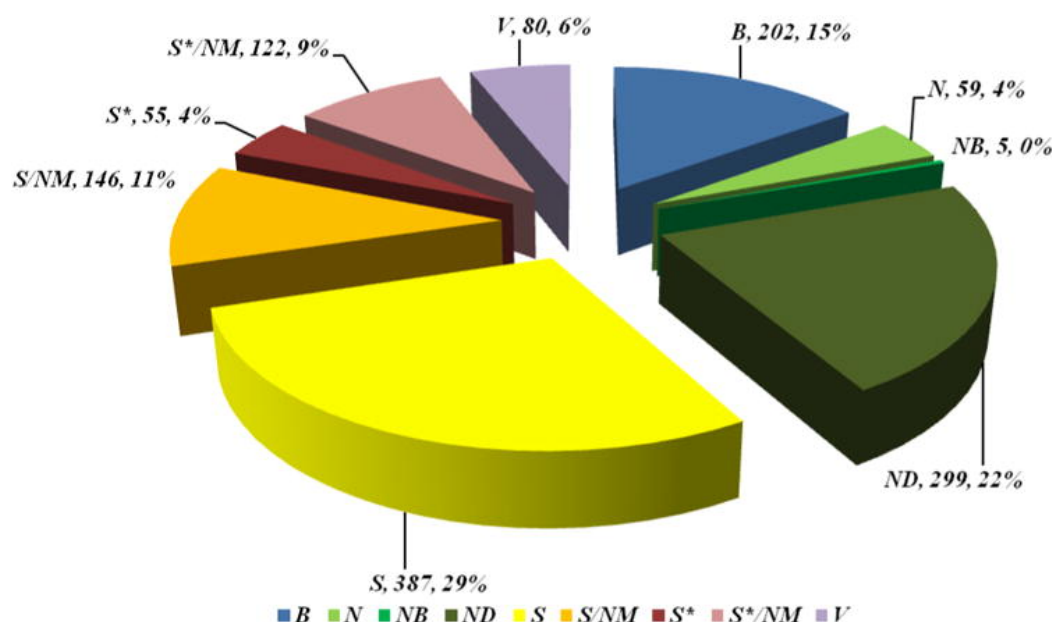


Figure 4. Natural products are the lead source of drugs over the past 30 years.

Source of small molecule drugs, 1981–2010, major categories, $N = 1355$ (in percentages). Major categories are as follows: “B”, biological, usually a large peptide or protein isolated from an organism/cell line or produced by biotechnological means in surrogate host; “N”, natural product; “NB”, natural product „Botanical“; “ND”, derived from a natural product and usually a semisynthetic modification; “S”, totally synthetic drug often found by random screening/modification of an existing agent; “S*”, made by total synthesis, but the pharmacophore is/was from a natural product. Figure from Newman and Gragg (76).

Natural products can be prepared from plants, algae, fungi, bacteria or animals by different methods and used to for various purposes. Methods of extraction can be different and yield different products from the same source. The extracts can then be purified by various methods to yield a mixture of only a few similar components or a pure compound (79). Historically, the most important natural sources for drugs have been plants but more recently there has been a trend towards exploration of marine organisms as a source of drug candidates (72).

The main focus in screening for new drugs in plants and other organisms has been on secondary metabolites. Secondary metabolites are chemicals that are not required for the immediate survival of the organisms. These chemicals often function to protect the organism from microorganisms and pests, such as parasites, rather than being directly involved in normal growth and development like primary metabolites (80, 81). Plants produce over 100,000 low molecular-mass bioactive secondary metabolites and the ability of these compounds to improve human health and cure symptoms seems to be extraordinary (82).

The macromolecules in natural products are not less interesting than the metabolites. Many macromolecules from plants, such as proteins and polysaccharides, have been shown to have immunomodulatory effects (83, 84). For example, pectin, the major component of plant cell walls, has been shown to affect cytokine production *in vitro* (85). Therefore, screening for bioactive compounds in plants and organisms is an interesting subject.

1.3.1 Medicinal plants of Iceland

Medicinal herbs have been used in Iceland since the country's settlement in 874 AD. Old Icelandic manuscripts mention various herbs and many Icelandic places are named after herbs. This fact suggest that various herbs were abundant at the time, but also that they were highly thought of. For example, the highest peak of Iceland, Hvannadalshnjúkur, is named after Angelica, or Hvönn, in the Icelandic language.

Screening and testing of Icelandic plants and herbs for immunological effects has only recently been started and have been rising in the past years. Polysaccharides have been isolated from lichens and they have been shown to have immunostimulating (86, 87) and immunosuppressing activity (88-90). Research from ethanol extract from birch bark also suggests immunomodulating activity, which could be used to treat autoimmune diseases (91). Furthermore, results from experiments on *Angelica archangelica* leaf extract demonstrate anti-proliferative and anti-tumor activity, both *in vitro* and *in vivo* (92, 93).

Adaption to Iceland's unique conditions may have encouraged plants and other organisms to develop biological compounds with different properties, as organisms evolve to survive under diverse conditions. Yet, there are a number of unexplored Icelandic plants, which will be an exciting subject to follow in the future.

1.3.2 Natural products and the immune system

As mentioned, the immune system represents our ability to fight against infections and diseases. Dysfunctional immune system increases risks of opportunistic infection and uncontrolled tumor growth, while a hyperactive immune system results in afflictions, such as persistent inflammation, allergy and autoimmunity. However, allopathic medicine has provided limited tools for treatment, especially for prevention.

An unbalanced immune system hampers good health and quality of life. Hence, the immune system is a good target to select in the fight against inflammation and thereby prevent chronic diseases (94). Many compounds, isolated from natural products, have been shown to have immunoregulatory, anti-inflammatory and other biomedically effects (95-99).

1.3.3 Polysaccharides

Polysaccharides are long carbohydrate molecules of repeated mono- or disaccharide units, joined together by glycosidic bonds. Polysaccharides can either be homoglycans or heteroglycans. Homoglycans contain only one type of monosaccharide, but heteroglycans more than one type (100). They can be classified by their origin: vegetal origin, such as pectin, algal origin, such as alginate, microbial origin, such as dextran, and animal origin, such as chitosan (101).

Polysaccharides represent the most abundant biomolecule group in nature with important role in a wide range of processes in living systems (102). Polysaccharides are essential to every single organism and are involved in various vital activities. They have been found in marine organisms, fungi and plants. Polysaccharides can be complicated in their structure because of a number of conjunctives between different types of monosaccharides. Their shape depends on the ordering of the monosaccharides and their molecular weight. In plants, the storage polysaccharide is starch and the structural polysaccharide is cellulose (103).

Many natural polysaccharides from plants have recently gained much attention, especially for their physical properties, chemical and physical modification and their utility (104). They have also attracted a great deal of attention in the biomedical arena because of their broad spectrum of therapeutic properties and relatively low toxicity (105). Polysaccharides from plants have, for example, been found to have immunomodulating activities and also anti-tumour and anti-viral activities, both *in vitro* as well as *in vivo* (90, 103, 106-108). The knowledge of the mechanism by which polysaccharides have their effects is still quite limited. It appears that one of the main mechanism involves nonspecific induction of the immune system and is thought to occur via macrophage stimulation and modulation of the complement system (105, 109, 110). Certain polysaccharides, such as β -glucan, can affect the regulation of immune responses during the progression of infectious diseases via interactions with T cells, monocytes, macrophages and polymorphonuclear cells (111, 112).

1.3.4 Yarrow, *Achillea millefolium*

Yarrow, or vallhumall in Icelandic, is a flowering plant that belongs to the family of *Asteraceae*. Yarrow is perennial and grows mostly in dry fields and sands (113). The plant grows wild all around Europe, Asia, North Africa and North America (114). Yarrow is often considered to be a weed, but is well known as a medicinal herb.

The plant has gone by many names throughout the centuries but its Latin name, *Achillea millefolium* is derived from the Trojan War hero, Achilles. He is said to have used Yarrow's medicinal powers to stop his soldiers from bleeding to death. Many other common names of the plant allude to its ability to stop bleeding and healing wounds, such as Soldier's woundwort, Knight's milfoil, Bloodwort and Nose-bleed (115).

Yarrow has been used in folk medicine for centuries widely around the world, including Iceland. It is considered being good herbal remedy, used to treat various symptoms. It is believed to be restorative and softening and has been used in teas, broths and lotions. The broth is thought to have a positive effect on cold, high blood pressure, gastrointestinal disorders and rheumatoid arthritis. The lotion from the Yarrow's leaves is thought to be very healing and softening and has been used as hand cream and for skin rashes, inflammation and wounds (113, 116, 117).

Yarrow has been shown to have immunomodulatory effects *in vitro* (118, 119). Anti-inflammatory effects of a water extract from Yarrow on DCs were described in a recent publication from the laboratory of my advisors (120).

However, more studies are needed to examine effects on the immune system and diseases and how these effects are regulated.

1.3.4.1 Yarrow's active compounds

More than 120 chemical compounds have been identified in Yarrow. The major phenolic compounds present in Yarrow are the flavonoids, apigenin and quercetin, and the phenolic acid, caffeoylquinic acid (121). Antioxidant molecules, such as tocopherols and ascorbic acid, have also been identified in the plant and found to be present in considerable amounts (122).

Chemical composition of wild Yarrow samples has been identified. Carbohydrates, followed by proteins are the major macronutrients in Yarrow. Table 1 shows the composition of macronutrients, free sugars and organic acids in the plant.



Figure 5. Yarrow, *Achillea millefolium*.
Photograph: Guðrún
Tryggvadóttir ©Náttúran.is

Table 1. Macronutrients, free sugars and organic acids in wild *Achillea millefolium* L. Table adjusted from Dias *et.al* (123).

Nutrients	Wild sample
Fat (g/100 g dw)	5.20
Proteins (g/100 g dw)	12.53
Ash (g/100 g dw)	6.43
Carbohydrates (g/100 g dw)	75.84
Energy (kcal/100 g dw)	400.28
Fructose	1.11
Glucose	0.66
Sucrose	0.80
Trehalose	0.42
Raffinose	0.15
Total sugars (g/100 g dw)	3.14
Oxalic acid	1.08
Quinic acid	0.69
Malic acid	1.64
Shikimic acid	0.02
Citric acid	0.83
Succinic acid	0.27
Fumaric acid	0.03
Total organic acids (g/100 g dw)	4.55
<i>dw = dry weight</i>	

2 Aim

Yarrow has been used for centuries to treat human diseases. This project is a continuation of Guðbjörg Jónsdóttir's master project, where anti-inflammatory effects of water extract from Yarrow on DCs were described (120). The aim of this study was to isolate and determine the effects of polysaccharides from Yarrow on immune responses in THP-1 monocytes:

Specific aims:

- a) To isolate polysaccharides from Yarrow.
- b) To determine the effects of polysaccharides from Yarrow on cytokines secretion by THP-1 monocytes.
- c) To investigate how polysaccharides from Yarrow affect intracellular pathways in THP-1 monocytes.

3 Material and methods

3.1 Isolation of the polysaccharide

Bioguided fractionation was used to isolate a polysaccharide from *Achillea millefolium*. Between all steps in the isolation process, the fractions were tested in cell culture and cytokine secretion determined (Figure 7).

3.1.1 Plant

The Yarrow was collected at Hvanneyri in early July 2008. The plants were allowed to dry at room temperature (RT) and were stored in a dry, dark place until used (120).

3.1.2 Preparation of water extract

Water extracts were prepared by grinding the whole, dried plant and shaking 300 g of the material in 4.5 L of 85° distilled water for two hours, followed by filtration. The filtered refuse was dried and not used in this project. The supernatant was freeze-dried and called water extract.

All Yarrow samples were dissolved in RPMI 1640 medium at RT an hour before they were used in experiments.

3.1.3 Dialysis

Between all steps of the fractionation, the extracts, fractions and polysaccharides were dialyzed (Figure 6). Dialysis was performed using Spectra/Por® membrane, which allows compounds with molecular weight less than 6-8 kDa to pass through it. Most polysaccharides have molecular weight between 15-2000 kDa and therefore do not pass through the membrane.

Dialysis membranes containing the extract, fraction or polysaccharide(s) were added to a container of distilled water and kept there for four days at RT. Magnetic stirrer was used to stir and the water was changed each day.

The dialyzed water extract was freeze-dried and dissolved in medium before being added to the cells.

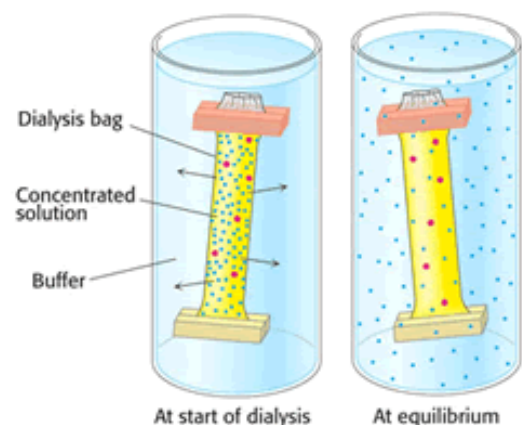


Figure 6. Dialysis

Separation based upon molecular size. Figure from Berg *et al.* (124).

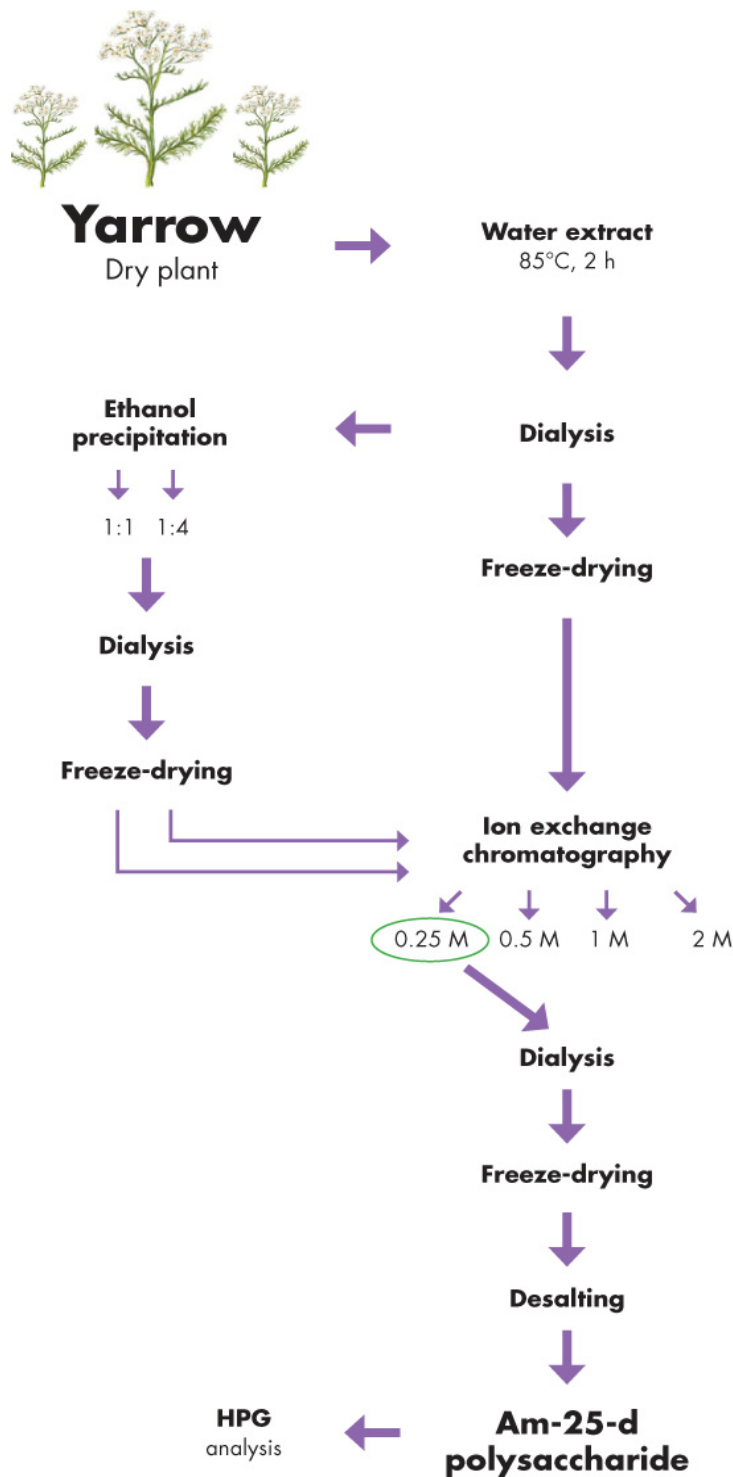


Figure 7. Overview of the isolation process of polysaccharide(s) from Yarrow

Dry and milled yarrow was swirled in 85°C distilled water for 2 h, then filtered and the supernatant called water extract. This extract was then dialyzed and added to ion exchange column, followed by another dialysis, resulting in four fractions. 0.25 M polysaccharide was then desalted and used for further experiments. Ethanol precipitation was also performed on the dialyzed extract in a separate run, resulting in two fractions, which were then dialyzed, lyophilized, re-dissolved in higher concentration and added to ion exchange column, followed by dialysis and lyophilization. Between every step in the process, the extracts/fractions/polysaccharides were tested in cell culture. Authors image.

3.1.4 Ethanol precipitation

Ethanol precipitation is used to separate polysaccharides by their solubility. First dried, dialyzed water extract was dissolved in distilled water. Ethanol was then added in a 1:1 ratio and the mixture incubated overnight at 2-4°C. The precipitated polysaccharides were then collected and freeze-dried. For further precipitation, ethanol was added to the supernatant in the ratio of 1:4 and the mixture incubated again overnight at 2-4°C. The solution was then centrifuged and the precipitated polysaccharides collected and freeze-dried.

3.1.5 Ion exchange chromatography

Ion exchange chromatography is a process that allows the separation of molecules or groups of molecules that have only a slight difference in charge. Separation is based on the reversible interaction between a charged molecule and a proper charged medium (125). Ion exchange chromatography can either be cationic or anionic. Anionic exchange chromatography was used in this project (Figure 8).

The column (DEAE sepharose fast flow; 2.6x40 cm) was packed with Sepharose Fast Flow positively charged ion exchange media (GE-Healthcare). The flow rate was set to 15 ml/min according to the GE Healthcare instructions (126). When packing was successful, the column was equilibrated by pumping 750 mL of start buffer (Milli-Q water) at a flow rate of 8 ml/min. Both packing and sample runs were conducted at 4°C.

First the sample containing 300 mg of dialyzed and freeze-dried Yarrow water extract dissolved in 50 ml of distilled water was added to the column. Water, the first mobile phase, was passed through the column to wash off uncharged molecules. Then 0.25 M, 0.5 M, 1 M and 2 M sodium chloride (NaCl) buffer was passed through the column to release molecules with different charge from the column. Different concentration of the NaCl mobile phase will flush off polysaccharides with different pH value, i.e. highest concentration will flush off the most acidic polysaccharides. The flow rate for the mobile phases was 60 mL/hour. Fraction collector was used to collect the samples from the column. In the end, 1 L of water was passed through the column and then 20% ethanol solution for the gel for storage. Fractions from the column were dialyzed and freeze-dried. This resulted in four fractions, including the Am-25 polysaccharide (obtained from the 0.25 M NaCl elution).

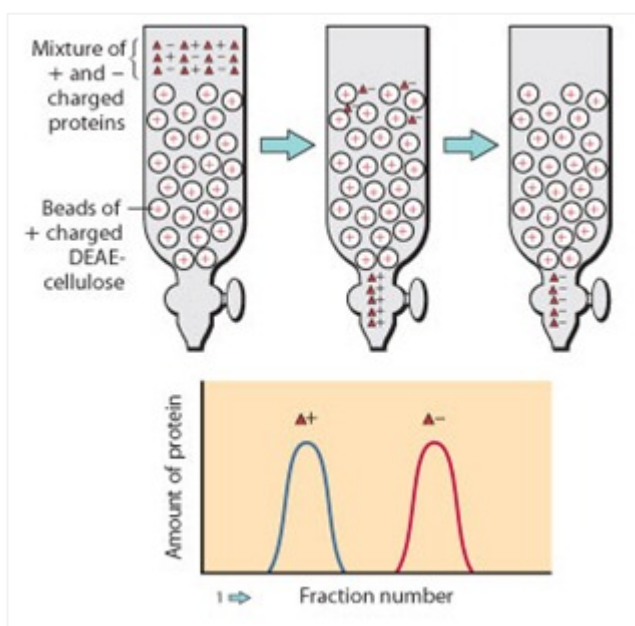


Figure 8. Anion exchange chromatography.

Anion exchangers have positively charged groups that attract negatively charged anions. ©1999 John Wiley & Sons

3.1.6 Desalting

Small PD-10 desalting column (GE Healthcare), was used to desalinate the fractions from the ion exchange chromatography. The column contains Sephadex G-25 Medium, which allows rapid group separation of high molecular weight substances from low molecular weight substances. The largest molecules are excluded from the matrix and are eluted first. Smaller molecules (<5 kDa) are eluted later.

The column was used according to the manufacturer's protocol. The sample containing 10 mg of Am-25 polysaccharide (0.25 M fraction from the ion exchange column) was dissolved in 2.5 mL of distilled water and added to the PD-10 column. Then water was added in 3.5 mL portions. Overall, 12 mL were collected from the column into 3 tubes. The first 2 mL of the flow-through were discarded. The next 4 mL contained the polysaccharide(s) and the last 6 mL of the flow-through were smaller compounds, such as NaCl. The samples containing the polysaccharide(s) were then freeze-dried.

3.1.7 High-performance gel permeation chromatography

High-performance gel permeation chromatography (HP-GPC) is a chromatographic method that separates components on the basis of molecular weight.

A SUPREMA Linear XL gel column (Polymer Standard Service) was used for the HP-GPC. First 200 mL of water (Milli-Q) were used to rinse the column and then a buffer containing 10 mM NaCl was used as mobile phase to run the samples. The samples were loaded on the column which was connected to RI detector. The flow rate was 0.5 mL/min, the sensitivity of the detector was $4-8 \times 10^{-5}$ RIU/F.S and paper rate was 2 mm/min. Afterwards, water was used to remove the rest of the mobile phase and then 20% ethanol was used to store the column. Dextran standards T-10, T-40, T-70, T-500 and T-2000 (Amersham Biosciences GE Healthcare) at concentration of 10 mg/mL and glucose at 2.5 mg/mL were used to generate a standard curve and the molecular mass of the polysaccharides derived from the standard curve.

Standard curve were plotted according to the molecular mass of the standards with log MW on the x-axis and the distribute index, K_{av} on the y-axis. The following equation was used to calculate the K_{av} :

$$K_{av} = (V_e - V_0) / (V_t - V_0)$$

V_t = the value of the standard that remain longest on the column, in this case glucose.

V_0 = the value of the standard that remains shortest on the column, in this case Dextran T-2000.

V_e = the value for indicated standard or sample.

3.2 Determination of the immunomodulatory effects of the polysaccharide

3.2.1 Cell culture

3.2.1.1 THP-1 cells

The THP-1 cells were obtained from The German Collection of Microorganisms and Cell cultures, Braunschweig, Germany (DSMZ). The cells were used as undifferentiated, activated monocytes. RPMI 1640 medium with 2,05mM L-glutamine, supplemented with 10% heat inactivated fetal calf serum (FCS), penicillin (100 U/mL) and streptomycin (100 µg/mL) (all from Gibco, Invitrogen) was used to maintain and culturing the cells. The cell line was maintained in 75 cm² EasYFlasks™ plastic cell culture flasks (Nunc). The cells were divided twice a week by centrifugation of the cell suspension at 300x g for 10 min and re-suspension in fresh medium. The cells were counted using Trypan blue staining and a Countess® cell counter (Invitrogen) and their concentration set to 2.7-2.8 x 10⁵ cells/mL before each passage. Each cell batch was passaged 15 to 20 times maximum.

The cells were primed with 100 U/mL IFN γ (R&D Systems) for 3 h and stimulated with 0.75 µg/mL of bacterial LPS (Sigma-Aldrich). The concentrations and times used for stimulating the cells were determined following generation of time and concentration curves by previous students in the lab. Yarrow-samples were added at a concentration indicated for each experiment simultaneously with the LPS. The cells were then cultured at 37°C, 5% CO₂ and 95% humidity for the time given for each experiment.

For determining the viability of the cells, the cells at density of 5 x 10⁵ cells/mL, 1 mL per well in 48 well plates, were stimulated with LPS in the absence or presence of the polysaccharide, Am-25-d (100 µg/mL). After incubation, the cells were counted as before and the percentage of life cells calculated.

3.2.1.2 Dendritic cells

DCs were generated from human blood monocytes collected from healthy volunteers from buffy coat obtained from the Icelandic blood bank (ethical approval number 06-068). Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coat from three donors using Ficoll-Paque PLUS medium, which separates cells according to density. The PBMCs were then washed three times with magnetic activated cell sorting (MACS) buffer (PBS with 0.5% bovine serum albumin (BSA) (Millipore, USA) and 2 mM EDTA). The cells were counted using trypan blue and Neubauer chamber.

CD14⁺ positive monocytes were isolated by adding microbeads-conjugated antibodies against CD14 to the PBMCs, which were then run through LS⁺/VS⁺ MACS column placed in a high magnetic field. Unstained cells were washed through, thereafter the column was removed from the magnetic field and the CD14⁺ monocytes released. Then the cells were counted and diluted in fresh RPMI 1640 medium in the concentration of 0.5 x 10⁶ cells/mL and seeded in 48 well plate, 1 mL each well. The monocytes were then stimulated with IL-4 (R&D Systems) and GM-CSF (R&D Systems) that stimulate monocyte differentiation into immature DCs (imDCs). The cells were incubated at 37°C, in 5% CO₂ and 95% humidity for 7 days.

At day 7 the imDCs were collected and counted as before. The imDCs were diluted in fresh RPMI 1640 medium to a final concentration of 2.5×10^5 cells/mL and seeded in 48 well plate, 0.5 mL in each well. IL-1 β (R&D Systems) and TNF- α (R&D Systems) and LPS (Sigma-Aldrich) were added to each well to stimulate the imDCs. The Yarrow samples at concentrations indicated for each experiment were added at the same time, and the cells cultured for 48 h.

On day 9, the imDCs had matured into DCs and were harvested. The cells were centrifuged and the supernatants collected and stored at -80°C for determination of cytokine concentration by ELISA.

3.2.2 Assays

3.2.2.1 Cytokine determination

THP-1 cells were seeded at a density of 5.0×10^5 cells/mL in RPMI 1640 medium, 1 mL per well in 48 well plates. The cells were always pre-treated with 100 U/ml of IFN- γ for three hours. Then Yarrow samples at various concentrations were added simultaneously with LPS at 0.75 μ g/mL to the wells and the cells cultured for 48 hours. The cells were then collected, centrifuged (at 300x g for 10 min) and supernatants collected and stored at -80°C until used to determine the concentration of various cytokines.

3.2.2.1.1 ELISA

DuoSet® sandwich ELISA (R&D Systems) was used to measure concentration of the cytokines IL-1 β , IL-6, IL-8, IL-10 and IL-12p40, IL-23, IL-27 and TNF- α in the supernatants from the THP-1 cells according to the manufacturer's protocol. Maxisorp plates (Nunc) were coated with cytokine-specific capture antibody dissolved in PBS and incubated overnight at RT. The following day the solution was removed and the blocking solution (1% BSA, 5% sucrose, 0.05% NaN₃ in PBS) was added to block unbound binding sites on the plates for one hour at RT. The plates were then washed four times with wash buffer (PBS, 0.05% tween-20) and standards and supernatants added. Sample dilution was different for each cytokine tested (Table 2), but ELISA buffer (1% BSA in PBS) was used for all dilutions. The plates were then incubated for two hours at RT followed by another wash. Next the detection antibody, diluted in ELISA buffer, was added and the plates incubated for two hours at RT. For IL-12p40 measurements, 2% normal goat serum was added to the ELISA buffer. The plates were then washed again four times and incubated with Streptavidin-horseradish peroxidase (HRP) for 20 minutes in a dark place at RT. The plates were washed again and substrate solution (TMB from Kem-En-Tec) added. When the color had developed, the reaction was stopped by adding 0.18 M sulphuric acid (H₂SO₄) to each well. The absorbance was measured at 450 nm using a micro-plate spectrophotometer.

Each sample was measured in duplicate and the average concentration calculated. The concentration of the cytokine in the supernatant samples was calculated from a standard curve and the results expressed as pg/mL or secretion index (SI) where the cytokine concentration in supernatants from THP-1 cells cultured in the presence Yarrow samples was divided by cytokine concentration in supernatant by THP-1 cells cultured without Yarrow samples. The ratio of the SI for

IL-12p40 and IL-10 was calculated to determine whether the overall effect of the Yarrow samples on cytokine secretion were pro- or anti-inflammatory.

Table 2. DuoSet® ELISAs

Duonet	Cat#	Dilution	Supplier
Human IL-1 β /IL1F2	DY201	1:2	R&D systems
Human IL-6	DY206	1:5	R&D systems
Human CXCL8/IL-8	DY208	1:10	R&D systems
Human IL-10	DY217B	None	R&D systems
Human IL-12p40	DY1240	1:2	R&D systems
Human IL-23	DY1290	None	R&D systems
Human IL-27	DY2526	None	R&D systems
Human TNF- α	DY210	1:5	R&D systems

3.2.2.2 Expression of surface molecules

Expression of TLR4, CD14, CD40, CD54, CD80, CD86 and HLA-DR was determined using flow cytometry.

THP-1 cells were primed with 100 U/mL IFN- γ for 3 hours and then stimulated with 0.75 μ g/mL LPS for 24 hours. Am-25-d at the concentration 100 μ g/mL was added along with the LPS.

Cells were collected and centrifuged at 300 g for 10 min, at 4°C. The supernatants were discarded and the cells resuspend in 450 μ L of staining buffer (PBS with 0.5% BSA, 2 mM EDTA and 0.1% NaN₃). The cells were divided into 5 FACS tubes and 2 μ L of blocking solution (1:1 normal mouse serum and normal rat serum) added to each tube and incubated for 10 min on ice. Next 5 μ L of fluorescence-labeled monoclonal antibodies were added and appropriate isotype-specific antibodies were used as controls (Table 3). Cells were incubated with the antibodies for 20 min, on ice and in the dark and then washed with 2 mL staining buffer and centrifuged at 1200 rpm for 5 min at 4°C. Then the cell pellets were resuspended and fixed with 300 μ L of 1% paraformaldehyde in PBS. Cells were collected using FACScan (BD Bioscience) and the results were expressed as percentage positive cells and mean fluorescence intensity (MFI) of positive cells.

Table 3. Fluorescence-labeled monoclonal antibodies used in flow cytometry

Antibody	Cat#	Isotype	Conjugate	Dilution	Supplier
HLA-DR	L243	Mouse IgG2a	PE	none	eBioscience
TLR4	12-9912-41	Mouse IgG2a	PE	1:2	eBioscience
CD14	Mca596	Mouse IgG2a	APC	1:10	AbD Serotec
CD40	Mca1590	Mouse IgG2a	PE	none	AbD Serotec
CD54	Mca1615	Mouse IgG1	FITC	1/10	AbD Serotec
CD80	Mca2071	Mouse IgG1	FITC	1/10	AbD Serotec
CD86	Mca1118	Mouse IgG1	FITC	none	AbD Serotec
FITC isotype control	Mca928	Mouse IgG1	FITC	1/10	AbD Serotec
PE isotype control	Mca929	Mouse IgG2a	PE	1/5	AbD Serotec
APC isotype control	Mca929	Mouse IgG2a	APC	1/10	AbD Serotec

3.2.2.3 Intracellular pathway examination

For Western blot analysis the cells were seeded in 6 well plates at a density of 1×10^6 cells/mL, 2 mL per well. The cells were primed with 100U/ml IFN- γ for 3 hours and then stimulated with 0.75 μ g/mL LPS for 1 h. Yarrow samples were added along with the LPS. After 1 h incubation, cells were collected and lysed to generate whole cell lysis or the cytoplasmic and nuclear fractions were separated as described below.

3.2.2.3.1 Whole cell lysis

Whole cell lysates of treated cells were prepared to determine phosphorylation of Akt, MAP kinases (ERK 1/2, p38) and the NF- κ B inhibitor I κ B α .

Cells were collected into pre-cooled Eppendorf tubes and centrifuged at 450x g for 5 min at RT and the supernatants discarded. The cells were washed with 1 mL ice cold PBS and centrifuged again. Then the PBS was carefully pipetted off and the cell pellets lysed in 150 μ L Western Lysis Buffer (25 mL 1M Tris-HCl pH 7.4, 15 mL 5M NaCl, 5 mL NP-40, 334 mL dH₂O) with protease (Mini-Tab, Roche) and phosphatase (PhosStop, Roche) inhibitors added. Samples were then sonicated on ice in Bioruptor® sonicator (Diagenode) for 6 min (30 sec sonication, 30 sec off). The lysed cells were put on ice for 25 min and then centrifuged at 1400 rpm for 5 min at 4°C. Whole cell lysate supernatant was collected and stored at -20°C until used.

3.2.2.3.2 Nuclear extraction

Nuclear lysates were prepared to determine levels of expression and phosphorylation of the NF- κ B transcription factor.

Nuclear Extract Kit from Active Motif was used according to the manufacturer's instructions. Cells were collected into pre-cooled 15 mL tubes and centrifuged at 500 rpm for 5 min at 4°C. The cells were then washed with PBS containing protease inhibitor and centrifuged again. Then the cell pellets were gently re-suspended in Hypotonic buffer and transferred to a 1.5 mL Eppendorf tube and

incubated on ice for 15 min. Next, detergent was added to the samples and they vortexed for 10 sec and then centrifuged at 16,000 rpm for 1 min at 4°C. Then the supernatants were transferred to another 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 followed by incubation for 30 min on ice and rocking platform at the highest speed. Finally, the samples were vortexed for 30 sec and centrifuged at 16,000 rpm for 10 min at 4°C and the supernatants transferred to a 500 µL microcentrifuge tube. The samples were stored at -80°C until used in Western blot.

3.2.2.3.3 Western blot

Whole cell lysis or nuclear lysis samples were prepared by adding equal amount of lysate and 2x sample buffer (Table 4) into a PCR tube and all stored on ice. The samples were heated at 95°C for 5 min to denature the proteins. Then the samples were loaded onto 8-10% SDS-PAGE gels using the Mini-PROTEAN Tetra Cell equipment from Bio-Rad® and electrophoresed for 2-3 hours at 100-110 volts and 4°C.

The proteins in the gels were blotted onto a nitrocellulose membrane, either using iBlot® dry transfer system (Invitrogen) or wet electrotransfer. For the iBlot dry transfer, the gels were placed into distilled water, for a wash after the electrophoresis, and then placed on a bottom buffer matrix covered with a nitrocellulose membrane in the iBlot® gel transfer device. The gels were covered with wet filter papers, the top buffer matrix and a sponge (Figure 9, left). The proteins were transferred to the membrane with 5:40-6:20 min transfer. The membranes were cut out according to the size of the gel and placed into blocking buffer (5% skimmed milk powder in TBS) for 1 h on a rocker at RT.

For wet transfer a kind of 'sandwich' was built on a clip with foam, 3 filter papers, the gel, polyvinylidene fluoride (PVDF) membrane (Macherey-Nagel), again 3 filter papers and foam (Figure 9, right). Every item was placed into transfer buffer (25 mM Tris base, 190 mM glycine and 0.1% SDS) before stacking. Next, the 'sandwich' was added to a transfer bucket and the electrotransfer performed at 340 mA for 1.5-2 hours at 4°C. The membranes were then placed into a blocking buffer for 1 h on a rocker.

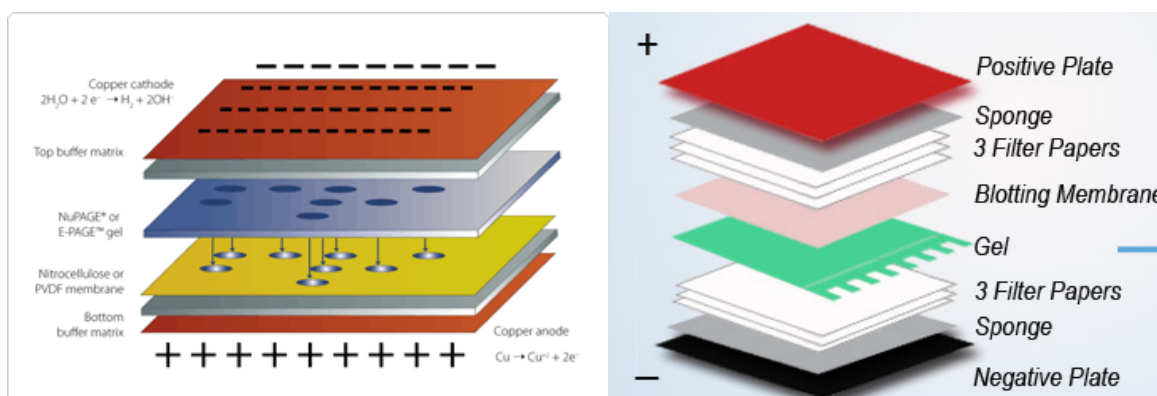


Figure 9. Set up for protein transfer

Set up for iBlot® dry transfer (left) and wet transfer (right). Figure from LifeTechnologies (127).

After the blocking, the membranes were incubated with primary antibodies (Table 5) diluted in 5% BSA or non-fat dried milk in 0.1% TBS-Tween at 4°C on a rocker overnight.

The following day, the membranes were washed three times with 0.1% TBS-Tween for 5 min and then incubated with near-infrared (NIR)-labeled secondary antibodies (Table 6) diluted in 0.1% TBS-Tween with 0.01% SDS for 40-60 min at RT. The membranes were then washed again as before in addition to a wash with TBS for 5 min. Odyssey Infrared Imaging System (Li-Cor Biosciences) was used to scan the membranes.

To analyze the results, Image Studio software from Li-Cor Biosciences was used, and intensity of each band examined. A ratio between the signal intensity of the phosphorylated protein and the total protein was calculated. In case of I κ B α and NF- κ B, the ratio of the intensity of the total protein and the control protein (β -actin or LaminB) was calculated. Results are shown as the ratio between the signal intensities of a particular protein for treatment versus a stimulated control.

Table 4. 2x Sample buffer

2x Sample buffer
4 mL 1M Tris-HCl pH 7.0 (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 dH₂O

Table 5. Primary antibodies used for Western blotting

Antibody	Cat#	Isotype	Dilution	Supplier
B-actin	4967	Rabbit	1:2000	Cell Signaling
IκBα	4814	Mouse IgG1	1:1000	Cell Signaling
ERK1/2 (p44/42)	4696	Mouse IgG1	1:2000	Cell Signaling
Phospho-ERK 1/2 (p44/42)	4370	Rabbit IgG	1:2000	Cell Signaling
P38	9212	Rabbit	1:2000	Cell Signaling
Phosphor-p38	9216	Mouse IgG1	1:2000	Cell Signaling
Akt	2920	Mouse IgG1	1:2000	Cell Signaling
Phospho-Akt	4060	Rabbit IgG	1:2000	Cell Signaling
LaminB1	33-2000	Mouse IgG1	1:1000	Invitrogen
NF-κB p65	Sc-372	Rabbit IgG	1:500	Santa Cruz Biotech.

Table 6. Secondary antibodies used for Western blotting

Antibody	Cat#	Label	Dilution	Supplier
Anti-rabbit IgG	926-68021	IRDye 680LT	1:15000	Li-Cor Biosciences
Anti-mouse IgG	926-32210	IRDye 800CW	1:15000	Li-Cor Biosciences

3.2.2.4 Coomassie blue staining

To determine the amount of proteins left on the gel after transfer to the membrane, the gels were stained with coomassie blue staining solution (0.1% coomassie R250, 10% glacial acetic acid and 40% methanol in dH₂O) on a rocker, overnight at RT. The following day, the gel was washed 2-3 times with de-staining solution (20% glacial acetic acid and 40% methanol in dH₂O) for 3-5 min on a rocker. Next the gel was incubated in microwave for 20-40 sec and washed again with de-staining solution 2-3 times.

3.2.3 Statistics

Results are expressed as mean \pm standard error of the means (SEM). ANOVA, followed by Tukey's post hoc test was used to compare more than two groups and paired t-test when comparing only two groups. P-value of $p < 0.05$ was considered statistically significant and labeled with * for $p < 0.05$, ** for $p < 0.01$ or *** for $p < 0.001$. GraphPad Prism was used for all statistical calculations.

4 Results

4.1 Bioguided isolation of a polysaccharides from Yarrow

Bioguided fractionation was used to isolate polysaccharides from Yarrow. The ratio of the secreted pro-inflammatory cytokine IL-12p40, and the anti-inflammatory cytokine IL-10 in THP-1 monocytes was used to indicate whether the effects were pro- (IL-12p40/IL-10 >1) or anti- (IL-12p40/IL-10 <1) inflammatory.

4.1.1 The effects of water extract from Yarrow on cytokine secretion by THP-1 monocytes

THP-1 monocytes stimulated in the presence of water extract from Yarrow at 100 µg/mL secreted more IL-10 than THP-1 monocytes stimulated in the absence of the water extract. The increase was about three fold (Figure 10A). There was a tendency towards more IL-12p40 being secreted by THP-1 monocytes stimulated in the presence of both concentrations of the water extract (10 and 100 µg/mL) when compared with the control (Figure 10B).

The ratio between IL-12p40 and IL-10 in the supernatants from THP-1 monocytes stimulated in the presence of water extract from Yarrow at the concentration 100 µg/mL was about 50% lower than the ratio in supernatants from THP-1 monocytes stimulated in the absence of the water extract (Figure 10C).

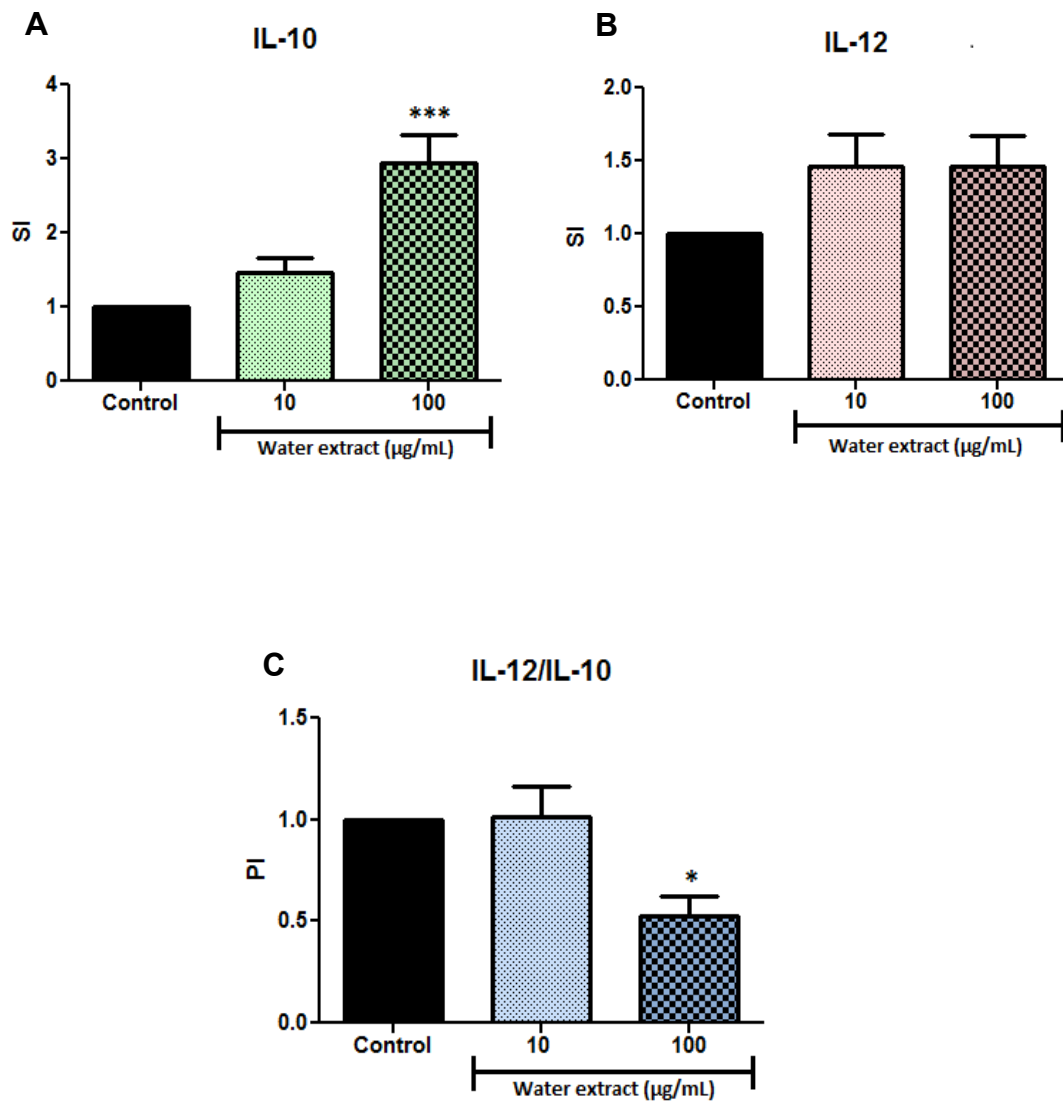


Figure 10. Effects of water extract from Yarrow on IL-12p40 and IL-10 secretion by THP-1 monocytes.

THP-1 monocytes were primed with IFN- γ for 3 h. The cells were then stimulated with LPS for 48 h (control). Water extract from Yarrow was added to the cells at the indicated concentrations simultaneously with the LPS. **A.** IL-10 concentration in the supernatants. **B.** IL-12p40 concentration in supernatant. **C.** Ratio between secreted IL-12p40 and IL-10. Results are shown as the mean of the SI (cytokine concentration with extract / cytokine concentration without extract) \pm SEM, or the mean of the PI (SI for IL-12p40 / SI for IL-10) \pm SEM, $n=3$. Statistical significance is indicated with * for $p<0.05$ or *** for $p<0.001$.

4.1.2 The effects of dialyzed water extract from Yarrow on cytokine secretion by THP-1 monocytes

THP-1 monocytes stimulated in the presence of dialyzed water extract from Yarrow at 50 and 100 µg/mL secreted more IL-10 than THP-1 monocytes stimulated in the absence of the dialyzed water extract. The increase in IL-10 was dose dependent, with the highest dose (100 µg/mL) causing about six fold increase in IL-10 secretion (Figure 11A). THP-1 monocytes stimulated in the presence of dialyzed water extract at 50 and 100 µg/mL had a tendency towards secreting more IL-12p40 than THP-1 monocytes stimulated in the absence of dialyzed extract (Figure 11B).

Stimulation of THP-1 monocytes in the presence of dialyzed water extract from Yarrow decreased the ratio between IL-12p40 and IL-10 dose dependently. The highest concentration of dialyzed water extract led to more than 60% decrease in the IL-12p40/IL-10 ratio compared with that in supernatants from THP-1 monocytes stimulated in the absence of the dialyzed water extract (Figure 11C).

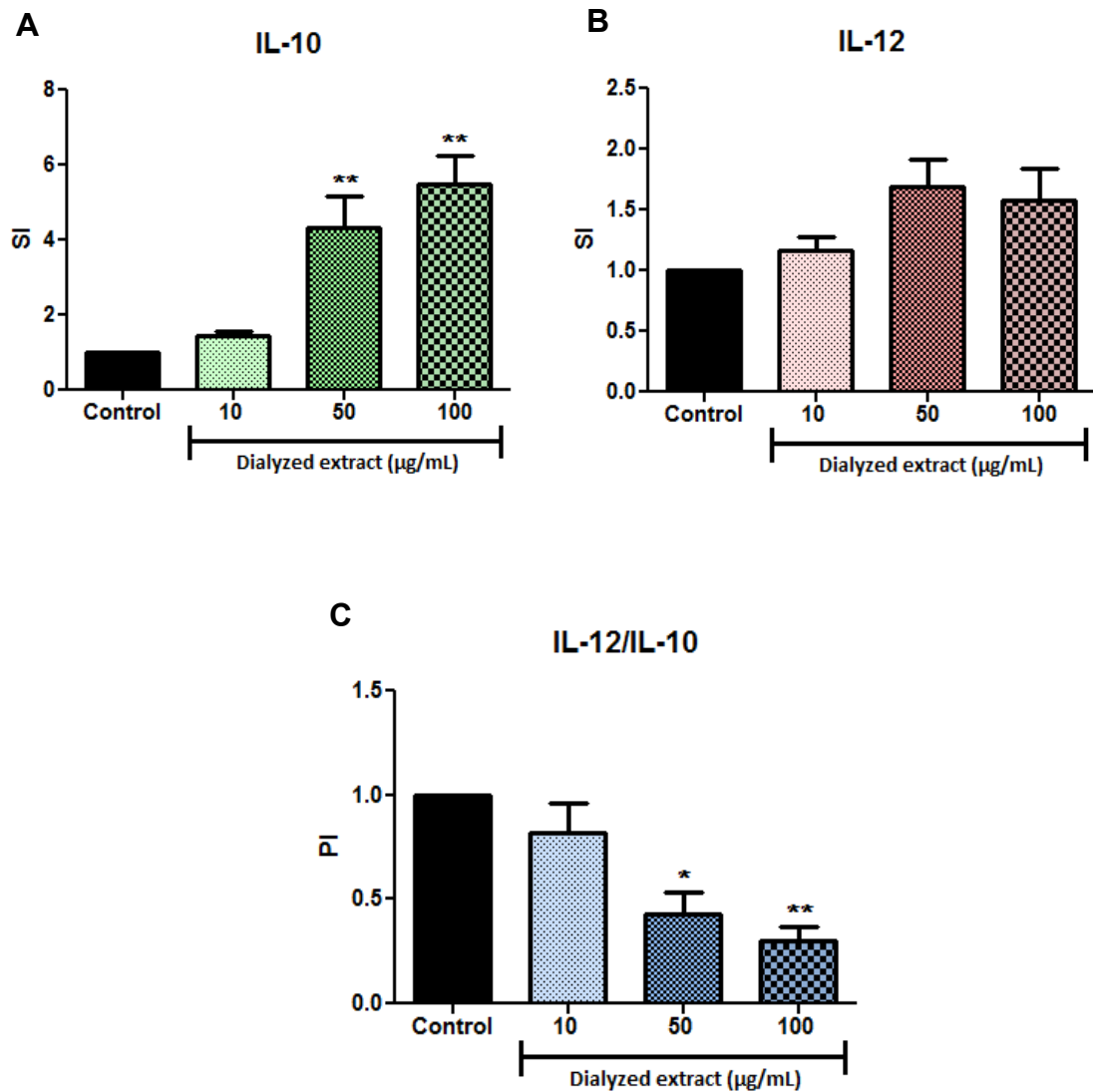


Figure 11. Effects of dialyzed water extract from Yarrow on IL-12p40 and IL-10 secretion by THP-1 monocytes.

THP-1 monocytes were primed with IFN- γ for 3 h. The cells were then stimulated with LPS for 48 h (control). Dialyzed water extract from Yarrow was added to the cells at the indicated concentrations simultaneously with the LPS. **A.** IL-10 concentration in the supernatants. **B.** IL-12p40 concentration in supernatant. **C.** Ratio between secreted IL-12p40 and IL-10. Results are shown as the mean of the SI (cytokine concentration with extract / cytokine concentration without extract) \pm SEM, or the mean of the PI (SI for IL-12p40 / SI for IL-10) \pm SEM, $n=3$. Statistical significance is indicated with * for $p<0.05$ or ** for $p<0.01$.

4.1.3 The effects of ion exchange chromatography fractions of dialyzed water extract from Yarrow on cytokine secretion by THP-1 monocytes

The dialyzed water extract from Yarrow was applied to an ion exchange column and the different fractions (0.25 M, 0.5 M, 1.0 M and 2.0 M) added to the THP-1 monocytes. The 1 M and 2 M fractions were only tested in a pilot run and as they had very little effect on IL-10 and IL-12p40 secretion (Appendix 1) they were not included in further cytokine measurements.

THP-1 monocytes stimulated in the presence of 100 µg/mL of the 0.25 M fraction from the ion exchange column secreted six times more IL-10 than THP-1 monocytes stimulated without the fraction (Figure 12A). There was a tendency towards a decrease in the ratio between IL-12p40 and IL-10 in cultures from THP-1 monocytes stimulated in the presence of the 0.25 M fraction (Figure 12C). IL-10 concentration was also higher in THP-1 monocytes stimulated in the presence of the 0.5 M fraction, also leading to a tendency towards a decrease in the IL-12p40/IL-10 ratio (Figure 12A and Figure 12C).

THP-1 monocytes stimulated in the presence of the 0.25 M and the 0.5 M fractions had tendency towards increased IL-12p40 secretion (Figure 12B).

Due to the higher IL-10 concentration from THP-1 monocytes stimulated in the presence of 0.25 M fraction, and greater tendency towards decrease in IL-12p40/IL-10 ratio for 0.25 M fraction from the ion exchange column than when stimulated in the presence of 0.5 M fraction, 0.25 M was chosen for further isolation and termed Am-25.

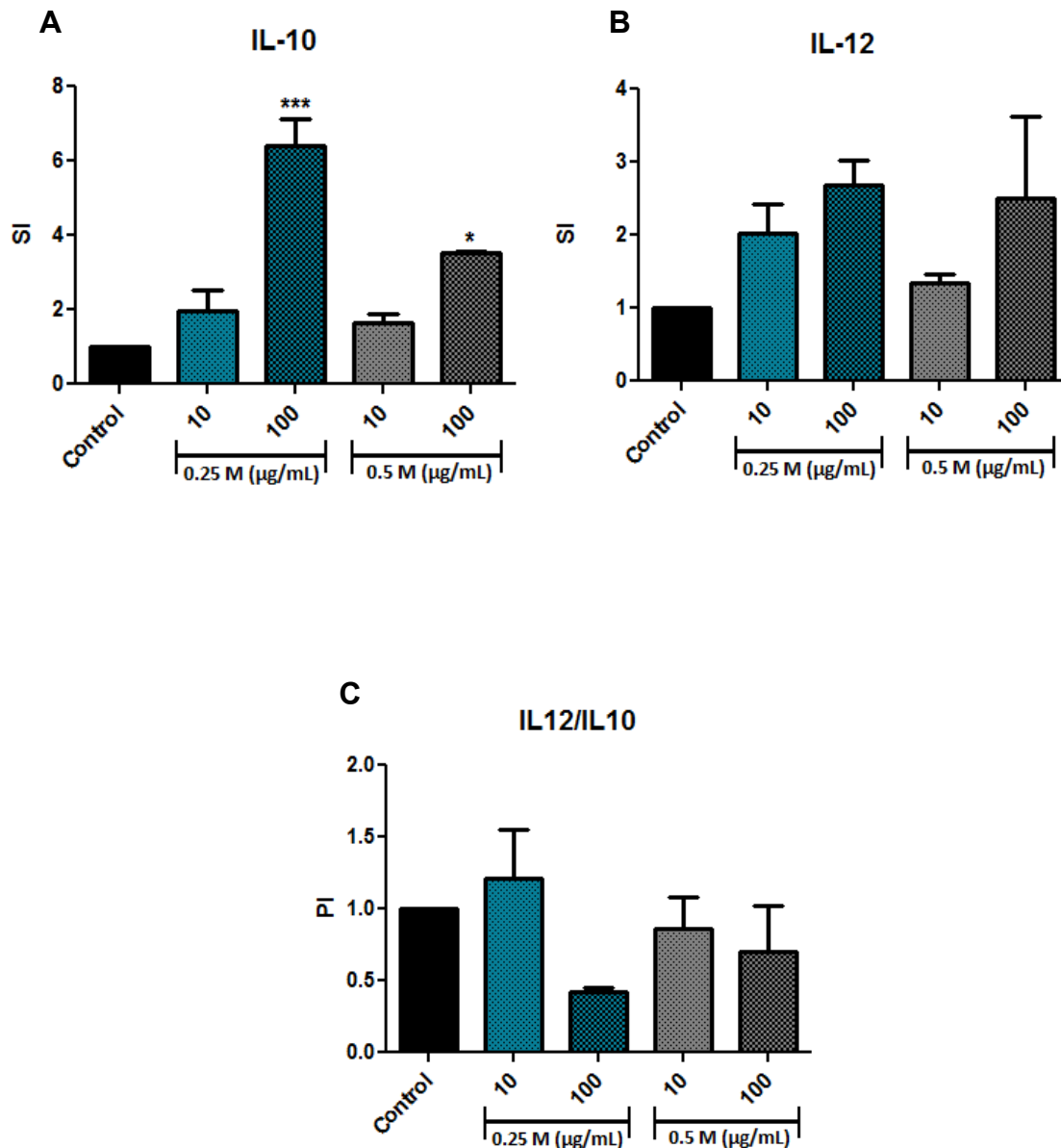


Figure 12. Effects of ion exchange column fractions on IL-12p40 and IL-10 secretion by THP-1 monocytes.

THP-1 monocytes were primed with IFN- γ for 3 h and then stimulated with LPS for 48 h (control). Fractions from the ion exchange column were added to the cell culture at indicated concentrations simultaneously with the LPS. **A.** IL-10 concentration in the supernatants. **B.** IL-12p40 concentration in supernatant. **C.** Ratio between secreted IL-12p40 and IL-10. Results are shown as the mean of the SI (cytokine concentration with extract / cytokine concentration without extract) \pm SEM, or the mean of the PI (SI for IL-12p40 / SI for IL-10) \pm SEM, $n=3$. Statistical significance is indicated with * for $p<0.05$ or *** for $p<0.001$.

4.1.4 The effects of Am-25 and desalted Am-25 (Am-25-d) on cytokine secretion by THP-1 monocytes

The 0.25 M fraction from the ion exchange column (Am-25) was desalinated and results from a HP-GPC analyzes indicated that it was likely a polysaccharide which will be termed Am-25-d from now on.

THP-1 monocytes stimulated in the presence of both Am-25 and Am-25-d secreted more IL-10 than THP-1 monocytes stimulated in the absence of Am-25 and Am-25-d (Figure 13A). IL-12p40 concentration increased by THP-1 monocytes stimulated in the presence of Am-25-d compared with THP-1 monocytes stimulated in the absence of Am-25-d. Am-25 treated THP-1 monocytes had also tendency towards more secretion of IL-12p40 than THP-1 monocytes stimulated in the absence of Am-25 (Figure 13B). THP-1 monocytes stimulated in the presence of Am-25 and Am-25-d had tendency towards a decrease in the IL-12p40/IL-10 ratio compared with THP-1 monocytes stimulated in the absence of Am-25 and Am-25-d (Figure 13C).

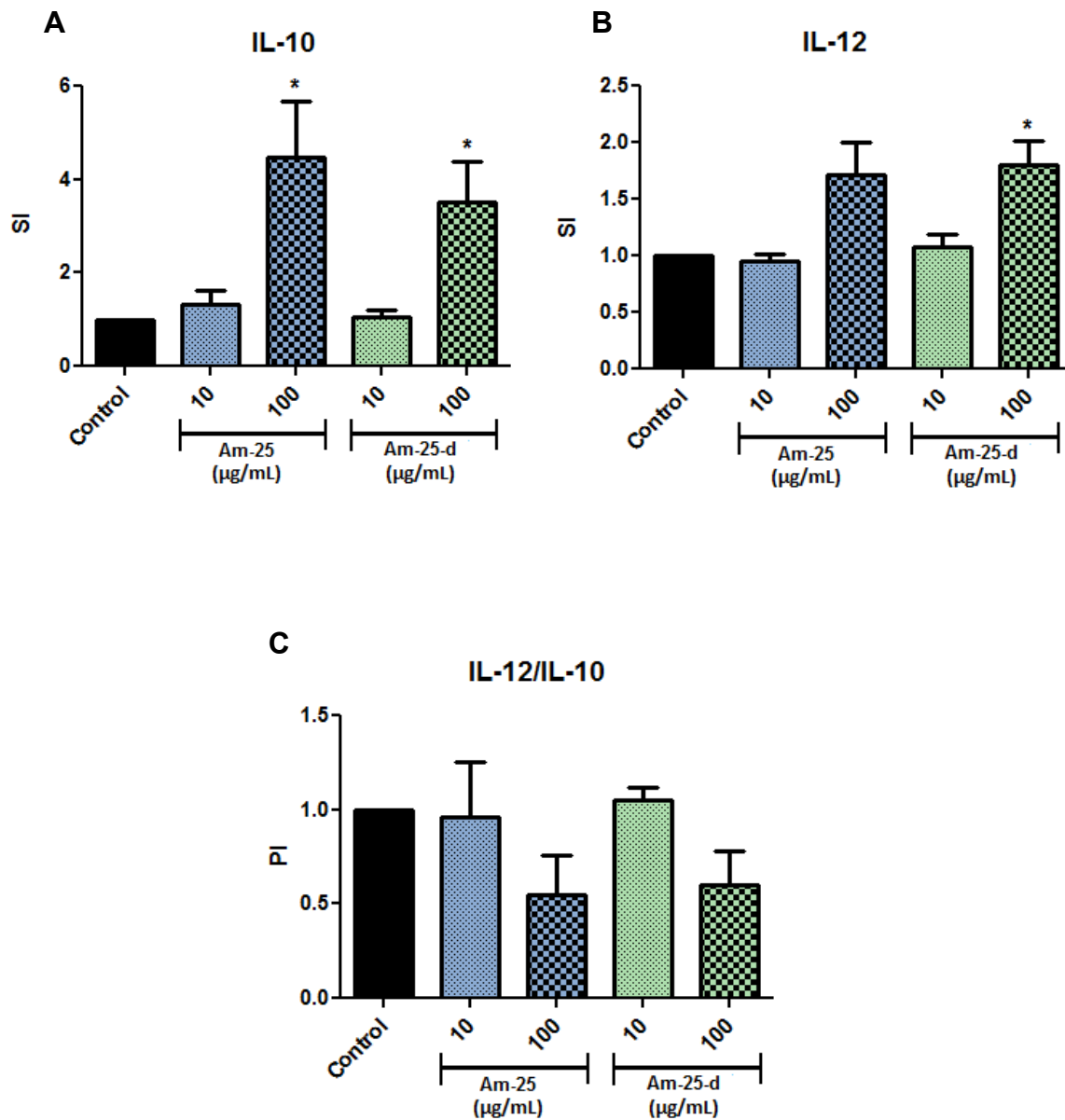


Figure 13. Effects of Am-25 and Am-25-d on IL-12p40 and IL-10 secretion by THP-1 monocytes.

THP-1 monocytes were primed with IFN- γ for 3 h and then stimulated with LPS for 48 h (control). Either Am-25 or Am-25-d polysaccharide was added to the culture at indicated concentrations simultaneously with the LPS. **A.** IL-12p40 and IL-10 concentration in the supernatant. **B.** Ratio between secreted IL-12p40 and IL-10. Results are shown as the mean of the SI (cytokine concentration with extract / cytokine concentration without extract) \pm SEM, or the mean of the PI (SI for IL-12p40 / SI for IL-10) \pm SEM, $n=4$. Statistical significance is indicated with * for $p<0.05$.

4.1.5 Molecular weight determination of Am-25-d

A molecular weight determination was performed on Am-25-d using HP-GPC analysis. Values for Dextran standards are listed in Table 7. K_{av} was calculated for Am-25-d from its values and standards V_0 and V_t . The molecular weight of Am-25-d was then determined from the best fit equation from the standard curve (Table 7, Figure 14).

Two peaks were detected when Am-25-d was added to the column. The molecular weight of the prior top was estimated around 270 kDa, whereas the latter top was estimated to have molecular weight around 0.5 kDa (Table 7).

Figures from the HP-GPC analysis can be found in Appendix 6.

Table 7. Molecular weight and values for Dextran standards used for K_{av} and standard curve calculations and for Am-25-d to determine its molecular weight.

Standard	MW (Da)	V_e (cm)	V_0 (cm)	V_t (cm)	K_{av}	log MW
T 2000	2000000	3,5	3,5	5,0	0,000	6,301
T 500	500000	3,7	3,5	5,0	0,133	5,699
T 70	70000	4,0	3,5	5,0	0,333	4,845
T 40	40000	4,1	3,5	5,0	0,400	4,602
T 10	10000	4,3	3,5	5,0	0,533	4,000
Glucose	180	5,0	3,5	5,0	1,000	2,255
Am-25-d	267300,6	3,8	3,5	5,0	0,200	5,427
	535,8	4,8	3,5	5,0	0,867	2,729

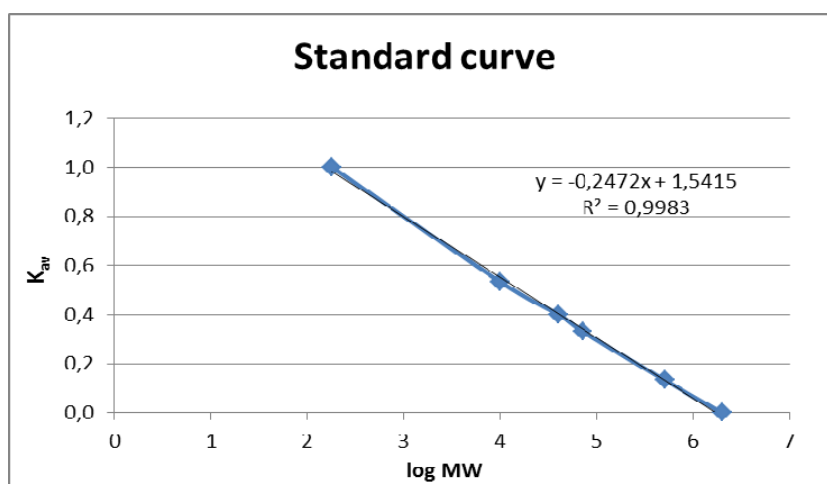


Figure 14. Standard curve for Dextran standards used to determine molecular weight.

$K_{av} = (V_e - V_0) / (V_t - V_0)$, where V_t is the value of the standard that remain longest on the column, in this case glucose. V_0 is the value of the standard that remains shortest on the column, in this case Dextran T 2000. V_e is the value for indicated standard or sample.

4.2 Effects of Am-25-d on THP-1 monocytes

Am-25-d was chosen for further analyzes and viability, further cytokine secretion, expression of surface molecules and intracellular pathways examined.

4.2.1 The effect of Am-25-d on the viability of THP-1 monocytes

Viability test was used to determine if Am-25-d had cytotoxic effects. There was no difference in viability of the THP-1 monocytes stimulated in the presence of Am-25-d and THP-1 monocytes stimulated in the absence of Am-25-d (Figure 15).

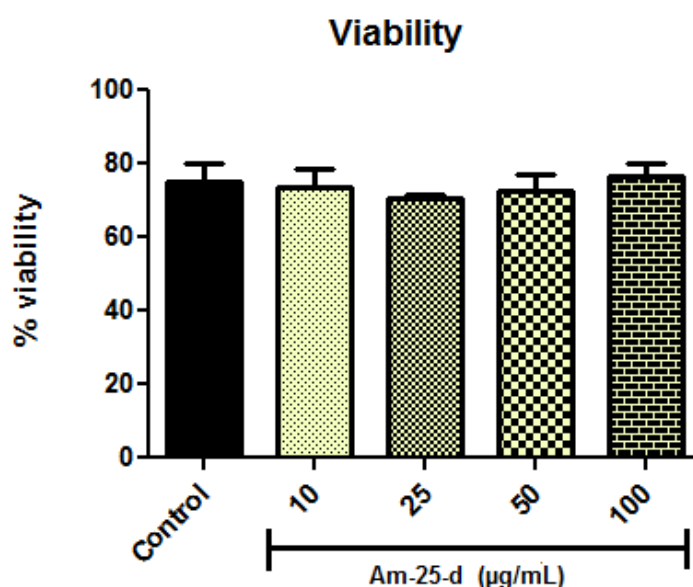


Figure 15. Effects of Am-25-d on the viability of THP-1 monocytes.

THP-1 monocytes were primed with IFN- γ for 3 h. The cells were then stimulated with LPS for 48 h (control). Am-25-d was added to the cells at the indicated concentrations simultaneously with the LPS. Results are shown as a mean percentage of live cells \pm SEM, n=4.

4.2.2 Effects of Am-25-d on appearance of THP-1 monocytes

After 48 h culture in medium alone, unstimulated THP-1 monocytes were rather equally spread (Figure 16, A) and were not attached to each other. THP-1 monocytes primed with IFN- γ and stimulated with LPS formed clusters that are a typical sign for stimulated monocytes (Figure 16, B). A slight difference was observed in the appearance of THP-1 monocytes that were stimulated in the presence of Am-25-d as they formed smaller clusters than those stimulated in absence of Am-25-d (Figure 16, C).

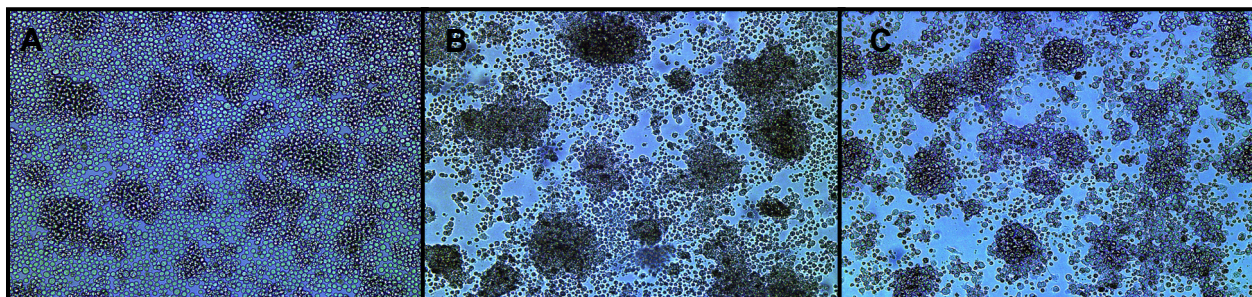


Figure 16. Effects of Am-25-d on appearance of THP-1 monocytes.

A. Unstimulated THP-1 monocytes cultured for 48 h. B. THP-1 monocytes primed with IFN- γ for 3 h and stimulated with LPS for 48 h. C THP-1 monocytes primed with IFN- γ for 3 h and stimulated with LPS for 48 h with Am-25-d added simultaneously with the LPS. Photographic images of cells, taken using Leica DMI 3000B Inverted Microscope at 10 x magnification.

4.2.3 The effects of Am-25-d on cytokine secretion by THP-1 monocytes

When stimulated in the presence of Am-25-d at concentrations 50 and 100 µg/mL, THP-1 monocytes secreted more of IL-10 compared with THP-1 monocytes stimulated in the absence of Am-25-d (Figure 17). THP-1 monocytes stimulated in the presence of Am-25-d had tendency towards increase in IL-27 secretion (Figure 17).

THP-1 monocytes secreted more IL-12p40, IL-23, IL-8, IL-1β and TNF-α when stimulated in the presence of 100 µg/mL of Am-25-d, compared with those stimulated in the absence of the polysaccharide. There was also a tendency towards increase in IL-6 secretion by THP-1 monocytes stimulated in the presence of Am-25-d, compared with untreated control (Figure 18).

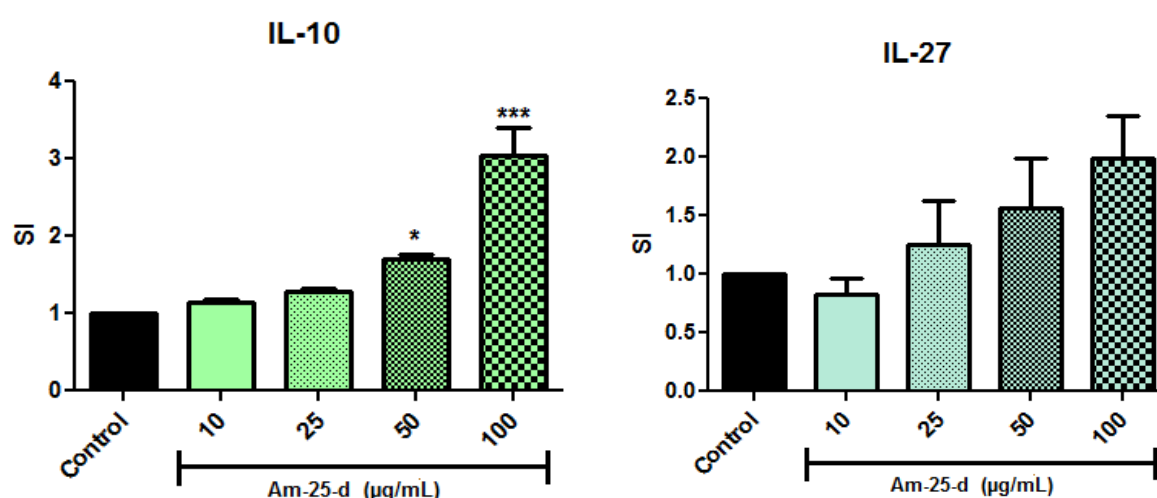


Figure 17. Effects of Am-25-d on anti-inflammatory cytokines secretion by stimulated THP-1 monocytes.

THP-1 monocytes were primed with IFN-γ for 3 h and then stimulated with LPS for 48 h (control). Am-25-d was added to the culture at indicated concentrations simultaneously with the LPS. Results are shown as the mean of the SI (cytokine concentration with extract / cytokine concentration without extract) ± SEM, n=6. Statistical significance is indicated with * for p<0.05 or *** for p<0.001.

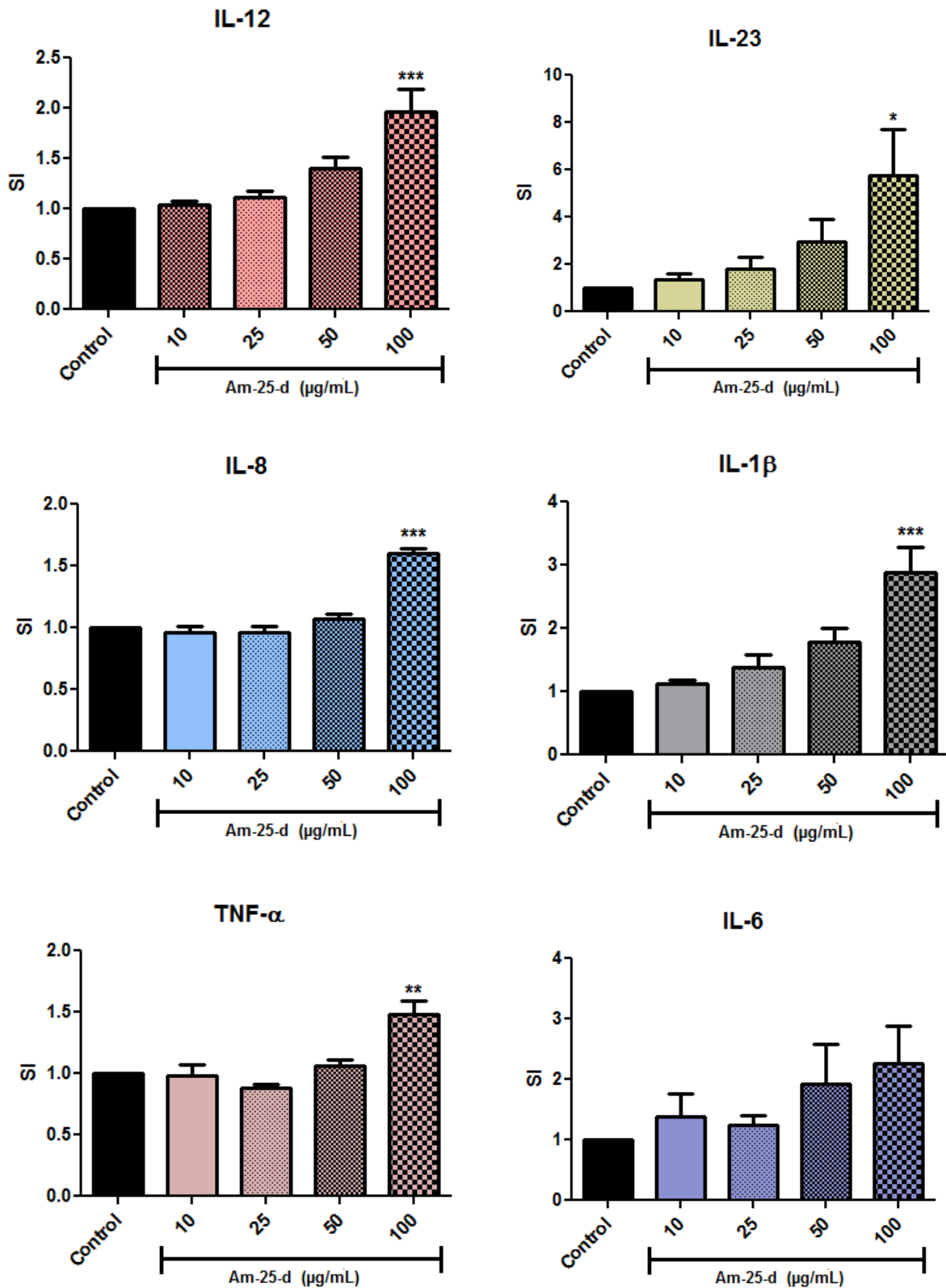


Figure 18. Effects of Am-25-d on pro-inflammatory cytokines secretion by stimulated THP-1 monocytes.

THP-1 monocytes were primed with IFN-γ for 3 h and then stimulated with LPS for 48 h. Am-25-d was added to the culture at indicated concentrations simultaneously with the LPS. Results are shown as the mean of the SI (cytokine concentration with extract / cytokine concentration without extract) ± SEM. For IL-12p40, IL-8, IL-1β, TNF-α and IL-6 n=4, for IL-23 n=6. Statistical significance is indicated with * for p<0.05, ** for p<0.01 or *** for p<0.001.

4.2.4 The effects of Am-25-d on expression of surface molecules on THP-1 monocytes

No difference was detected in expression of the surface molecules, CD14, TLR4, CD40, CD54, CD80, CD86 and HLA-DR, between THP-1 monocytes stimulated in the presence or absence of Am-25-d, neither in percentage of positive cells nor the MFI of positive cells (Figure 19).

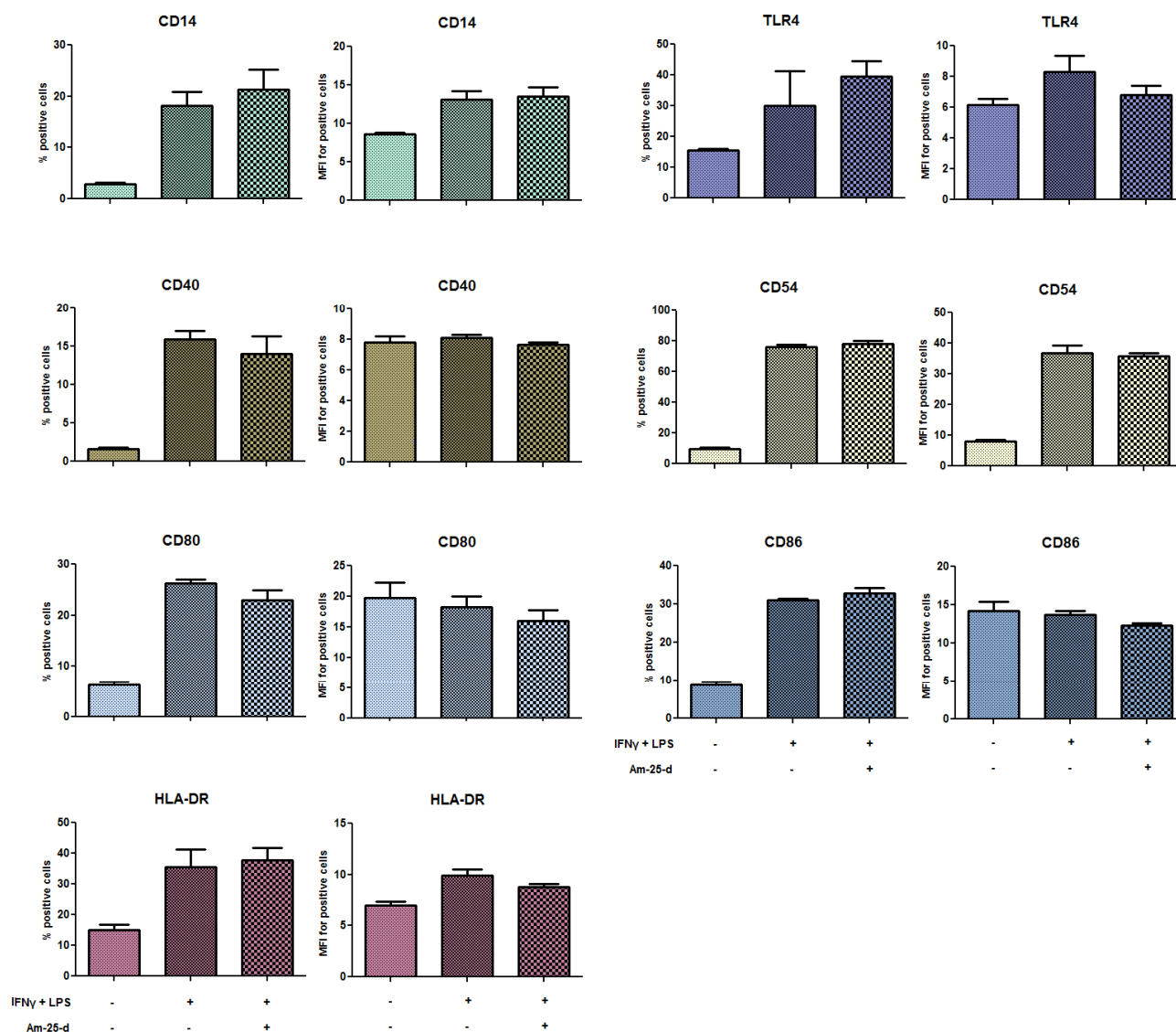


Figure 19. Effects of Am-25-d on expression of surface molecules by stimulated THP-1 monocytes.

THP-1 monocytes were left unstimulated or primed with IFN- γ for 3 h and then stimulated with LPS for 24 h (control). Am-25-d was added to the culture at 100 μ g/mL simultaneously with the LPS. Results are shown as mean percentage of positive cells or mean fluorescence intensity (MFI) of positive cells \pm SEM. For CD14 n=5, TLR4 n=3, CD40 n=2, CD54 n=5, CD80 n=5, CD86 n=2 and HLA-DR n=5.

4.2.5 The effects of Am-25 on activation of intracellular signaling pathways in THP-1 monocytes

Activation of several intracellular signaling pathways was examined to determine whether the effects of Am-25-d on cytokine secretion were mediated by an effect on these pathways.

4.2.5.1 The effects of Am-25-d on MAP kinase activation in THP-1 monocytes

Stimulating THP-1 monocytes in the presence of Am-25-d led to less phosphorylation of ERK1/2 kinase than that in THP-1 monocytes stimulated without Am-25-d. However, Am-25-d did not affect phosphorylation of p38 in THP-1 monocytes when compared with stimulated control (Figure 20).

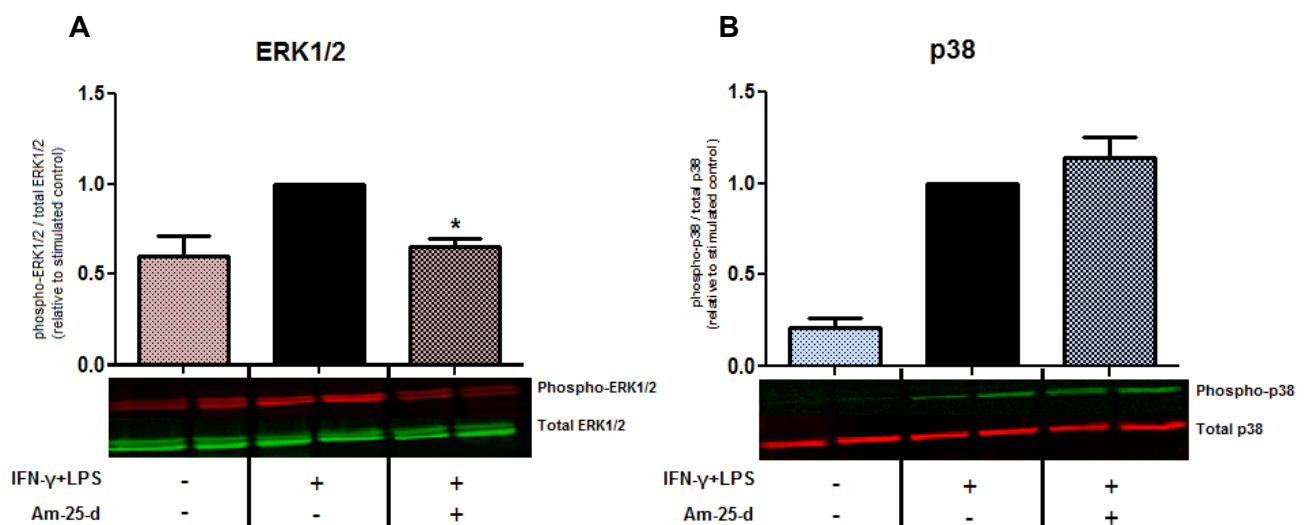


Figure 20. Effects of Am-25-d on phosphorylation of ERK1/2 and p38 in THP-1 monocytes.

THP-1 monocytes were left unstimulated (left) or primed with IFN- γ for 3 h and stimulated with LPS for 1 h (middle). 100 μ g/mL of Am-25-d was added to the cells simultaneously with the LPS (right).

A. Phosphorylation of ERK1/2 kinase. **B.** Phosphorylation of p38 kinase. Results are shown as the ratio of the mean signal intensity for phosphorylated protein versus total protein, relative to the same ratio for the stimulated control (black) \pm SEM, $n=3$. The blots are representative of three independent experiments with a duplicate of each sample. Statistical significance is indicated with * for $p<0.05$.

4.2.5.2 The effects of Am-25-d on PI3K/Akt pathway in THP-1 monocytes

Akt kinase is known to be downstream from PI3K. Am-25-d decreased the activation of Akt kinase in stimulated THP-1 monocytes compared with THP-1 monocytes stimulated without Am-25-d, which indicates a decrease in PI3K activation as well (Figure 21).

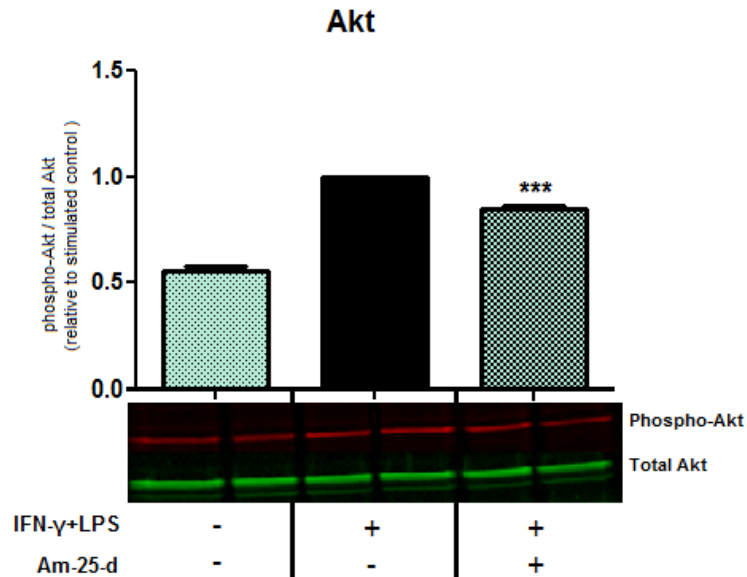


Figure 21. Effects of Am-25-d on Akt kinases phosphorylation in THP-1 monocytes.

THP-1 monocytes were left unstimulated (left) or primed with IFN- γ for 3 h and then stimulated with LPS for 1 h (middle). 100 μ g/mL of Am-25-d was added to the cells simultaneously with the LPS (right). Results are shown as the ratio of the mean signal intensity for phosphorylated protein versus total protein, relative to the same ratio for the stimulated control (black) \pm SEM, n=3. The blots are representative of three independent experiments with a duplicate of each sample. Statistical significance is indicated with *** for p<0.001.

4.2.5.3 The effects of Am-25-d on NF- κ B pathway in THP-1 monocytes

I κ B α is an inhibitor for the NF- κ B transcription factor. THP-1 monocytes stimulated in the presence of Am-25-d did not affect the breakdown of I κ B α compared with THP-1 monocytes stimulated without it (Figure 22, A). A small tendency towards decrease was detected on the nuclear localization of NF- κ B in THP-1 monocytes stimulated in the presence of Am-25-d, compared with those stimulated in the absence of it (Figure 22).

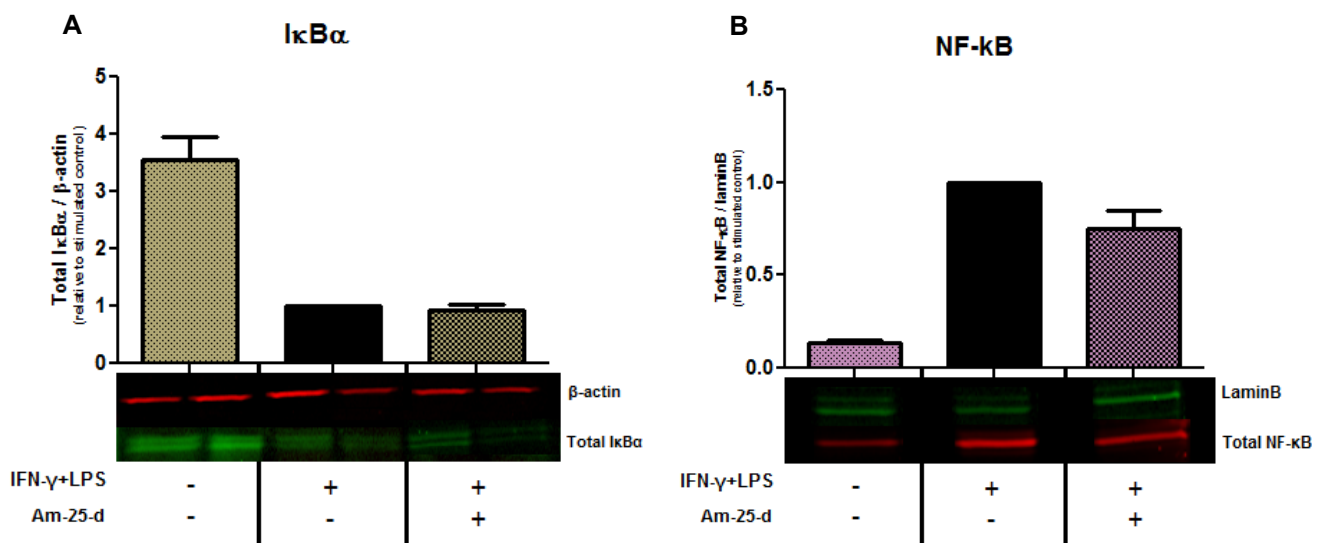


Figure 22. Effects of Am-25-d on I κ B α breakdown and nuclear localization of NF- κ B in THP-1 monocytes.

THP-1 monocytes were left unstimulated (left) or primed with IFN- γ for 3 h and then stimulated with LPS for 1 h (middle). 100 μ g/mL of Am-25-d was added to the cells simultaneously with the LPS (right). Results are shown as the ratio of the mean signal intensity for; **A**. Total I κ B α versus β -actin and **B**. Total NF- κ B versus LaminB, relative to the same ratio for the stimulated control (black) \pm SEM, n=3. The blots are representative of three independent experiments.

5 Discussion

The present study shows that a polysaccharide, Am-25-d, isolated from dry *Achillea millefolium* affected cytokine secretion and activation of intracellular signaling pathways in THP-1 monocytes. Am-25-d increased secretion of the anti-inflammatory cytokine IL-10 and had a tendency towards increasing secretion of IL-27, which is also considered an anti-inflammatory cytokine. Am-25-d also increased secretion of the pro-inflammatory cytokines IL-12p40, IL-23, IL-8, IL-1 β and TNF- α . Based on the ratio between the secreted IL-12p40 and IL-10, which was increased when Am-25-d was added to the cells, the effects of Am-25-d on THP-1 monocytes could be considered anti-inflammatory. Am-25-d decreased phosphorylation of both the MAP kinase ERK1/2 and the Akt kinase in THP-1 monocytes. Although Am-25-d did not affect breakdown of the NF- κ B inhibitor, I κ B α , there was small tendency towards a decrease in nuclear localization of NF- κ B.

5.1 Isolation of the polysaccharide

As a water extract from *Achillea millefolium* was shown to have immunomodulatory effects on DCs in a previous MS project in the lab (120) the first aim of this project was to isolate the active component from the plant.

After confirming that a water extract from *A. millefolium* had similar effects on IL-10 and IL-12p40 secretion in THP-1 monocytes in the present study as it did in the DCs in the previous study, the THP-1 monocytes were used for bioguided isolation of the active component in the present study.

After dialysis of the water extract, which removed small molecules from the extract, the dialyzed extract had more immunomodulatory effects than the original extract. The dialyzed extract was then fractionated with anion-exchange chromatography and subsequent analysis in the THP-1 monocytes revealed that both the 0.25 M NaCl and the 0.5 M NaCl fractions increased IL-10 secretion and showed a tendency towards an increase in IL-12p40 secretion in THP-1 monocytes. Ion-exchange chromatography is widely used to isolate polysaccharides and many polysaccharides from plants have anionic groups (128, 129). The 0.25 M and 0.5 M fractions are most likely composed of acidic (anionic) groups, although 0.25 M is considered less anionic than the 0.5 M fraction. Because the effects of the 0.25 M fraction were more pronounced than the effects of the 0.5 M fraction, the 0.25 M fraction was chosen for further purification by desalination and called Am-25-d. Desalination of the 0.25 M fraction did not affect its immunomodulatory properties.

Am-25-d is a water-soluble compound and considering the isolation process used, is most likely a polysaccharide. To confirm that hypothesis, the next step would be to determine the monosaccharide content of the preparation and the type of linkage between them, followed by a detailed structure elucidation by 1D and 2D nuclear magnetic resonance spectroscopy (NMR). The HP-GPC-analysis, which was used to determine the mean molecular weight of the polysaccharide, showed two peaks. The first one was estimated to be around 270 kDa and the latter around 0.5 kDa. The compound in the latter peak has molecular weight that is far too small for a polysaccharide. Thus, the prior top was considered contain the active polysaccharide. The latter peak could possibly represent several mono-

and oligosaccharides that have been hydrolysed from the polysaccharide in sample preparation or on the ion-exchange column.

5.2 Effects of Am-25-d

THP-1 monocytes stimulated in the presence of Am-25-d secreted more, or had a tendency towards secreting more, of all the cytokines tested in the project, than THP-1 monocytes stimulated in the absence of Am-25-d.

Am-25-d increased secretion of the anti-inflammatory cytokine IL-10 by stimulated THP-1 monocytes and also had a tendency towards increasing secretion of IL-27, which is considered to possess both pro- and anti-inflammatory properties (66) and be mainly produced by macrophages and DCs. Recent findings have, for example, suggested that IL-27 may be a potential therapeutic target for rheumatoid arthritis (130). Based on these results the effects of Am-25-d could be considered anti-inflammatory. However, Am-25-d also increased secretion of the pro-inflammatory cytokines IL-12p40, IL-23, IL-8, IL-1 β and TNF- α and had a tendency towards increasing secretion of IL-6, which is most often considered a pro-inflammatory cytokine, although it has also been shown to have anti-inflammatory activities (57). The ratio of the pro-inflammatory cytokine IL-12p40 and the anti-inflammatory cytokine IL-10 is sometimes used to determine whether effects of a compound are pro- or anti-inflammatory, and in the case for Am-25-d this ratio was lower when the THP-1 monocytes were stimulated in the presence of Am-25-d compared with that when they were stimulated without it. That suggests that the effects of Am-25-d are more anti- than pro-inflammatory, but it seems that the effects of Am-25-d are not purely anti- or pro-inflammatory but rather increase secretion of cytokines in general.

In a previous study in the lab, a water extract from *A. millefolium* decreased IL-12p40 secretion by DCs (120). Therefore, it was surprising that Am-25-d increased secretion of IL-12p40 by the THP-1 monocytes in the present study. Thus, in the present study a comparison was made of the effects of Am-25-d on cytokine secretion by DCs and THP-1 monocytes. The results showed that when stimulated in the presence of Am-25-d, DCs secreted less IL-12p40 than when they were stimulated without Am-25-d (Appendix 5) confirming the result from the previous study (120). However, when using THP-1 monocytes to test the water extract in the first step of the isolation in the present study, IL-12p40 secretion was increased when compared with monocytes stimulated without the extract. These results indicate that there is a difference in how the water extract and the Am-25-d affect cytokine secretion in the two cell types, i.e. DCs and THP-1 monocytes. THP-1 monocytes have been shown to differ from monocytes and peripheral blood mononuclear cells with respect to cytokine release after stimulation with LPS (10) and perhaps this difference in the response of the THP-1 monocytes and the DCs to the water extract and the Am-25-d is explained by a difference in their functions.

In both the THP-1 monocytes and the DCs, the water extract and the polysaccharide Am-25-d decreased the IL-12p40/IL-10 ratio, indicating an anti-inflammatory effect in both cell types. However, one cannot set aside the increase in all the other pro-inflammatory cytokines. Therefore, whether Am-25-d is having anti- or pro-inflammatory effects in THP-1 monocytes remains not clear.

Although Am-25-d increased secretion of pro-inflammatory cytokines by THP-1 monocytes, it attenuated LPS-induced phosphorylation of the MAP kinase, ERK1/2. Other natural products have also been shown to inhibit phosphorylation of the ERK1/2 pathway and, thereby, inhibit secretion of pro-inflammatory cytokines, such as IL-1 β , IL-6 and TNF- α (131). Thus, the decrease in phosphorylation of ERK1/2 in the present study was surprising in light of the increased secretion of the pro-inflammatory cytokines by stimulated THP-1 monocytes treated with Am-25-d. Previous studies have shown that regulating the ERK1/2 pathway plays a role in regulating the production of IL-1 β (131), IL-6 (132), IL-8 (133), IL-10 (134), TNF- α (135, 136) and IL-23 (137). As decreased activation of ERK1/2 would be expected to attenuate secretion of these cytokines, the increase in secretion of the pro-inflammatory cytokines by THP-1 monocytes when stimulated in the presence of Am-25-d in the current study cannot be explained by the decreased ERK1/2 activation.

LPS-induced phosphorylation of the Akt kinase was also decreased by Am-25-d. Stimulation of the PI3K/Akt pathway has been shown to result in pro-inflammatory events (36, 37), although most studies suggest anti-inflammatory effects of activation of Akt (33-35, 138). The PI3K/Akt pathway is reported to reverse LPS-induced inflammation (139), therefore, the decrease in phosphorylation of Akt should cause pro-inflammatory effects (34), which could explain the increase in pro-inflammatory cytokines in present study. In a recent study, decreased phosphorylation of Akt in THP-1 derived-macrophages was shown to induce secretion of IL-1 β , TNF- α and IL-6 (140), which lines up with the increased secretion of these pro-inflammatory cytokines in the current study.

However, the increase in IL-10 secretion by THP-1 monocytes treated with Am-25-d cannot be explained by the decrease in phosphorylation of Akt, as Akt activation has been shown to promote IL-10 production (139).

Am-25-d did not alter the breakdown of the NF- κ B inhibitor I κ B α . Nonetheless, translocation of NF- κ B to the nucleus had a small tendency towards being decreased when the THP-1 monocytes were stimulated in the presence of Am-25-d. Several natural products have been shown to inhibit the NF- κ B pathway (131). Decreasing transcription of NF- κ B has been shown to attenuate secretion of many pro-inflammatory cytokines and is considered to be an optimal target in treatment of inflammatory diseases (53, 58, 59). The increase in cytokine secretion in present study can therefore not be explained by the effects of Am-25-d on the NF- κ B pathway. A possible explanation for the tendency towards a decrease in translocation of NF- κ B to the nucleus when the THP-1 monocytes were stimulated in the presence of Am-25-d is that Am-25-d may have inhibited phosphorylation of the NF- κ B subunit, p65. Although the tendency towards decrease of translocation of NF- κ B to the nucleus was not significant in present study and should not be over-interpreted, this effect is in concordance with research that showed that an extract from *A. millefolium* impaired NF- κ B signaling in endothelial cells (118).

Whether Am-25-d is having anti- or pro-inflammatory effects in THP-1 monocytes is not clear and many questions are left unanswered. A possible explanation for Am-25-d increasing secretion of all the cytokines tested could be that the Am-25-d preparation contains endotoxin impurity. However, there are several facts that contradict that. Firstly, if that was the case, phosphorylation of the ERK1/2 kinase and translocation of NF- κ B to the nucleus would be expected to increase with addition of Am-25-d, rather than decrease, as endotoxin increases phosphorylation of ERK1/2 and translocation of NF- κ B to the nucleus. Secondly, IL-12p40 secretion would also have been expected to be increased with addition of Am-25-d rather than decrease in stimulated DCs, as endotoxin increases IL-12p40 secretion by DCs. Thirdly, Am-25 did not affect any of the surface markers investigated in this study, whereas endotoxin by itself did. Therefore, it is unlikely that Am-25-d contain levels of endotoxin that are affecting the cells although this possibility cannot be excluded.

Another possible explanation of the simultaneous pro- and anti-inflammatory effects of the Am-25-d on the THP-1 monocytes is that different parts of the Am-25-d were having opposing effects, with one or more parts having pro-inflammatory effects, possibly through the PI3K/Akt pathway while the other part or parts were having anti-inflammatory effects by increasing secretion of IL-10. Through which signaling pathway IL-10 secretion is being mediated is unclear though.

More purification of the Am-25-d would be preferable to remove the free monosaccharaides, detected in the HP-GPC analysis, as well as confirming that Am-25-d is a single polysaccharide. Further experiments on the immunomodulatory effects of Am-25-d could include investigating other signaling pathways, such as the AP-1 and the JAK-STAT pathways. In addition, determination of its effects on secretion of pro-inflammatory cytokines, other than IL-12p40, in DCs would be of interest.

Various experimental models and approaches have been used to study immunomodulatory effects of natural products. In the present study, the THP-1 monocytic cell line was used as a model resembling stimulated monocytes. This model is often used for screening for and testing effects of natural products on immune responses (141, 142). There are both pros and cons for using a cell line instead of primary cells, for example using a cell line is less expensive and less time consuming than using primary cells. The variation between experiments using cell lines are smaller than in experiments where primary cells from different donors are used, which can also be a disadvantage as the variation can be an important part of detecting the overall effects. However, results from *in vitro* experiments do not necessarily represent what is occurring *in vivo*. After testing effects of natural products *in vitro*, for both the innate and the adaptive immune responses, the overall effects should eventually be tested in animal models and human clinical trials.

6 Conclusion

The polysaccharide, Am-25-d, induced secretion of both pro- and anti-inflammatory cytokines by THP-1 monocytes, without affecting the expression of several surface molecules linked with monocyte stimulation, making it impossible to determine whether Am-25-d is having pro- or anti-inflammatory effects on THP-1 monocytes. Am-25-d reduced activation of the ERK1/2 MAP kinase pathway and the PI3K/Akt pathway and had a tendency towards reducing translocation of NF- κ B to the nucleus. The increased secretion of pro-inflammatory cytokines may be mediated through the PI3K/Akt pathway, but not ERK1/2 kinase pathway. Through which pathway the increase in the anti-inflammatory cytokine IL-10 is mediated remains to be examined. Therefore, the immunomodulatory effects of Am-25-d have to be studied in more detail before it can be decided whether it has a potential for being used for promoting health.

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Appendix 1

Ion exchange column 1

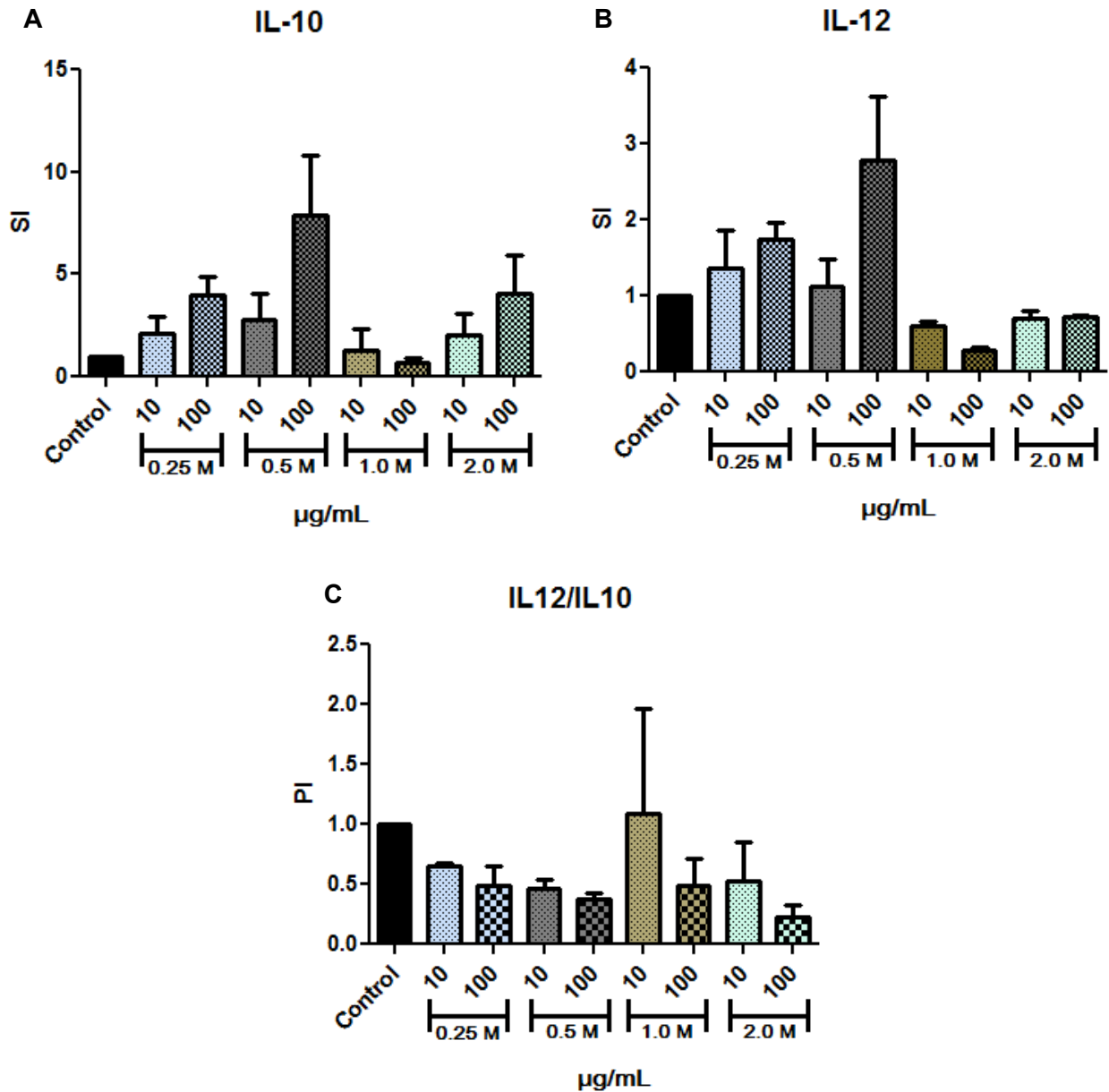


Figure A 1. Effects of ion exchange column fractions on IL-12p40 and IL-10 secretion by THP-1 monocytes.

THP-1 monocytes were primed with IFN- γ for 3 h and then stimulated with LPS for 48 h (control). Fractions from the ion exchange column were added to the cell culture at indicated concentrations simultaneously with the LPS. **A.** IL-10 concentration in the supernatants. **B.** IL-12p40 concentration in supernatant. **C.** Ratio between secreted IL-12p40 and IL-10. Results are shown as the mean of the SI (cytokine concentration with extract / cytokine concentration without extract) \pm SEM.

Appendix 2

Ethanol precipitation

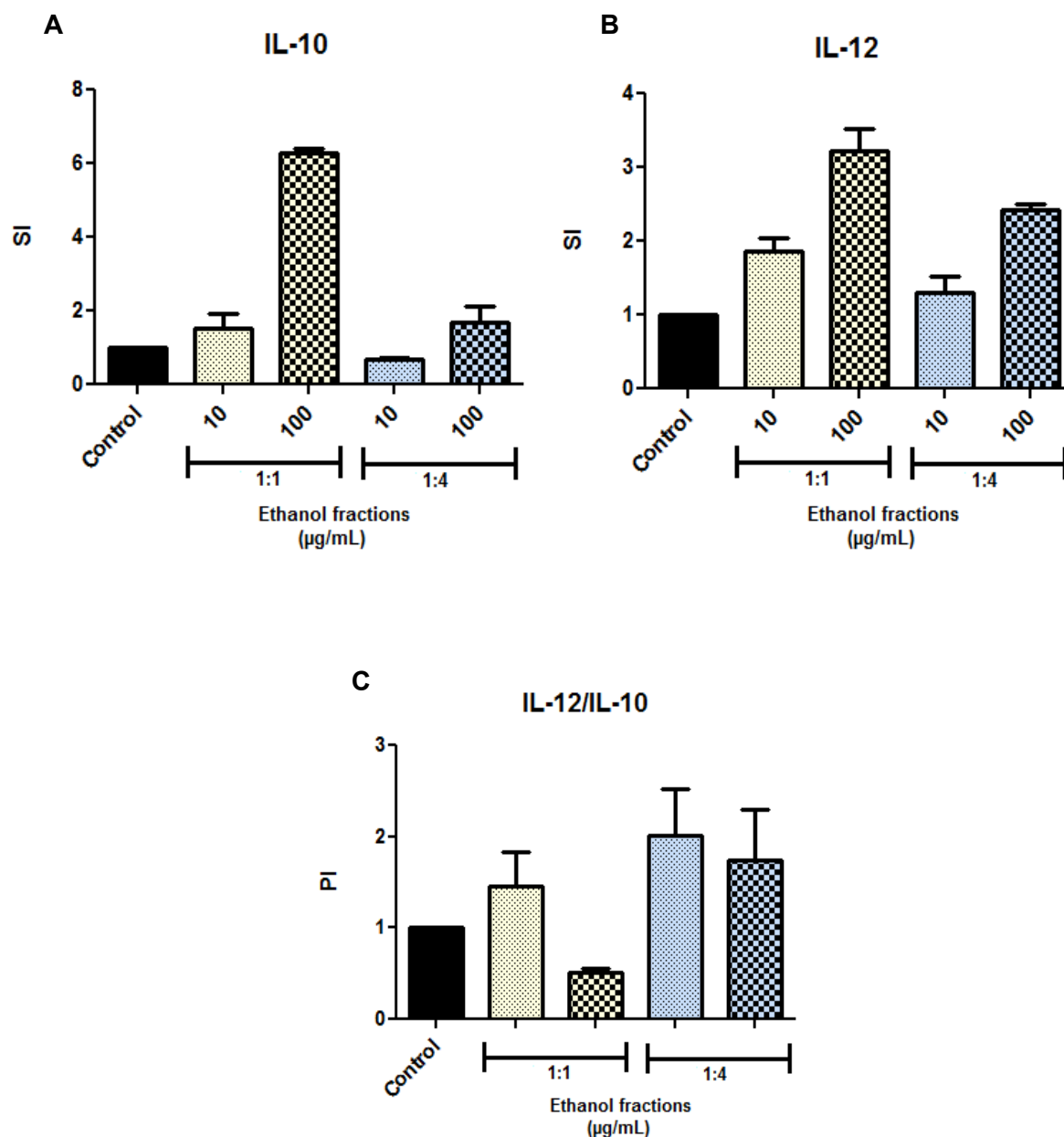


Figure A 2. Effects of ethanol precipitation fractions on IL-12p40 and IL-10 secretion by THP-1 monocytes.

THP-1 monocytes were primed with IFN- γ for 3 h and then stimulated with LPS for 48 h (control). Fractions from ethanol precipitation were added to the cell culture at indicated concentrations simultaneously with the LPS. **A.** IL-10 concentration in the supernatants. **B.** IL-12p40 concentration in supernatant. **C.** Ratio between secreted IL-12p40 and IL-10. Results are shown as the mean of the SI (cytokine concentration with extract / cytokine concentration without extract) \pm SEM, n=3.

Appendix 3

Ion exchange columns from ethanol precipitated fractions

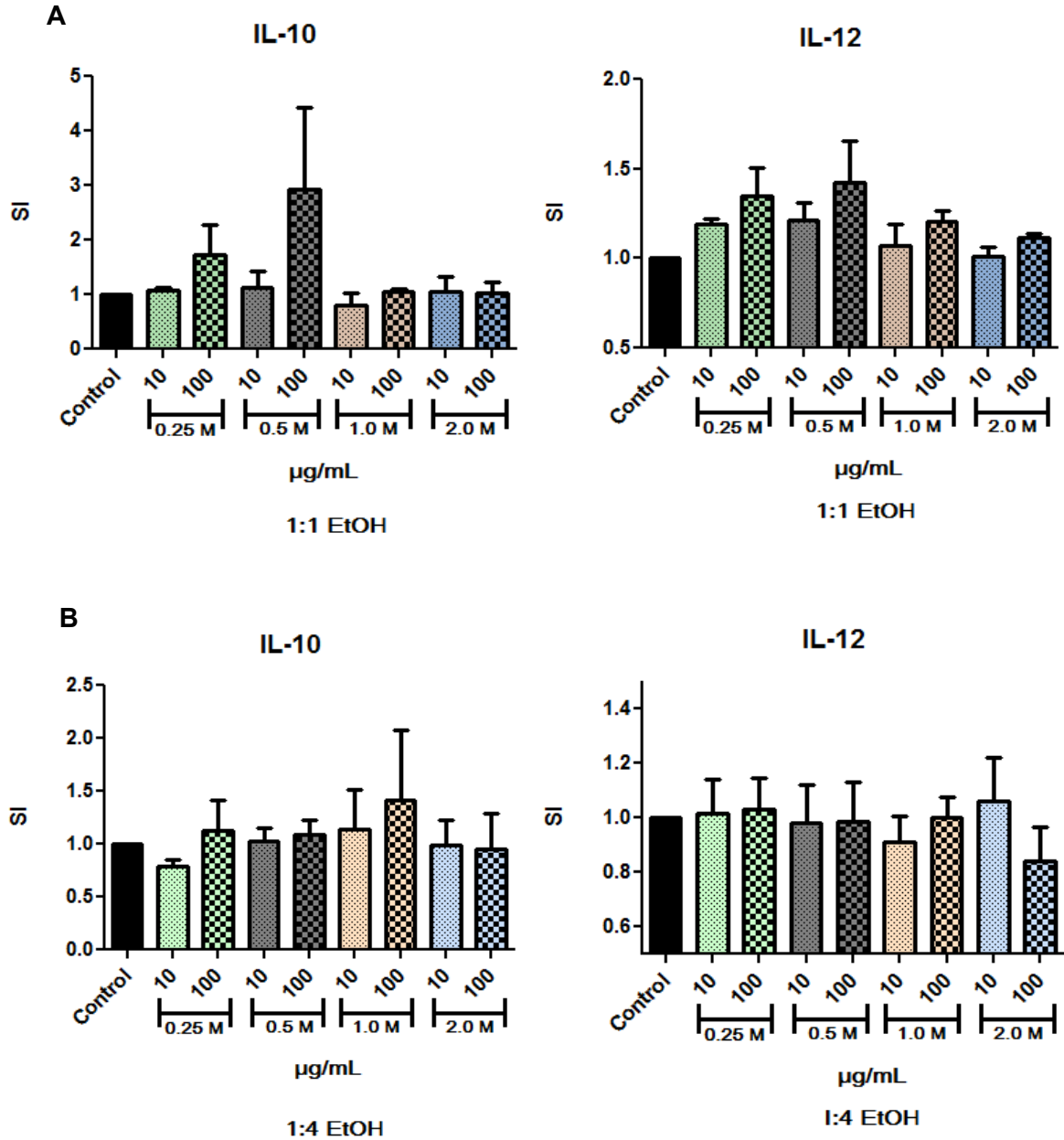


Figure A 3. Effects of ion exchange column from ethanol precipitation fractions on IL-12p40 and IL-10 secretion by THP-1 monocytes.

THP-1 monocytes were primed with IFN- γ for 3 h and then stimulated with LPS for 48 h (control). Fractions from the ethanol precipitation were added to ion exchange column. Fractions from the EtOH column were added to the cell culture at indicated concentrations simultaneously with the LPS. **A.** 1:1 EtOH precipitated fractions. IL-10 and IL-12p40 concentrations in the supernatants. **B.** 1:4 EtOH precipitated fractions. IL-10 and IL-12p40 concentrations in supernatant. Results are shown as the mean of the SI (cytokine concentration with extract / cytokine concentration without extract) \pm SEM, n=3.

Appendix 4

Fractions from EtOH precipitation and ion exchange column on DCs

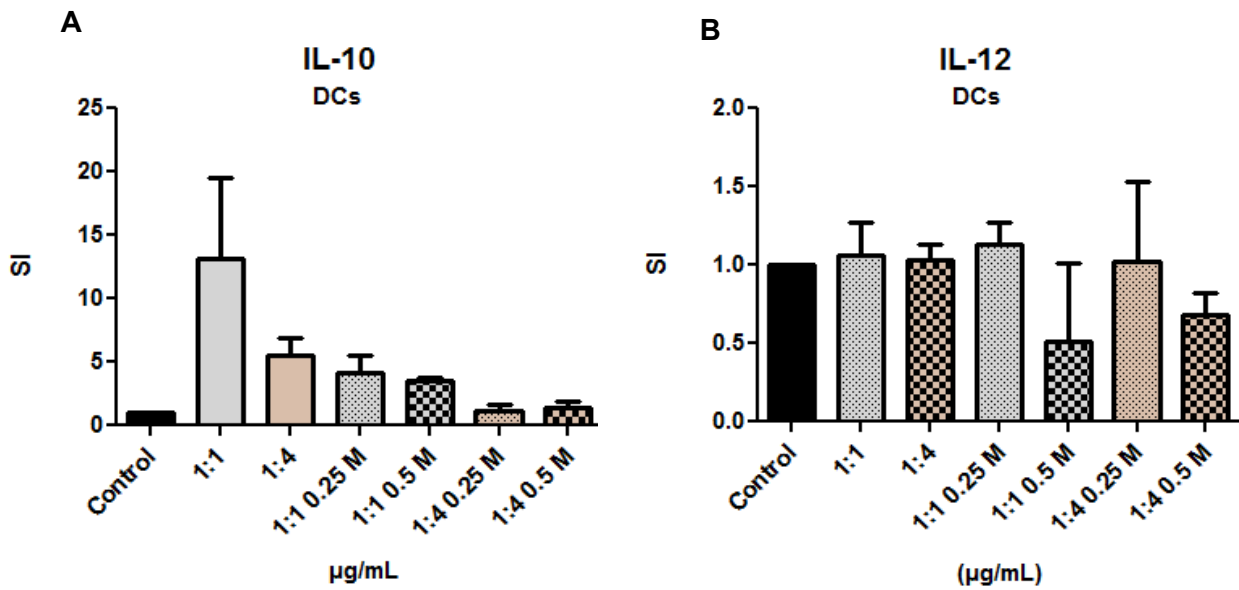


Figure A 4. Effects of ethanol precipitated fractions and ion exchange column from ethanol precipitation fractions on IL-12p40 and IL-10 secretion by DCs.

DCs were stimulated with LPS for 48 h (control). Fractions from the ethanol precipitation and ion exchange chromatography were added to ion exchange column. Fractions from the EtOH column were added to the cell culture at indicated concentrations simultaneously with the LPS. **A.** IL-10 concentration in the supernatants. **B.** IL-12p40 concentration in supernatant. Results are shown as the mean of the SI (cytokine concentration with extract / cytokine concentration without extract) \pm SEM, \pm SEM, n=3.

Appendix 5

Am-25 and Am-25-d comparison in DCs

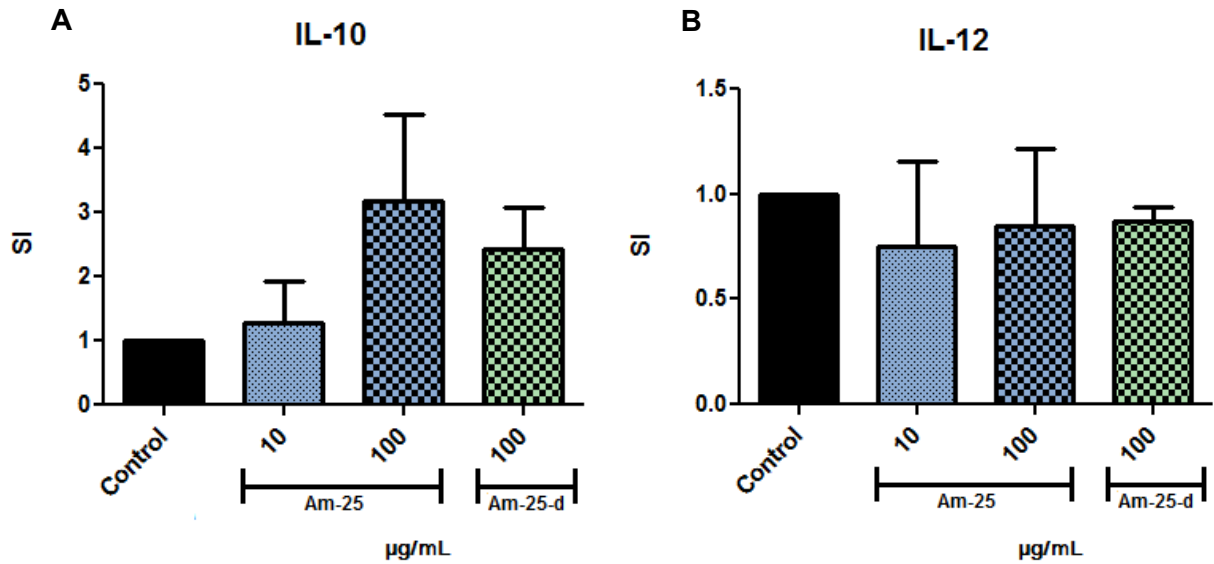


Figure A 5. Effects of Am-25 and Am-25-d on IL-12p40 and IL-10 secretion by DCs.

DCs were stimulated with LPS for 48 h (control). Either Am-25 or Am-25-d polysaccharide was added to the culture at indicated concentrations simultaneously with the LPS. **A.** IL-12p40 and IL-10 concentration in the supernatant. **B.** Ratio between secreted IL-12p40 and IL-10.

Results are shown as the mean of the SI (cytokine concentration with extract / cytokine concentration without extract) \pm SEM, n=4.

Appendix 6

HP-GPC spectrum

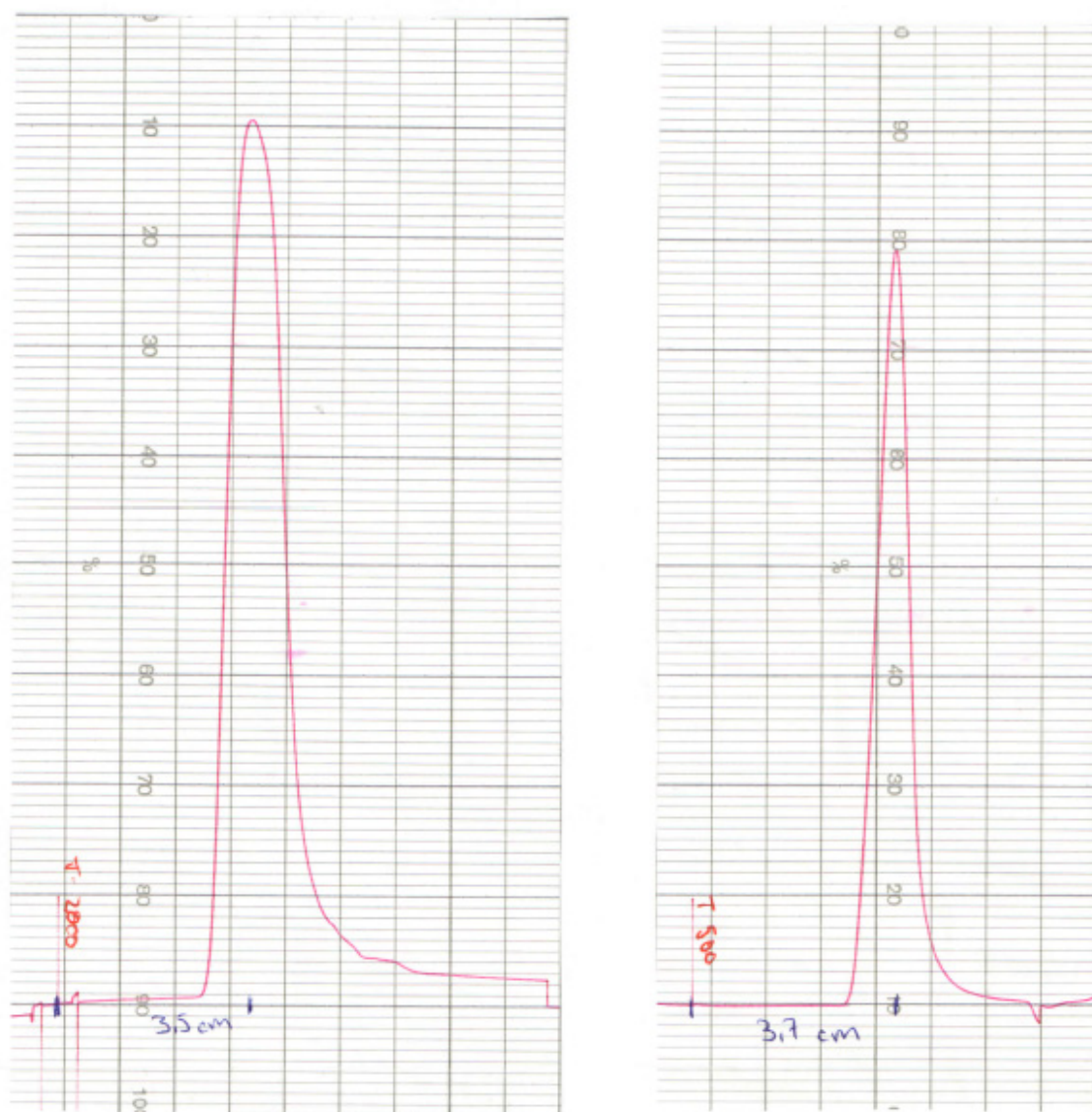


Figure A 6. HP-GPC spectrum for Dextran standards.

1% Dextran T2000, MW = 2,000,000 Da (left) and T500, MW = 500,000 Da (right).

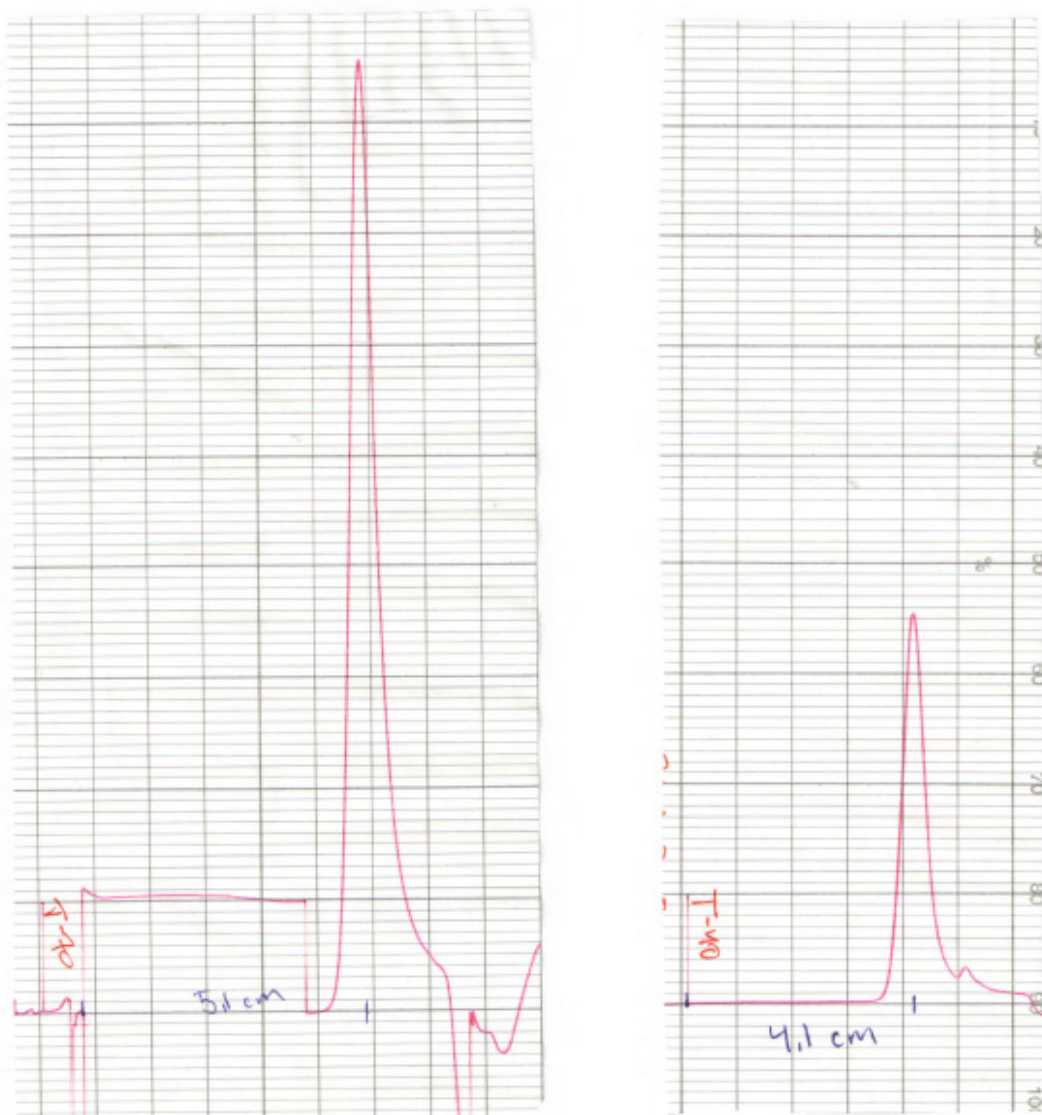


Figure A 7. HP-GPC spectrum for Dextran standards.

1% Dextran T70, MW = 70,000 Da (left) and T40, MW = 40,000 Da (right).

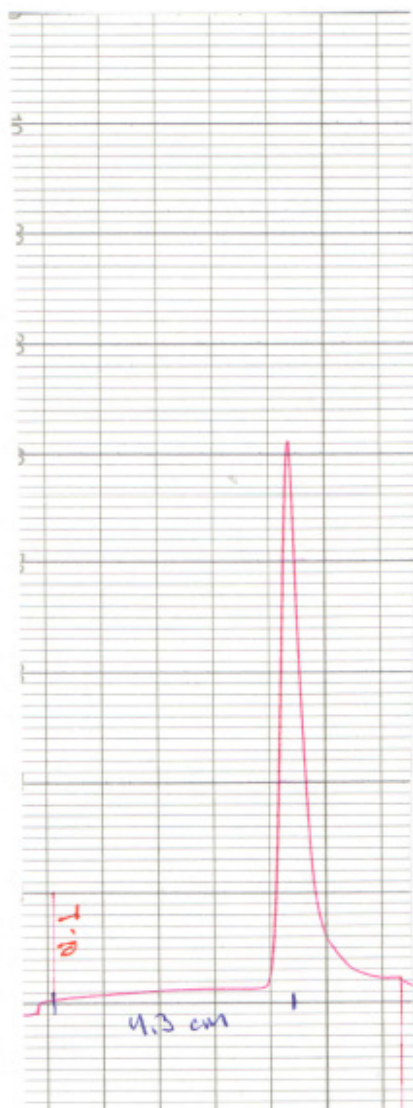


Figure A 8. HP-GPC spectrum for Dextran standards and glucose.

1% Dextran T10, MW = 10,000 Da (left) and glucose, MW = 180 Da (right).



Figure A 9. HP-GPC spectrum for Am-25-d.

Am-25-d, MW = 535 - 267,000 Da

