The effect of cystatin C amyloid and T-ChOS on THP-1 cells

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Thesis for the degree of Master of Science

University of Iceland
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HÁSKÓLI ÍSLANDS

Áhrif cystatín C mýlildis og T-ChOS á THP-1 frumur

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ÁGRIP

Það er mikilvægt að rannsaka áhrif mögulega lífvirkra efna við vel skilgreindar aðstæður. Ein leið til þessa er að rannsaka áhrif á frumur ónæmiskerfisins hvort sem um jákvæð eða neikvæð áhrif er að ræða á t.d. ónæmissvörun. Í þessari rannsókn voru áhrif tveggja ólíkra lífvirkra efna skoðuð á THP-1 mónócýta frumulínuna; cystatín C mýlildi annars vegar og T-ChOS kítófáliðan, hins vegar. Einnig var notast við æðaþelsfrumur einangraðar úr naflastreng. Áhrif beggja þessara efna á THP-1 frumulínuna voru rannsökuð, svo sem á frumusérhæfingu, tjáningu mikilvægra gena í ónæmissvörun og gena viðloðunarpróteina.

Vitað var að cystatín C mýlildi hefur eitrandi áhrif á frumur en ekki er vitað um nein eitrandi áhrif T-ChOS á frumur. Cystatín C mýlildið er einangrað úr heilum sjúklinga sem látist hafa úr arfgengri heilablæðingu af íslensku gerðinni, sjúkdómi sem veldur ótímabærum dauða. Kítófáliður eru leiddar af fjölsykrunni kítíni sem talið er að séu lífvirkar í m.a. mannslíkamanum.

Cystatín C mýlildi olli því að mónócýtarnir sérhæfðust yfir í makrófaga. THP-1 mónócýtar og makrófagar brutu niður cystatín C mýlildi. THP-1 frumur loðuðu við, og átu líklega upp cystatín C mýlildi á þekjuglerjum og staðfest var að uppleyst cystatín C mýlildi hvarf úr frumurækt. Áhrif cystatin C mýlildisins eru svipuð og annars mýlildis, sem bendir til þess að meðferðir sem nýtast í öðrum mýlildisjúkdómum sem hafa áhrif á æðakerfið, gætu nýst sjúklingum. Aukin tjáning gensins *TNF-α*, sem er mikilvægt í bólgusvörun, er sérlega áhugaverð, en hún bendir til þess að hluta af meinafræði sjúkdómsins mætti útskýra með bólgusvörun í æðakerfinu, en þá svörun mætti mögulega hindra og þannig auka batahorfur sjúklinga.

T-ChOS hafði, á hinn bóginn, engin áhrif á sérhæfingu í THP-1 frumum en breytileg áhrif á genatjáningu kítínasans *CHIT-1* og kítínasa-líka próteinsins *YKL-40* í makrófögum.

T-ChOS hafði jákvæð áhrif á gen viðloðunarpróteina í æðaþelsfrumum og cystatín C mýlildi hafði einnig áhrif á gen viðloðunarpróteina í THP-1 frumum. Þetta bendir til þess að bæði efnin geti aukið viðloðun milli fruma, en viðloðun mónócýta við æðaþelsfrumur er eitt af fyrstu skrefunum í ónæmissvari líkamans.

ABSTRACT

Studying the effects of potentially bioactive compounds under well defined conditions is of importance. Whether pro or anti stimulatory, the effects can be studied on the cells of the immune system. In this study, two distinct bioactive compounds were analyzed, cystatin C amyloid and T-ChOS, a chitooligosaccharide. Endothelial cells isolated from umbilical cord were also used. The effects of these two substances on the THP-1 cell line were studied, e.g. monocyte differentiation and the expression of genes coding for inflammation and adhesion proteins.

Cystatin C amyloid is known to be cytotoxic but the same has not been reported for T-ChOS. The amyloid can be isolated from brains, *post mortem* of patients that have died of Hereditary Cerebral Hemorrhage with Amyloidosis, Icelandic type (HCHWA-I), a disease which causes premature death. Chitooligosaccharides (ChOS) are derivatives of chitosan, both regarded as being bioactive physiologically.

Cystatin C amyloid induced the differentiation of monocytes to macrophages. It was degraded by the THP-1 monocytes, as well as the macrophages. THP-1 cells adhered to, and likely phagocytosed cystatin C amyloid on cover slips and the disappearance of cystatin C amyloid from cell culture was confirmed. The effects of the cystatin C amyloid are similar to other amyloids, indicating that treatments beneficial in other amyloid vascular diseases could be beneficial to HCHWA-I patients. Of great interest was the large response in the pro-inflammatory gene *TNF-α* indicating that some of the disease pathology can be explained due to inflammation in the vascular system. The potentially damaging inflammation might be inhibited leading to increased prognosis.

T-ChOS showed no effect on the differentiation of THP-1 cells. It showed variable effect on the gene expression of the chitinase *CHIT-1*, and the chitinase-like protein (CLP) *YKL-40,* in macrophages.

Both T-ChOS and cystatin C amyloid had an effect on adhesion proteins in HUVEC cells and THP-1 cells, respectively, indicating that the substances might be able to induce cell contact between these two cell types, but the adherence of monocytes to endothelium is one of the first steps in the inflammatory process.

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I would like to thank a few people that have contributed to the making of this project.

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ABBREVIATIONS

Aβ	Amyloid β
AD	Alzheimer's disease
AMCase	Acidic Mammalian Chitinase
βAPP	β Amyloid Precursor Protein
CAA	Cerebral Amyloid Angiopathy
CHIT-1	Chitotriosidase
ChOS	Chitooligosaccharides
CLPs	Chitinase-like proteins
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
ddH_2O	Double distilled water
dH_2O	Deionized water
FBS	Fetal Bovine Serum
GlcN	Glucosamine
GlcNAc	β(1→4)linked *N*-acetyl-*D*-glucosamine
Gln	Glutamine
HCHWA-D	Hereditary Cerebral Hemorrhage with Amyloidosis-Dutch type
HCHWA-I	Hereditary Cerebral Hemorrhage with Amyloidosis-Icelandic type
HUVEC	Human Umbilical Vascular Endothelial Cells
ICAM	Intercellular adhesion molecule
IL-6	Interleukin-6
IL-8	Interleukin-8
Leu	Leucine
PBS	Phosphate Buffered Saline
PMA	Phorbol 12-myristate 13-acetate
RealTime PCR	Real Time polymerase chain reaction
SDD-AGE	Semidenaturing detergent agarose gel
SDS PAGE	SDS polyacrylamide gel
T-ChOS	Therapeutic chitosan oligosaccharides
THP-1	Human acute monocytic leukemia cell line
TNF-α	Tumor necrosis factor-α
VCAM	Vascular cell adhesion molecule

I AIMS OF THE STUDY

The aim of this project was to examine the effects of two distinct bioactive compounds on the differentiation and gene expression in the THP-1 cell line, a well established model for human monocyte studies. The compounds used were cystatin C amyloid isolated *post mortem* and kindly supplied by Dr. Finnbogi Þormóðsson, and T-ChOS, generated from shrimp shells by the company Genís ehf.

The primary hypothesis was that cystatin C amyloid, the first compound tested, would elicit a similar response in THP-1 cells as other amyloids, such as the extensively studied Aβ amyloid, a principal characteristic of Alzheimer's disease (AD).

Specific aims:

1. Determine the composition of the isolated cystatin C amyloid using Western blotting and Thioflavin T staining.
2. To study the effect of the amyloid on differentiation/adherence of the THP-1 cells in culture and determine whether the cells take up the amyloid or degrade it in some manner.
3. To study the effect of cystatin C amyloid on the up/downregulation of specific inflammation markers.

The second compound tested, is a derivative of chitin. The company Genís ehf. has developed a mixture of chitooligosaccharides (ChOS) called T-ChOS (therapeutic chitosan oligosaccharides) that likely have bioactivity in the body. The hypothesis was that when THP-1 cells were exposed to T-ChOS differentiation might be affected and chitin binding proteins would be induced. Also, another goal of this study was to determine the effect of T-ChOS on primary human endothelial cells. The hypothesis was that the endothelial cells might express adhesion proteins in response to the T-ChOS.

Specific aims:

1. To study whether T-ChOS can affect differentiation/adherence of the THP-1 cells.
2. To compare the expression of chitinases and CLPs in differentiated and undifferentiated THP-1 cells.
3. To study the effect of T-ChOS on the expression of adhesion proteins in HUVEC cells.

II INTRODUCTION

2.1 Cystatin C

2.1.1 Cerebral amyloid angiopathy

Cerebral amyloid angiopathy (CAA) is the collective term used to define pathological amyloid deposition in brain vasculature, reviewed in (1). The most common type of CAA is caused by the amyloid β (Aβ) protein which is the primary hallmark of Alzheimer's disease (AD), a neurodegenerative condition characterized by the presence of senile plaques, composed of Aβ amyloid, in the brains of afflicted patients (2). CAA is found in 90% of brains from patients with AD (3), but CAA also exists in other disorders, e.g. Down's syndrome and in the elderly (1). Rare forms of hereditary CAA have been identified, where the amyloid is made up of a different variant of Aβ, e.g. Hereditary Cerebral Hemorrhage with Amyloidosis – Dutch type (HCHWA-D). The amyloid formation in HCHWA-D is caused by a mutation at codon 693 of the β Amyloid precursor protein (βAPP) gene (4). Hereditary Cerebral Hemorrhage with Amyloidosis – Icelandic type (HCHWA-I) is another hereditary form of CAA where the amyloid forming protein is the protease inhibitor cystatin C (5).

2.1.2 Hereditary Cerebral Hemorrhage with Amyloidosis-Icelandic type

Hereditary Cerebral Hemorrhage with Amyloidosis–Icelandic type (HCHWA-I) is an autosomal dominant genetical disorder leading to cerebral hemorrhage early in life (3, 6-9). It has only been found in Iceland with one exception, a Croatian man with CAA was found to have the same mutation (10). Multiple strokes cause the development of dementia and paralysis, and generally, death from cerebral hemorrhage before the age of 40 years, characterized by the disappearance of the cerebral vascular smooth muscle cells (3, 9). Smooth muscle cells in the brains of patients disintegrate and show progressive loss as cystatin C amyloid accumulates (**Figure 1**) (3, 11). Fibrosis has also been suggested to play a major role in the pathology (Birkir Bragason, personal communication).

In 1983, amyloid fibrils were isolated from leptomeningeal blood vessels from patients that had died of HCHWA-I, verified by congo red staining, and determined to be composed of the cystatin C protein (12). Congo red staining of tissue sections had previously revealed amyloid deposits in the arteries of the central nervous system

(CNS) (9), but the type of amyloid-forming protein is used to categorize the specific CAA disorder (1). In HCHWA-I, the protein is a mutated form of cystatin C found only in HCHWA-I patients (5, 13).

Cystatin C amyloid can be isolated from the leptomeninges of HCHWA-I patients, *post mortem,* by a modification of a method developed by Pras *et al.* (3, 14). Smooth muscle cells receiving soluble cystatin C amyloid in culture show morphological changes, such as pyknotic nuclei, retraction of cellular processes and cell body rounding, in concentration-dependent manner and a dramatic reduction in cell numbers with more than half the cells gone in 4 days (3). Hence, the *post mortem* isolated cystatin C amyloid is cytotoxic and can be used to study the molecular mechanisms behind the disease.

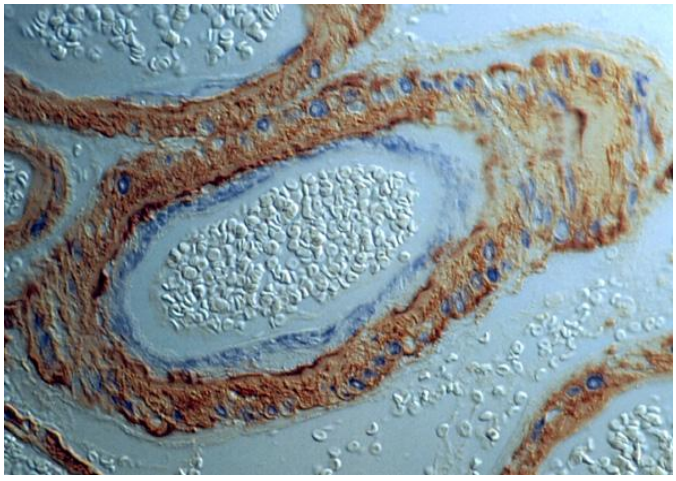

Figure 1. An artery of an HCHWA-I patient. α-actin of the smooth muscle cells is in blue and cystatin C is in brown. The majority of the smooth muscle cells have been buried in cystatin C amyloid, which is filling up the vessel wall. This image is courtesy of Dr. Finnbogi Þormóðsson.

2.1.3 Function

Cystatin C is a 13kD protein consisting of 120 amino acid residues (15). It is abundant in cerebrospinal fluid (CSF) and seminal plasma but can be found in all cells and tissues of the body (16) operating as a cysteine protease inhibitor, inhibiting e.g. cathepsin B and papain (17). Accumulation of amyloid fibrils made from a mutated form of cystatin C is a major factor of the pathogenesis of HCHWA-I (9). The cystatin C mutation is caused by a T→A point mutation resulting in a L68Q amino acid substitution in the cystatin C protein (13, 18). The amino acid substitution in the cystatin C mutant results in accelerated amyloid deposition, but the exact changes in the protein are unknown (19). The substitution does not affect inhibitory activity, both the mutant and wild type effectively inhibited the cysteine protease cathepsin B (19, 20). Upon dimerization, however, cystatin C loses its cysteine protease inhibitory activity indicating that the association may involve the active site (21).

2.1.4 Amyloid and cystatin C amyloid

All HCHWA-I patients tested have been heterozygous, producing both wild type and mutated cystatin C (8) and have the same mutation (5, 22). In human cell lines where mutated and non-mutated cystatin C was expressed, the variant cystatin C formed dimers at concentrations lower than those necessary for dimerization of the wild type protein (19). Dimer formation over a wide temperature range is favored in the mutated form causing it to precipitate and aggregate (8). This coincides with the acceleration of aggregation of the L68Q mutant with increasing temperature (20).

Once proteins are exposed to e.g. increased heat they can lose their native conformation more rapidly. The proteins then generally become unfolded by denaturation. The denatured state is thermodynamically unfavorable and unstable and therefore, the unfolded proteins tend to aggregate (**Figure 2**) (23). Relatively little is known about the process of cystatin C amyloid formation but extensive research has been conducted on the formation of Aβ amyloid, its aggregation state and its effect on cells and their response (11, 24). The assembly of monomeric Aβ into a fibrillar form has been studied where numerous species in the formation have been identified, according to their size, length, solubility and morphology (24).

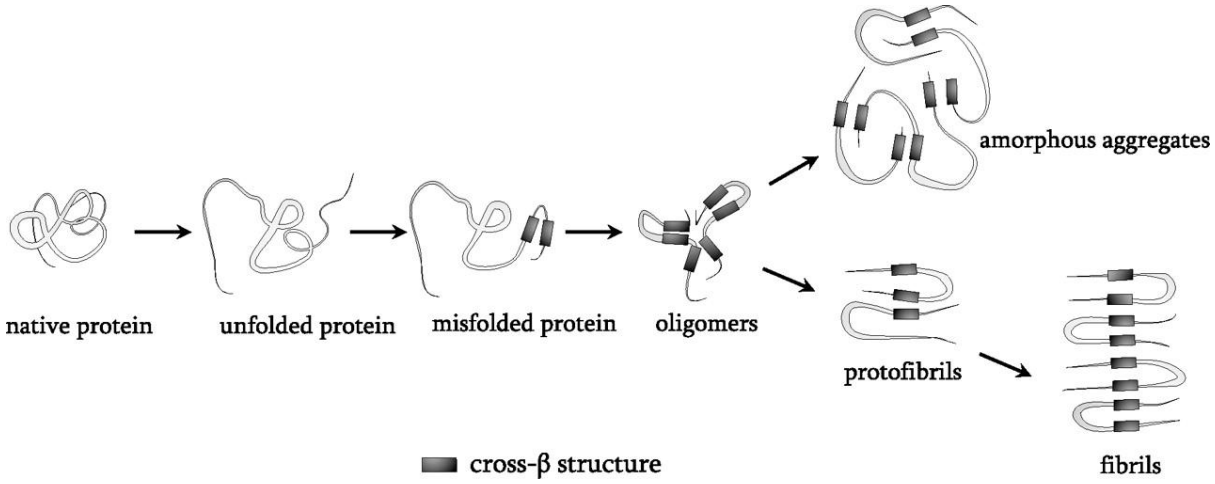

Figure 2. Protein misfolding and aggregation. Proteins can undergo conformational changes resulting in unfolding under certain circumstances e.g. pH or temperature change. During aggregation, proteins can obtain a range of different structures, generally enriched in β-sheets, including unordered amorphous aggregates to ordered fibrils, the amyloid. Image adapted from (23).

Why and how amyloid fibrils are formed is not completely understood. A common denominator of all amyloids is that they consist of polypeptide chains arranged in a β-

sheet. These polypeptides then give rise to amyloid deposits composed of accumulations of linear, nonbranching fibrils of indefinite length (25). A number of mechanisms have been proposed to explain the cytotoxicity of amyloids, reviewed in (26). However, the fibrillar, or amyloid structure itself, is the toxic element. For example, both Aβ and amylin fibrils cause comparable changes in inflammatory markers, indicating that cells are likely responding to the fibrillar structure of the proteins instead of to a specific sequence motif since amylin forms amyloid deposits in the pancreas but has little sequence homology to Aβ (27). Microglial cells of the brain phagocytose amyloid deposits in the CNS (28) and have also been reported to take up Aβ *in vitro* (29, 30). Other cell types have also been shown to degrade amyloid e.g. mouse astrocytes *in vitro* and *in situ* (31).

Activation of cells of the immune system leads to inflammation, a process which can be damaging in itself when chronic (32). It has long been known that inflammation occurs in the AD brain, partly brought on by Aβ amyloid (33). The role of inflammation in AD is complex. Significantly higher levels of the inflammation marker tumor necrosis factor- α (TNF-α) were found in AD serum compared to controls in a study (34), but high levels of TNF-α are known to be damaging to the vascular system (35, 36). The inhibition of TNF-α may hold promise as a potential approach to AD treatments (37). Little is known about the possible role of inflammation in HCHWA-I. One of the major goals of this study was to determine whether cystatin C amyloid induces the same response in THP-1 cells as reported for Aβ and whether the THP-1 cells are able to remove cystatin C amyloid. A key question in AD and HCHWA-I is why the amyloid accumulates despite being removed or why it is not completely eliminated. One explanation could be that the degradation of aggregates is reduced by age (in some AD patients) or that the mechanisms of degradation are defective (in HCHWA-I). If so, activating or assisting the degradation system could help control the damage caused by the amyloid forming proteins. Understanding the effect of cystatin C amyloid on THP-1 cells is the first step in this direction.

2.2 Chitin

Chitin is one of the most abundant polysaccharides found in nature, second only to cellulose. It is a linear polysaccharide consisting of β(1→4)linked *N*-acetyl-*D*-glucosamine (GlcNAc) residues, found in bacterial and fungal cell walls as a structural

component and in the exoskeletons of various animals such as beetles, ants, crabs, shrimp and lobsters (38). It is insoluble in water but can be broken down with various methods to form chitosan, reviewed in (39).

2.2.1 Chitosan

By partially deacetylating chitin, a heteropolymer of GlcNAc and D-Glucosamine (GlcN) is produced, called chitosan (**Figure 3**). The exact chemical composition of chitosan is less easily defined than chitins. Chitosan is most often characterized by the number of sugar units per polymer molecule (n), which defines the molecular weight, and the degree of acetylation (40). When characterizing a chitosan sample, its average degree of acetylation needs to be determined (41). Chitosan is soluble in dilute aqueous acid solutions (42) but the degree of acetylation affects the solubility. When the degree of deacetylation of chitin reaches about 50%, it becomes soluble and is called chitosan (41).

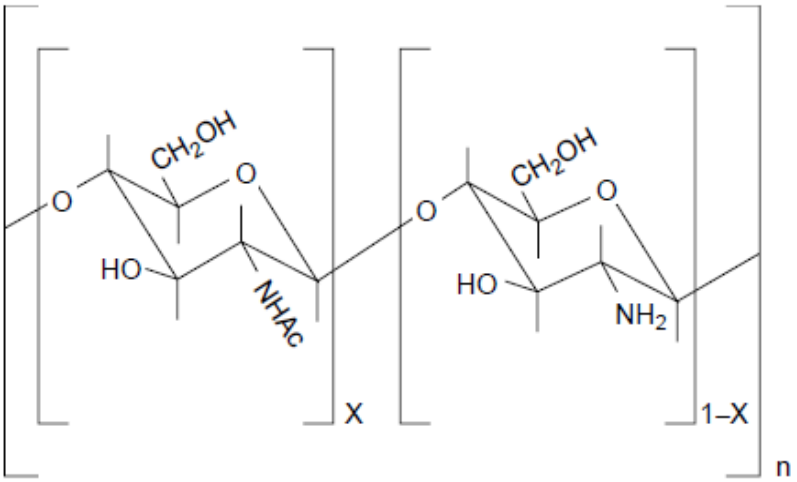

Figure 3. The chemical structure of chitosan. X: degree of deacetylation, n: number of sugar units per polymer (40).

Chitosan has garnered increasing interest over the years for use in e.g. cosmetics and medicine due to its interesting physiochemical and proposed biological properties as well as being generally considered non-toxic, biocompatible and biodegradable (reviewed in (39). It is largely used in different applications as solutions, gels, or films and fibers (41). Chitosan can be further broken down into chitooligosaccharides.

2.2.2 Chitooligosaccharides

Chitooligosaccharides (ChOS) are the degraded products of chitosan or chitin, produced either enzymatically (with glycosyl hydrolases such as chitinases or

chitosanases) or chemically (acid hydrolysis). The molecular weights of ChOS are generally 10 kDa or less (43).

Numerous reports on ChOS bioactivity can be found in literature, but the detailed molecular mechanisms have not yet been elucidated (43). Chitinase B, member of the family 18 chitinases, was found to directly interact with natural ChOS mixtures (44). Inhibiting family 18 chitinases is an emerging target in inflammatory therapy along with fungal control. ChOS have the ability to serve as such inhibitors (45). Genís ehf., an Icelandic company, has been producing biologically active ChOS from shrimp shells for the past few years. A well-defined mixture of ChOS called therapeutic chitosan oligosaccharides (T-ChOS) is one of Genís' main products. Since T-ChOS is a mixture of ChOS, it has been postulated that it can inhibit chitin binding enzymes, chitinases and chitinase-like proteins (CLPs) (Genís ehf., personal communication).

2.2.3 Chitinases and chitinase-like proteins

Despite there being more than one billion tons of chitin produced each year in nature, it does not accumulate in most ecosystems (46). The breakdown of chitin is carried on by enzymes termed chitinases belonging to the glycosyl hydrolase family 18. Studies have identified chitinases in mammals, including humans, despite the absence of chitin in the mammalian body (46). Some of these chitinases and chitinase-like proteins (CLPs) are YKL-40 (also known as chitinase 3-like-1) (47), chitotriosidase (CHIT-1) (48), YKL-39 (49), and acidic mammalian chitinase (AMCase) (50).

CHIT-1 and AMCase possess chitinase enzymatic activity whereas the other chitinases and CLPs, including YKL-39 and YKL-40, do not. In YKL-40, an essential glutamic acid residue has been substituted with leucine in the binding site for chitin, resulting in the absence of chitinase activity (47). However, YKL-40 can still bind chitin and ChOS with high affinity (51). Plasma CHIT-1 originates from activated macrophages (52) giving rise to CHIT-1 being established as a biochemical marker for lysosomal storage diseases (53).

2.3 THP-1 cells

Macrophages are a key cell type in immunity, derived from monocytes. Studies of human monocytes and macrophages are often restricted due to the limited amounts of human monocytes available for research (54). A monocyte cell line, the monocytic

leukemia cell line, THP-1, was isolated by Tsuchiya *et al.* (55) from a young boy suffering from acute monocytic leukemia. THP-1 cells have not been shown to have a specific genetic abnormality (56). Due to its close resemblance to human monocytes, the THP-1 cell line has been used extensively as a monocyte model.

2.3.1 THP-1 cells as a monocyte model

The THP-1 cell line resembles human monocytes in many ways, i.e. morphology, secretion profile and membrane antigens. By studying cell lines as opposed to using human primary cells from multiple donors, it is possible to base the investigation on a relatively homogenous groups of cells (54). Cells from the THP-1 cell line can be differentiated into macrophages, important phagocytes of the body.

2.3.2 Macrophages

Monocytes circulating the body can differentiate into a variety of tissue-resident macrophages all around the body as well as some specialized cells such as osteoclasts and dendritic cells. They originate in the bone marrow and are released into the peripheral blood where they circulate for a few days before entering tissues, replenishing the tissue macrophage populations (reviewed in (57)). However, this development and differentiation pathway is still relatively poorly understood *in vivo* (57).

In this study, the effect of T-ChOS on macrophage gene expression of chitinases and CLPs was investigated.

2.3.3 Differentiation

The THP-1 cell line is a well established model system for studying monocyte differentiation. A dramatic alteration in cell morphology is seen as THP-1 cells differentiate to macrophages with the development of an adherent cell phenotype. Cells acquire a variety of shapes after the initiation of the differentiation process and many phagocytic vacuoles can be recognized in their cytoplasm (54).

Various compounds have been used to differentiate THP-1 cells such as Vitamin D3 (58, 59), 7-ketocholesterol (60), iron chelator (61), phorbol 12-myristate 13-acetate (PMA) (62) and Aβ amyloid (24).

2.3.4 Monocytes and adhesion to endothelium

The cells of the immune system must be able to circulate through the body as nonadherent cells in blood and lymph and migrate through tissues and turn on proteins for adhesion and be able to cross endothelial barriers when needed. The transition between adherence and nonadherence is a key step in the rapid response of the immune system (63).

The interactions of white blood cells and endothelial cells play a central role in inflammation where different proteins mediate adhesion (64). A major part of the current knowledge of the endothelial function comes from the study of human umbilical vascular endothelial cells (HUVECs), *in vitro* (65). Amongst the surface adhesion proteins that have been studied are E-selectin, intercellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM). ICAM is expressed in low levels in vascular endothelium, increasing dramatically when inflammatory stimuli is present along with VCAM (66, 67). These adhesion proteins bind to receptors on monocytes, facilitating entry of cells into tissues. Monocytes have also been reported to respond to bacteria by expressing ICAM and VCAM (68), which, as a result, probably mediate cell-cell contact between monocytes.

2.3.5 The effect of Aβ amyloid on monocytes/macrophages

One of the aims of this study was to determine the effect of cystatin C amyloid, isolated *post mortem*, on the adhesion/differentiation of THP-1 cells. The effect of Aβ amyloid on various cell types is well established as well as its differentiation effect on THP-1 cells. Aβ can be considered a model amyloid as much is known about the composition and cytotoxicity of Aβ. Little is known about cystatin C amyloid and its cytotoxicity. As stated above, the general toxicity is expected to be the same for all amyloids. Cystatin C amyloid's effect on THP-1 cells was therefore expected to be similar to Aβ's effects. In this study, *ex vivo* amyloid was used while other studies usually use aggregates generated from peptides *in vitro*. This is of importance since it could be argued that the material used here, while more heterogenous, is a better model for *in vivo* bioactivity. This study is, as far as we know, the first one to measure the effect of *ex vivo* amyloid on THP-1 cells.

Effect on monocytes:

Aβ peptides cause human monocytes/macrophages to release increased amounts of pro-inflammatory cytokines and/or chemokines (69-71). Fiala *et al.* found Aβ to induce the secretion of pro-inflammatory cytokines TNF-α and interleukin-6 (IL-6) in human monocytes as well as stimulating the cells to adherent macrophages in a dose dependent fashion (70). Aβ amyloid enhances glial cell secretion of IL-1 and stimulates the proliferation and morphological transformation of microglia (69) as well as inducing the cells to produce TNF-α (71).

The deposition of extracellular aggregates of Aβ amyloid within senile plaques, as well as the intracellular accumulation of neurofibrillary tangles, is the main hallmark of AD (2).The presence of Aβ appears to encourage monocyte/macrophage infiltration to sites of Aβ accumulation as well as monocyte adhesion and differentiation into macrophages (28, 70, 72). El Khoury *et al.* found microglia to play a protective role in AD by mediating Aβ clearance, suggesting that strategies to enhance their accumulation to plaques may be beneficial for the treatment of early stages of the disease (72).

Effect on THP-1 cells:

Aβ induced adhesion of THP-1 monocytes as effectively as PMA following 6 hours of exposure, in a dose dependent manner (24). Ajit *et al.* sought to clarify which Aβ morphology or aggregation species induced the strongest pro-inflammatory response using THP-1 monocytes as a model system. They searched for the optimal Aβ aggregation state for the adhesion process and found the fibrillar precursor of an intermediate stage to be optimal for inducing a pro-inflammatory response in THP-1 monocytes (73). Udan *et al.* demonstrated that fibrillar Aβ triggers the innate immune response to a higher extent than Aβ amyloid, producing TNF-α in the THP-1 cell line (74).

2.3.6 The effect of chitin derivates on monocytes/macrophages

It seems plausible that chitin derivatives might affect either differentiation of monocytes or macrophage function e.g. because chitin containing parasites or fungal infections activate the immune response. Not much is known about the possible effect of chitin derivatives on the monocyte lineage, refer to (75, 76).

Chitosans with different chemical compositions were found to induce TNF-α production from human monocytes (77). High molecular weight chitosan upregulates the production of interleukin-1 (IL-1), TNF-α, and lL-6 in macrophages (78). Chitosan has been shown to induce increased levels of CHIT-1 with no difference in inflammation markers observed (76).

III MATERIALS AND METHODS

3.1 Cell Culture

3.1.1 THP-1 cells

The human acute monocytic leukemia cell line (THP-1) (a contribution from Magdalena Maria Stefaniak at The Blood Bank, University Hospital, Iceland, obtained from ACTT) was maintained in T-25 tissue bottle flasks (Falcon) in RPMI-1640 (Roswell Memorial Park Institute) media (Gibco) supplemented with 2% or 10% FBS (Fetal Bovine Serum (HyClone)). For maintenance, the cells were split every 3 days by removing half of the medium containing cells and adding equal amount of fresh media or by removing the cells, spinning for 3 min at 1500 rpm and splitting 1:2. Cells were seeded at a 5-7x10^5 cells/mL density for all experiments. The cells were cultured at 37°C in 95% humidified 5% CO_2 atmosphere.

3.1.2 THP-1 cell numbers and viability determined

A 10µL cell culture sample was added to 10µL of Trypan Blue (Invitrogen). Cell number and viability was determined with Countess (Invitrogen), an automated cell counter, by adding a 10µL sample from the Trypan blue cell mixture to a Countess cell counting chamber slide (Invitrogen). Total cell numbers are given in number of cells/mL.

3.1.3 HUVEC cells

Human Umbilical Vein Endothelial Cells (HUVEC) (a contribution from Bjarni Þór Sigurðsson) were maintained in T-25 tissue bottle flasks in EGM-2 BulletKit (Lonza-CC-3162) media and used at passage 3. The cells were cultured at 37°C in 95% humidified 5% CO_2 atmosphere.

3.2 Cover slips

3.2.1 Acid wash of cover slips

Round cover slips (15mm) (Assistent) were heated in a loosely covered glass beaker in 1M HCl at 50-60°C for 4-16 hours. After cooling, the cover slips were washed

extensively in deionized water (dH_2O), then double distilled water (ddH_2O) and stored in 70% EtOH solution until used. When used, the cover slips were sterilized further by flaming off the EtOH.

3.2.2 Coating cover slips for THP-1 cell adhesion

Three different coatings were used, 0.1% Polylysine, 0.1% Gelatin, both in phosphate buffered saline (PBS) (137mM NaCl, 2.7mM KCl, 8mM Na_2HPO_4, 2mM KH_2PO_4) or 0.05% Collagen I (Rat tail collagen I in 0.02M acetic acid). Each coating solution was added to cover slips and incubated at 37°C for 30 min.

3.3 Cystatin C amyloid

3.3.1 Cystatin C amyloid isolation

Cystatin C was isolated from the brains of HCHWA-I patients *post mortem* by modification of a method described by Pras *et al.* (14) and Vilhjalmsson *et al.* (3). The freeze dried cystatin C amyloid was kindly supplied by Finnbogi Þormóðsson. It was soluble in ddH_2O but needed to be vortexed thoroughly. The 200µM stock solution was stored at 4°C.

3.3.2 Fibrillar test for cystatin C amyloid using Thioflavin T

Cystatin C amyloid was stored at either 4°C or 37°C. Samples of the amyloid were taken at different time points and added to a 0.05mM Thioflavin T (Sigma) solution (in 10mM potassium phosphate and 150mM NaCl). The fluorescence intensities of the samples were compared by measuring in a Fluoromax (Spex) Spectrofluorometer by excitation at 440nm (5nm slit width) and emission at 480nm (5nm slit width). The emission at 480nm was used for comparison. Three scans were averaged.

3.3.3 Cystatin C amyloid dried on cover slips

A 10µL sample of 100µM cystatin C amyloid was placed on a 15mm round cover slip (previously acid washed) and dried overnight in an incubator at 37°C.

3.3.4 Thioflavin T staining

A Thioflavin T working solution (0.2% w/v) was prepared in 0.01M HCl and applied onto cover slips containing dried cystatin C amyloid for 10 min. Samples were washed by gentle immersion and incubated for 10 min with 0.1% (v/v) acetic acid and washed in PBS and dH_2O.

3.4 Differentiation of THP-1 cells

3.4.1 Differentiation of THP-1 cells into macrophages with PMA

Differentiation onto a plastic surface

THP-1 cells were seeded into a 24-well plate in RPMI-1640 media supplemented with 2% FBS. 50ng/mL of PMA (Phorbol 12-myristate 13-acetate (Sigma)) was added to the cell culture. Two days later, the cells were washed gently with PBS and reincubated with fresh PMA free media. Two days later, protein was extracted from the cells or they used for further experiments.

Differentiation onto glass cover slips

Cover slips (previously acid washed and sterilized) were placed in wells of a 12-well plate and THP-1 cells seeded in RPMI-1640 media supplemented with 2% FBS and 50ng/mL of PMA. Two days later the cover slips were removed from the cell culture plates and prepared for immunocytochemistry.

3.4.2 THP-1 adherence to cover slips with cystatin C amyloid

Cover slips with dried amyloid (see 3.3.3) were added to wells of a 12-well plate, amyloid side up, and THP-1 cells seeded. Two days later, the cover slips were removed from the cell culture plates and prepared for immunocytochemistry.

3.4.3 THP-1 adherence/differentiation to cell plates with various compounds

THP-1 cells were seeded to a 24-well plate or 96-well plate. Various compounds were added to the culture: i.e. T-ChOS, soluble cystatin C amyloid, chitosan or LPS. The cells were monitored for differentiation/adherence for 5 days. Chitosan obtained from Genís ehf. as Chitobiomer (G060724P).

T-ChOS is patented (79, 80). This design of T-ChOS^{TM} is based on years of research and development by Genís ehf., based on the hypothesis that biological activity of chitinous material is mediated through chitinases and chitinase-like proteins in the human body (79). Briefly, T-ChOS^{TM} is made by an extensive hydrolysis of randomly partly deacetylated chitin using family 18 chitinases (80).

3.5 Immunocytochemistry

Cover slips were washed twice with PBS and fixed in 4% formaldehyde (Sigma) for 15 min. The slips were then incubated with 0.1% Triton-X (Merck) for 7 min and blocked with 10% normal goat serum (NGS) for 10 min, washing twice with PBS between each step. The cover slips were then incubated with primary antibody (usually 1/1000 concentration) for 30 min with 5% NGS, washed thoroughly with PBS and incubated with secondary antibody (1/1000 concentration) for 20 min in the dark at room temperature. After washing with PBS and drying, the cover slips were mounted onto a microscope glass slide with Fluoromount (Sigma) and thoroughly sealed with clear nail polish.

3.6 Hematoxylin staining

Adhered THP-1 cells were washed with PBS and fixed in 4% formaldehyde for 15 min. The cells were then stained for 30 min with hematoxylin, previously filtered, and cells washed thoroughly with PBS.

3.7 Protein extraction

Cells were harvested from cell culture flasks, spun down (if suspended in the media) and washed in PBS twice. Ice cold RIPA buffer with protease inhibitors was added to the cells and stored on ice for 10 min, making sure the buffer covered the surface. If the cells had adhered, the cells were scraped with a pipet tip. The cells were then sonicated for 5 sec, if needed. The amount of protein was measured and stored at -20°C.

3.8 Western blotting

Polyacrylamide gels were prepared with 12.5% acrylamide concentration, see recipe in **Table 1**.

Table 1. Preparation of SDS-PAGE gels.

Reagents	Separating gel	Stacking gel
Water	3.1mL	3.4mL
30% Acrylamide	4.2mL	0.83mL
UTB	2.6mL	-
LTB	-	0.68mL
10% APS	100µL	50µL
Temed	5µL	5µL

*LTB: 1.5M Tris, 0.4% SDS, pH 8.8

*UTB: 0.5M Tris, 0.4% SDS, pH 6.8

Protein samples in sample buffer were loaded on the gel in running buffer (200mM Glycine, 0.1% SDS, 20mM Tris (all from AppliChem)). The ladder used was PageRuler™ Prestained Protein Ladder (Fermentas-SM0671). Electrophoresis was performed at a constant current of 20-40mA for 30-90 min. Proteins were then transferred from the gel to a PVDF membrane (Millipore), previously activated in MeOH, at 400mA for 1 hour in cold transfer buffer (25mM Tris, 200mM Glycine, 15-20%v/v MeOH). The membrane was then blocked for 30 min in milk solution (5% w/v non fat milk powder in PBS-T (PBS, 0.1% w/v Tween (Sigma)). After washing in PBS-T the membrane was incubated overnight at 4°C in milk solution containing primary antibody with constant shaking. The membrane was then washed extensively in PBS-T 3X and incubated in milk solution containing secondary antibody for 30-120 min and washed again in PBS-T 3X. The protein bands were visualized by using the enhanced chemiluminescence (ECL) system and detecting the signal with Amersham Hyperfilm (GE Healthcare).

3.9 SDD-AGE blot

THP-1 cells (total 1 million cells) were added to a 12-well plate. Mixing well, 300µL samples were taken at desired time points. The cells are spun down and 1µL of protease inhibitor (Sigma P8340) added at 4°C. Cells were washed 3X in PBS and

40µL RPB buffer (10mM Tris pH 7.5, 150mM NaCL, 1mM EDTA, 1% NP40, 0.5 % SDS) added to the cells with a protease inhibitor and stored at 4°C. Samples were mixed with loading buffer (150mM Tris pH 6.8, 33% glycerol, 1.2% SDS in Bromophenol blue) and run on 20V voltage in 0.1% SDS 1% agarose gel for 5 hours and blotted onto PVDF membrane with capilary transfer (81). This method of analyzing polymers was first described by Kryndushkin *et al.* (82). Western performed as usual.

3.10 Confocal microscopy

The microscope slides were examined in a confocal microscope (Zeiss LSM 5 Pascal), equipped with one Argon (wavelengths 458, 488 and 514nm), and two HeNe lasers (wavelengths 543 and 633nm). The program used to collect images was Pascal LSM 510 release version 4.2 SPI. The software used to view and process the confocal images was Zeiss LSM Image Examiner.

3.11 RealTime PCR

RNA was isolated with RNeasy (Qiagen) and cDNA made from RNA samples with RevertAid (Fermentas) with oligo(dT)18 primers. RealTime PCR was performed with TaqMan (ABI) primers or SYBRgreen (Fermentas) primers. The control genes used were GAPDH or 18sRNA. After the run, the 7500 Software v2.0 (Applied Biosystems) gives a Ct value for each sample. These are then used to calculate relative quantification values, or fold difference, relative to the selected house-keeping gene.

IV RESULTS

4.1 THP-1 cells

4.1.1 THP-1 cell adhesion

The THP-1 cell line was obtained and cultured according to the protocol described in 3.1.1. The cells are suspended in culture, a method of adhering them to 15mm glass cover slips for fixing and staining was desired for morphological studies under the microscope. To achieve adherence to the cover slips, different coating materials were tried, i.e 0.05% collagen I, 0.1% gelatin or 0.1% polylysine as described in sections 3.2.1 and 3.2.2 of materials and methods. After 2 days of incubation, the THP-1 cells had not adhered to the cover slips coated with polylysine or collagen. However, numerous THP-1 cells had adhered to the gelatin coated cover slips, see **Figure 5** (a-b) where the cells have been stained green with F-actin. The cells appear round and uniform and not showing filopodia. Gelatin is therefore an inducer of the adherence of THP-1 monocytes, but it does not lead to further differentiation.

4.1.2 THP-1 differentiation with PMA

THP-1 cells can be differentiated into macrophages by incubation with PMA, method described in 3.4.1. Modifications of the original PMA protocol have enhanced macrophage differentiation of THP-1 cells and resulted in differences in the PMA concentration used in published protocols (83). In order to establish the ideal concentration for differentiation for this study, PMA was added in a range of concentrations to THP-1 cells and incubated for 2 days, followed by 2 days in PMA free media. The PMA concentrations used were: 0, 6.25, 12.5, 25, 50, 100 and 200ng/mL. After monitoring the cells for adhesion and differentiation, the lowest concentration to ensure suitable differentiation of the cells was found to be 50ng/mL, and that concentration was therefore used for all further studies on THP-1 differentiation.

The effect of PMA on the differentiation of THP-1 cells in cell culture can be seen in **Figure 4**. THP-1 cells were treated with 50ng/mL of PMA, described in 3.4.1. The PMA treated cells adhered to the underlying plastic surface and acquired a spindle-like shape (a-b) with morphology that differs substantially from their original round shape

(c). The adhered cells in **Figure 4** are rather sparsely placed, and it should be noted that not all of the THP-1 cells adhered when exposed to PMA in culture.

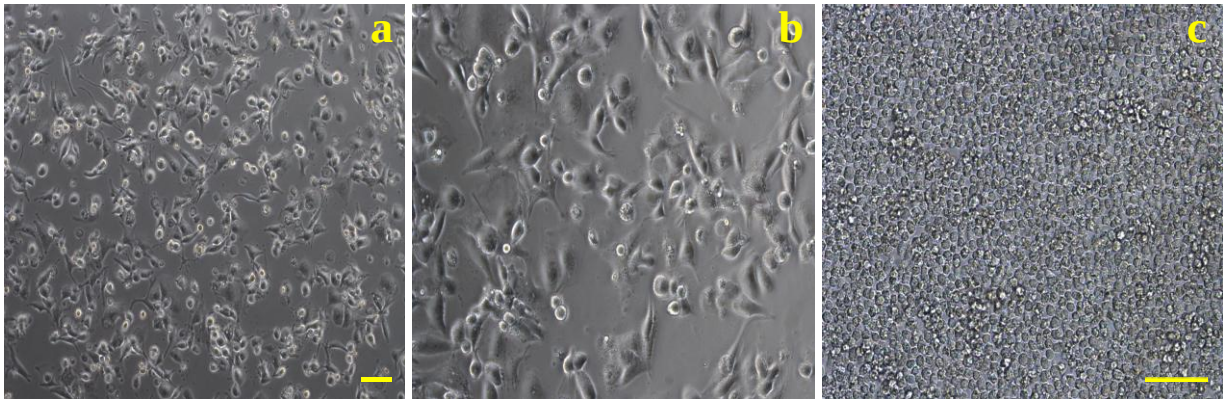

Figure 4. PMA differentiates THP-1 monocytes on plastic surfaces. THP-1 cells were treated with 50ng/mL of PMA for 2 days, washed with PBS and reincubated with fresh media without PMA for 2 additional days (a-b). THP-1 cells without PMA were used as control (c). Images were obtained from a phase contrast microscope. Magnification: a: 10x; b-c: 20x. Scale bars: 10µm.

For further studies on the morphology of differentiated THP-1 cells vs. control cells, confocal microscopy was applied. The cells were treated in the same way as described for **Figure 4**. In **Figure 5**, the cells have been stained green for F-actin, an actin polymer attached to the cell membrane. The control cover slips were coated with 0.1% gelatin for adherence of the cells as described in 3.2.2 and 4.1.1. The untreated control cells were round and uniform in shape as seen in **Figure 5** (a-b), whereas the PMA treated cells had acquired a variety of shapes (c-d). Protrusions, filopodia, from the main cell body can clearly be seen representing the characteristic signs of the transformation of a monocyte to a macrophage.

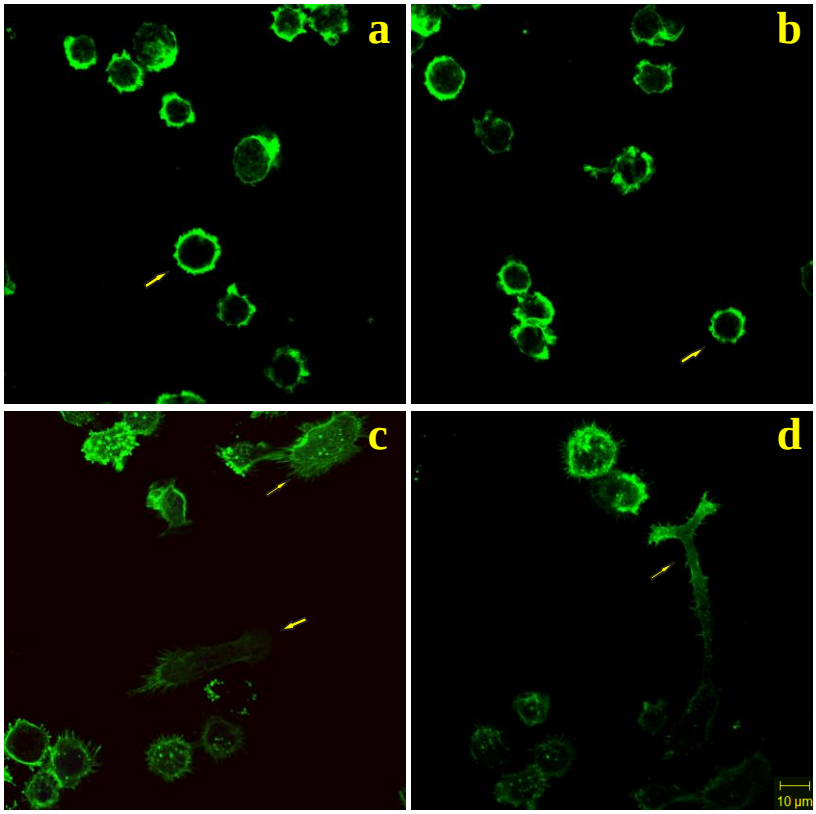

Figure 5. PMA differentiates THP-1 monocytes on glass cover slips. Cover slips were incubated with 50ng/mL PMA for 2 days in a THP-1 cell culture (c-d). Control was THP-1 cells without PMA (a-b). Control cover slips were coated with 0.1% Gelatin for 30 min in 37°C before use in the THP-1 cell culture. The arrows point to undifferentiated (a-b) and differentiated (c-d) THP-1 cells. Images were taken with a confocal microscope. Magnification: 64x. F-actin

4.2 Cystatin C

4.2.1 Characterization of soluble cystatin C amyloid

Cystatin C amyloid isolated from the brains of HCHWA-I patients was resolubilized in ddH_2O to an end concentration of 200µM, see 3.3.1. When a small sample of the solution was frozen, it led to its degradation, characterized by the disappearance of bands on a Western blot that had, prior to freezing, been visualized on the blot. Hence, the stock solution was stored at 4°C. No degradation was observed at this temperature for up to 6 months.

Amyloid fibrils are end products of the self-assembly of proteins occurring via an increase in internal order from soluble oligomers to protofibrils to fibrils. The assembly involves the combination of monomer to oligomer but disassembly occurs mainly via the release of the monomer (84). To determine the composition of the amyloid

solution, samples were analyzed by Western blotting. A 15µL sample of 4µM cystatin C amyloid mixed with 2X sample buffer was loaded on a 12.5% polyacrylamide gel and Western blotted, as described in 3.8. The primary antibody used was for cystatin C (Millipore) (see **Table 2**). The results of the Western blot can be seen in **Figure 6**. In the left lane, multiple bands are detected on the blot as monomers, dimers and oligomers representing the various aggregation states of the cystatin C proteins in the solution. The band at ~13kDa is the cystatin C monomer. The dimer is seen at ~26kDa and other oligomers appear at higher kDa. As a control, CSF from HCHWA-I patients was used, representing wild type cystatin C at 13kDa. Amyloid depositions are not found in the CNS of HCHWA-I patients, apparent in the CSF sample where only one band representing the monomer appears on the blot.

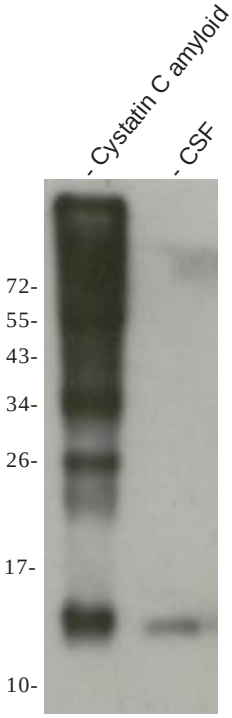

Figure 6. Soluble cystatin C amyloid visualized on a Western blot. Soluble cystatin C amyloid (4µM) (left lane). CSF as positive control for wild type cystatin C (right lane). Scale represents a protein ladder in kDa.

4.2.2 Determination of cystatin C fibrillar state

The soluble cystatin C amyloid consists of a crude mixture of cystatin C in different stages of aggregation, possibly with other molecules present (3). Some of these aggregates make up fibrils *in vivo* that appear as amyloids composed of polypeptide chains made up of β-sheets, characteristic of amyloid proteins, see **Figure 2**. The binding of Thioflavin T to these β-sheet structures induces fluorescence (25). In order

to confirm that the cystatin C amyloid solution contains or forms fibrils in β-sheets, different concentrations of samples were added to cover slips and dried overnight at 37°C and stained with Thioflavin T as described in 3.3.4. As seen in **Figure 7**, the Thioflavin T fluorescence of the cystatin C amyloid samples increases with increased amyloid concentration, as expected. No fluorescence can be seen in the control sample (a), but increased in the 25µM sample (b). The fluorescence of 50µM amyloid was also tested and appeared similar to the 25µM sample (data not shown). A stepwise increase in fluorescence intensity can be seen for the 100 and 200µM samples (c-d). Clumps of amyloid can be seen at all concentrations, but the overall fluorescence of the amyloid is more uniform in the samples of higher concentrations, where it forms a layer on the cover slips. This indicates that the soluble amyloid is in a fibrillar state as it dries. For all further experiments where cystatin C amyloid was dried on cover slips, a concentration of 100µM was used.

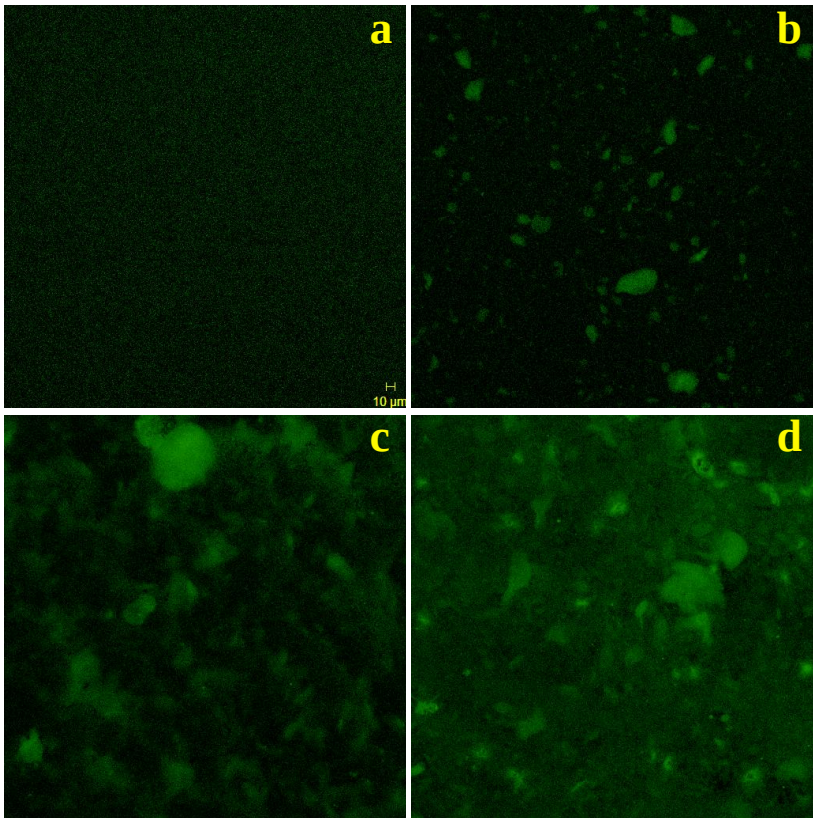

Figure 7. Soluble cystatin C amyloid is fibrillar when dried. Cystatin C amyloid was dried on cover slips (overnight, 37°C) at different concentrations and stained with 0.02% (w/v) Thioflavin T. The cystatin C amyloid concentrations were: a: 0, b: 25µM; c: 100µM, d: 200µM. Images were taken with a confocal microscope. Magnification: 20x.

The fibrillar state of Aβ in solution diminishes over time (73). Therefore, the stability of the cystatin C amyloid solution was tested by measuring its fibrillar state in solution over time and with increased temperature. Freshly soluble samples (1µM) of cystatin C amyloid were either stored at 4°C or 37°C and measured for fluorescence on days 0, 7 and 14 with a Spectrofluorometer as described in 3.3.2. The temperature of 37°C was used, since it is body temperature and used for human cell cultures. The original stock solution was kept at 4°C as well as the newly soluble sample. The results can be seen in **Figure 8**. Due to the rarity of the material, more time points were not added. The fluorescence of the 4°C solution had increased on day 7 but on day 14, the fluorescence was almost identical to the original state. Therefore, the cystatin C amyloid sample seems to be rather stable in solution at 4°C. The fluorescence of the 37°C solution decreased over time, however. This indicates that the fibrils in the cystatin C amyloid solution diminish over time when kept at 37°C. The heat is possibly disrupting some of the assembly of aggregates in the sample. This does not coincide with results from Ajit *et al.*, where the aggregation of an Aβ sample accelerated with increased temperature (73).

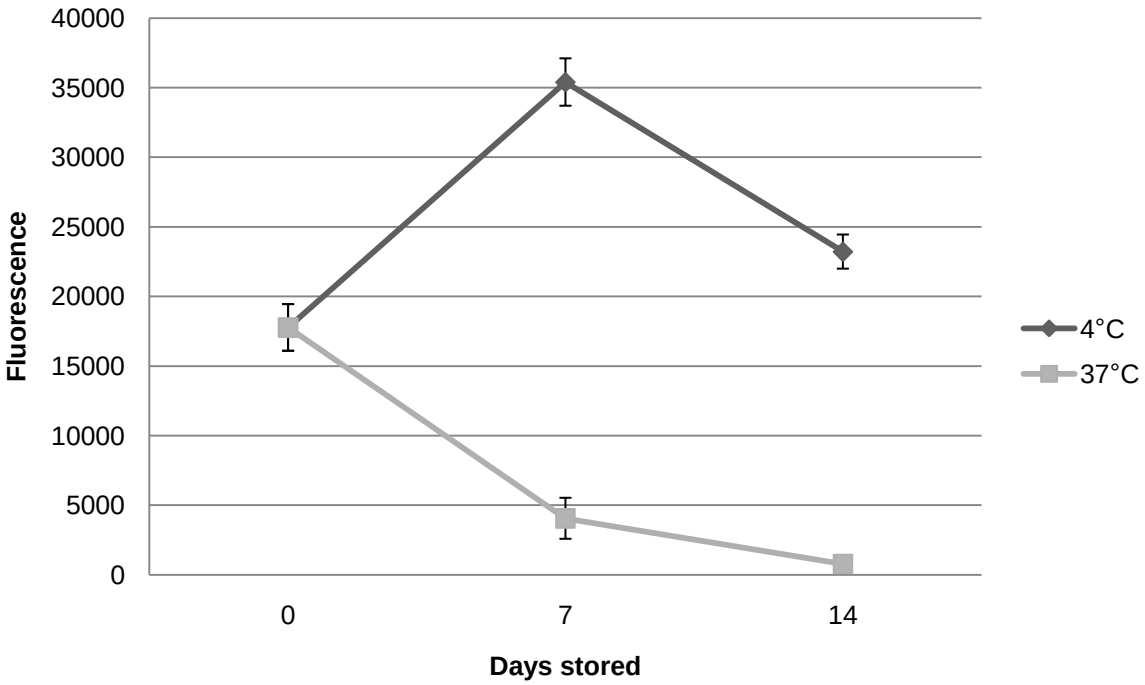

Figure 8. Soluble cystatin C amyloid contains fibrils. Cystatin C amyloid (1µM) was stored at 4°C and 37°C and measured at days 0, 7 and 14 in a 0.05mM Thioflavin T solution (in 10mM potassium phosphate and 150mM NaCl) in a Spectrofluorometer. Error bars show 95% confidence intervals (N=5). Fluorescence: arbitrary units.

Measuring the fibrillar state of boiled cystatin C amyloid samples was also of interest. The samples were boiled for 15 min and tested for fluorescence with Thioflavin T, but without any conclusive data. The amyloid seemed to precipitate out of solution when boiled making reliable measurements impossible. The aggregation state of the sample is probably accelerating to a point where the amyloid becomes insoluble and clusters together.

4.2.3 The effect of cystatin C amyloid on THP-1 cells

THP-1 cells are suspended in culture and do not adhere to uncoated surfaces, as described previously (4.1.1). A study by Crouse *et al.* revealed that Aβ amyloid causes THP-1 cells to adhere to the underlying surface in the cell culture (24). It was of interest to know whether the cystatin C amyloid would have the same effect on THP-1 cells.

A 100μM sample of cystatin C amyloid was added to cover slips and dried overnight as described in 3.3.3. The cover slips were incubated with THP-1 cells in culture for 2 days. The cells were subsequently stained green for F-actin and the amyloid layer stained red for cystatin C. The results are presented in **Figure 9**. On the control cover slips without any amyloid, very few or no cells adhered (a), whereas the cells adhered to the cover slip with an amyloid layer (b). Images were captured with a confocal microscope (N=12) and the cells counted manually. On average, ~41000 (±7600) cells had adhered to the amyloid cover slip versus none on the control cover slip. Cystatin C amyloid has therefore a clear effect on THP-1 cells causing them to adhere to the underlying amyloid covered surface in cell culture.

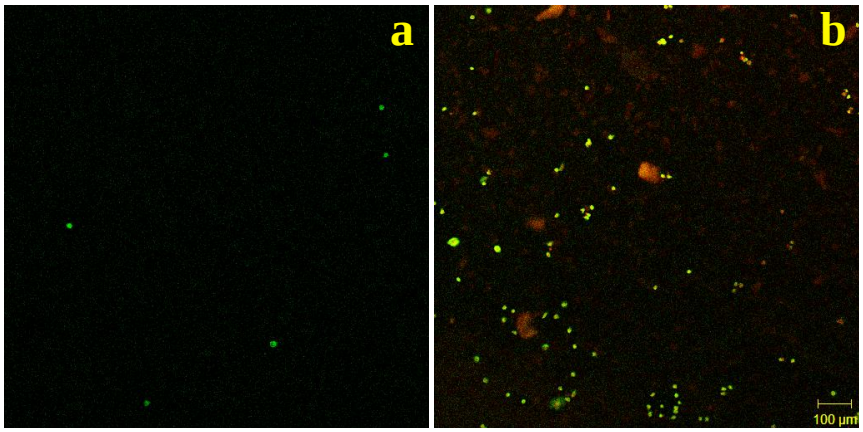

Figure 9. THP-1 monocytes adhere to a dried cystatin C amyloid layer. Cover slips with 100µM dried (overnight, 37°C) cystatin C amyloid were incubated for 2 days in a THP-1 cell culture (b). Control was cover slips without amyloid (a). Images were taken with a confocal microscope. Magnification: 10x. F-actin Cystatin C

The cells adhered when exposed to dry cystatin C amyloid as described above, thus the incubation of soluble cystatin C amyloid to a THP-1 culture was also examined. THP-1 cells were treated with 3µM of soluble cystatin C amyloid in the culture medium for 5 days as described in 3.4.3. The results are presented in **Figure 10**. The cells adhere to the plastic cell culture surface and have taken on a shape identical to the one seen after incubation with PMA (**Figure 4**).

It can be concluded that cystatin C amyloid stimulates the THP-1 cells to differentiate, causing them to adhere and differentiate into macrophages whether in solution or dried on an underlying surface. Earlier experiments showed that when treated with Aβ amyloid, THP-1 cells differentiated (24). Since mutated cystatin C does not share any sequence homology with Aβ and has three times its molecular weight (3), it can be elucidated that the structure of the amyloid seems to be the contributing factor to the differentiation.

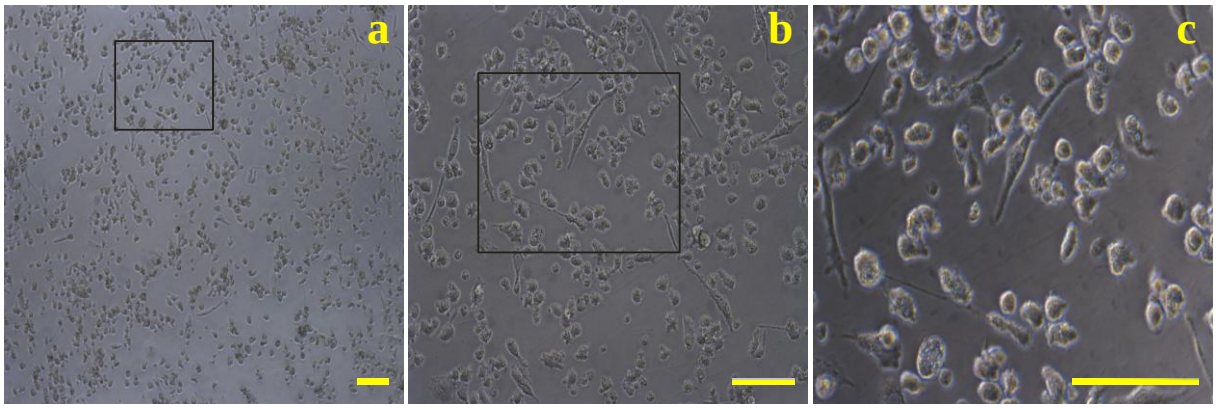

Figure 10. Soluble cystatin C amyloid differentiates THP-1 monocytes. Cystatin C amyloid (3µM) was added to a THP-1 cell culture and incubated for 5 days. Images were obtained from a phase contrast microscope. Magnification: a: 10x; b: 20x; c: 40x. Scale bars: 10µm.

Having shown that exposure to cystatin C amyloid makes THP-1 cells adhere/differentiate, the dose dependent effect was examined. THP-1 cells were incubated with 1.0; 0.5, 0.2 and 0.1µM cystatin C amyloid in the culture medium for 5 days. After incubation, the cells were fixed and stained with hematoxylin as described in 3.6, and counted manually (N=4). The results are presented in **Figure 11**. When exposed to 1µM and 0.5µM amyloid in solution the cells adhered to the cell culture surface, with no visible difference between the two concentrations. There was some adherence after incubation with 0.2µM and less adherence when incubated with 0.1µM. No adherence was seen when incubated with <0.1µM, data not shown. The dose dependent relationship is evident as decreased concentration of the sample resulted in fewer adherences of the cells. The results from the less diluted samples suggest the effect of cystatin C amyloid reaching a plateau above 0.5µM. A similar dose dependent relationship has also been reported for the differentiation effect of Aβ amyloid on THP-1 cells (24).

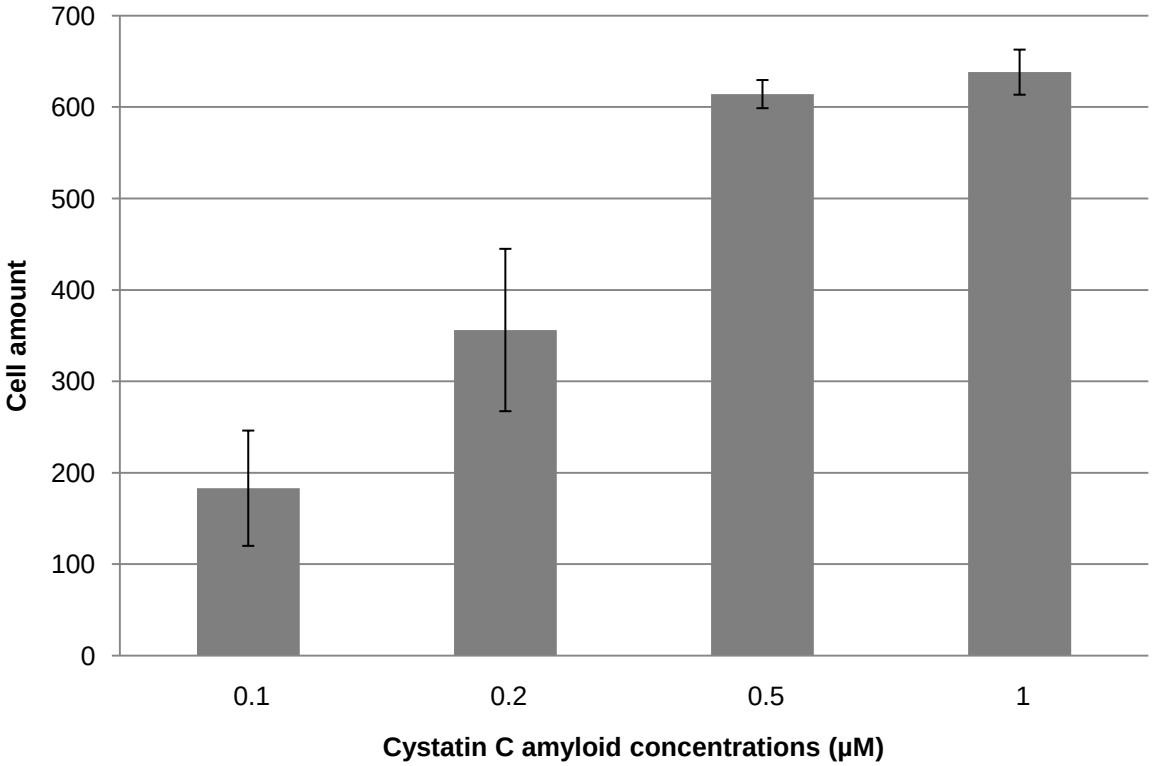

Figure 11. **The effect of soluble cystatin C amyloid on differentiation of THP-1 monocytes is dose dependent.** THP-1 cells were incubated with cystatin C amyloid concentrations of: 1.0; 0.5; 0.2 and 0.1µM for 5 days. Cells were fixed, stained with hematoxylin and counted. Error bars show 95% confidence intervals (N=4).

4.2.4 THP-1 cell degradation of dried cystatin C amyloid

THP-1 cells differentiate when exposed to cystatin C amyloid, going from a suspended monocyte to an adhered macrophage as seen in **Figure 10**. Macrophages are known to be able to phagocytose Aβ amyloid *in vivo* (85). Therefore, it was hypothesized that macrophages were also able to phagocytose cystatin C amyloid. To determine this, 100µM of cystatin C amyloid was added to cover slips and dried overnight at 37°C as described in 3.3.3. The cover slips were incubated with THP-1 cells in culture for 2 days. The cells were subsequently stained green for F-actin and the amyloid layer stained red for cystatin C. The results are presented in **Figure 12**. A uniform layer of amyloid can be seen stained red for cystatin C on the control cover slip without cells (a). On the cover slip incubated with THP-1 cells, the red staining for cystatin C is not as vivid (b-c). Visible in the amyloid layer are holes, which cannot be seen in the amyloid layer on the control cover slip, most likely caused by the THP-1 cells

phagocytosing the amyloid. There are no adhered THP-1 cells in (b-c), most likely explained due to a difference in serum concentration between the cultures in **Figure 9** and **12**. The THP-1 cells were supplemented with 10% FBS in the experiment represented by **Figure 12** whereas in other experiments where the cells did adhere to the amyloid layer, 2% FBS was used. The results indicate that at 10% FBS, the cells are weakly bound to the surface and probably phagocytosing the amyloid. The ability of the cells to adhere might decrease when there is more serum in the medium.

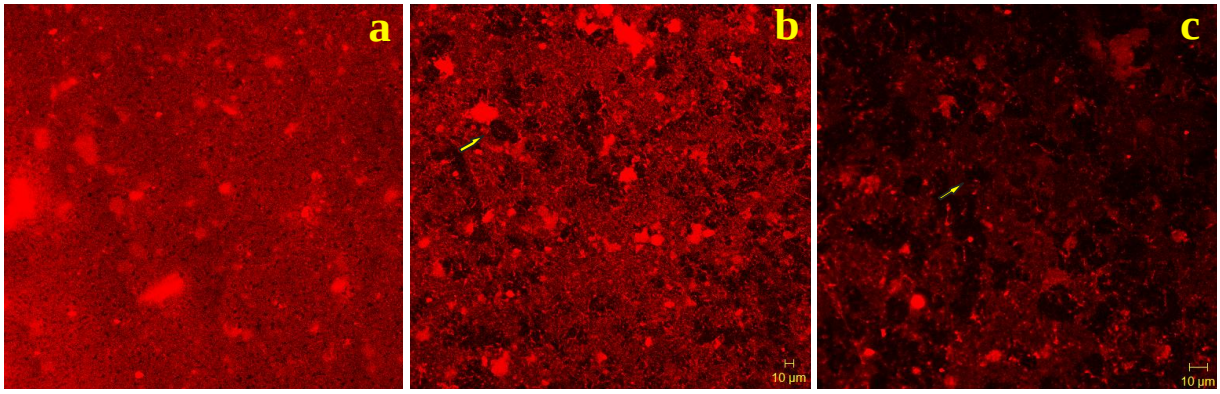

Figure 12. THP-1 monocytes leave holes in a dried cystatin C amyloid layer. Cover slips with 100µM dried (overnight, 37°C) cystatin C amyloid were incubated for 2 days in a THP-1 cell culture (b-c). Cover slips without THP-1 cells were used as control (a). Arrows point to holes in the amyloid layer (b-c). Images were taken with a confocal microscope. Magnification: a-b: 20x; c: 40x. Cystatin C

The holes in the amyloid layer on **Figure 12** might be caused by the THP-1 cells excreting enzymes that degrade the amyloid layer. To determine this, media was collected from a THP-1 cell culture and incubated with dried cystatin C amyloid on a cover slip for 2 days, described in 3.3.3. There was no difference observed in the amyloid layer compared to cell free media, data not shown. Based on these preliminary results, the cells are responsible for the holes in the amyloid, not any secretion products.

To better characterize the transformation of THP-1 monocytes into macrophages, cover slips with dried amyloid layer were incubated with THP-1 cells in culture as described in 3.3.3. Some cell cultures were also treated with PMA for 2 days to compare monocytes against macrophages on the amyloid layer. The cells were subsequently stained green for F-actin and the amyloid stained red for cystatin C. The results are presented in **Figure 13**. The amyloid caused the cells to take on various

shapes. Some retained their roundness (a), while others had taken on a completely different morphology, developing microspikes protruding from the cell bodies (b), much like the cells that received PMA (c). With the cystatin C staining, both the intracellular cystatin C as well as the amyloid layer is seen. A halo in the cystatin C layer around the cells is visible (c, middle panel), where the staining of cystatin C is absent. These halos follow the outline of cellular outgrowths. The halo is not as visible in (b, middle panel). Holes similar to the ones seen in **Figure 12** are visible next to undifferentiated THP-1 cells (a, middle and right panel) and are approximately the same size as the cells indicating phagocytosis.

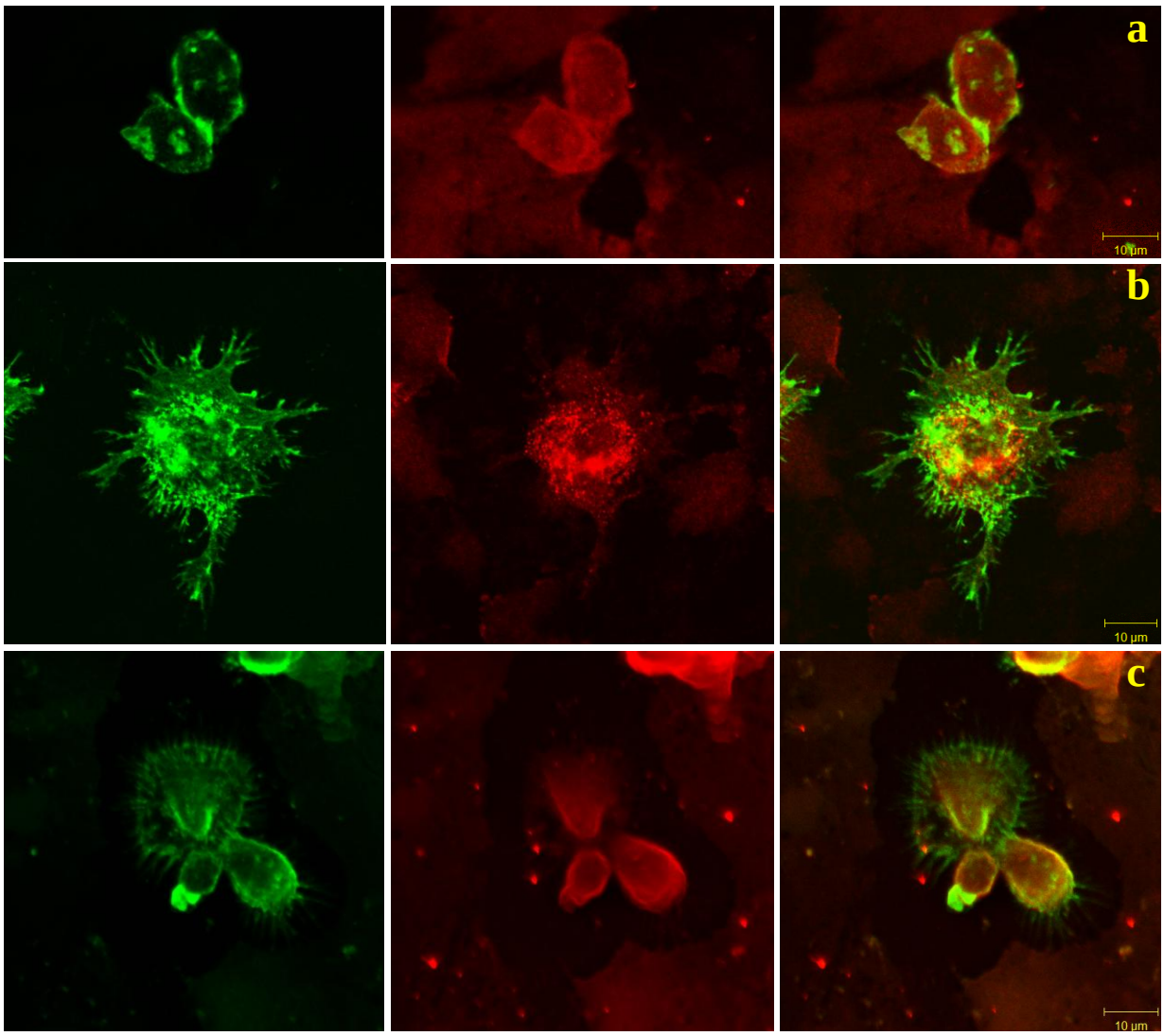

Figure 13. THP-1 monocytes and macrophages take on various shapes when adhered to a dried cystatin C amyloid layer. Cover slips with 100µM dried (overnight, 37°C) cystatin C amyloid were incubated for 2 days in a THP-1 cell culture (a-c). THP-1 cells in (c) were also treated with 50ng/mL PMA for 2 days. Images were taken with a confocal microscope. Magnification: 64x. F-actin Cystatin C

4.2.5 The cellular uptake of cystatin C amyloid in a THP-1 cell culture

To verify cellular uptake of soluble cystatin C amyloid, 10µM of the amyloid was added to a THP-1 cell culture. Samples were taken at 12hour intervals and analyzed on a 1% semidenaturing detergent agarose gel (SDD-AGE) described in 3.9. With the SDD-AGE method, protein polymers can be run on a gel without disassociation, which is not possible in a SDS-PAGE gel (82). Cells were washed in PBS and cellular proteins analyzed. The blot is represented on **Figure 14**. The primary antibody used was for cystatin C (DAKO) (see **Table 2**). Protein levels in the samples were measured and did not show variability confirming similar protein loading. The cystatin C monomer can be visualized at the bottom and the polymers or amyloid above the monomer. By the 48hour mark, there is no cystatin C monomer or amyloid visualized on the blot indicating cellular uptake of the aggregate. This coincides with the holes in the dried amyloid layer where the cells have probably phagocytosed the cystatin C amyloid (**Figure 12-13**).

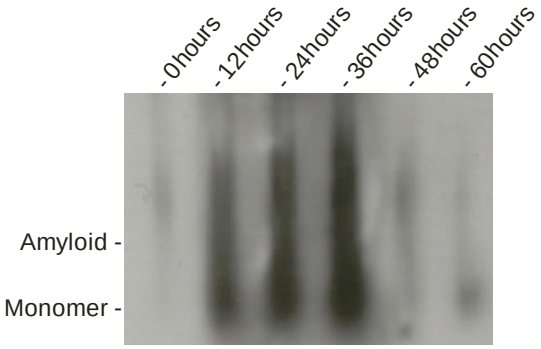

Figure 14. Soluble cystatin C amyloid from a THP-1 cell culture visualized on a SDD-AGE blot. THP-1 cells incubated with 10µM soluble cystatin C amyloid. Samples (300µL) were taken from the cell culture at 12hour intervals. The cystatin C monomer is visualized at the bottom of the blot with the amyloid forming a "tail" up from out of the monomer.

Cystatin C amyloid differentiates THP-1 monocytes to macrophages and the cells appear to take up or phagocytose the amyloid, see **Figures 10, 12-14**. With phagocytosis occurring, increased expression of lysosomal markers would be expected. Preliminary experiments were performed where the colocalization, in macrophages, of LysoTracker (Invitrogen), a lysosomal marker, and Lamp2, a lysosome-associated membrane protein (86), was studied. The two markers did not colocalize highly, indicating non-specificity of the Lamp2 antibody and/or the LysoTracker marker or that these two markers do not label the same structures. The

lysosomal activity of macrophages incubated with/without cystatin C amyloid from protein extractions was also analyzed by Western blotting, described in 3.7 and 3.8. The primary antibody used was Lamp2. After several attempts, the experiments were determined unsuccessful due to very faint and unclear bands representing Lamp2 on a Western blot. Future studies should include determining possible colocalization of cystatin C amyloid and a lysosomal marker, confirming phagocytosis.

4.2.6 The effect of cystatin C amyloid on inflammation proteins in THP-1 cells

Soluble cystatin C amyloid causes THP-1 cells to differentiate as shown in **Figure 10**. Since the differentiation of monocytes to macrophages *in vivo* is an inflammatory response, the transcription of pro-inflammation genes in THP-1 cells exposed to cystatin C amyloid was expected. It is well established that Aβ amyloid as well as amylin fibrils lead to an upregulation of pro-inflammatory genes (73, 74) in THP-1 cells, but parallel studies of cystatin C amyloid induction have not been performed previously. RNA was isolated from THP-1 cells treated with 1µM of soluble cystatin C amyloid for 4 hours and a RealTime PCR study performed according to 3.11. Representative target genes used to study the transcription of inflammation markers were *tumor necrosis factor-α* (*TNF-α*), *interleukin-6* (*IL-6*) and *interleukin-8* (*IL-8*), which are some of the key cytokines involved in the inflammatory process in the body. They are all upregulated by Aβ (73).

Soluble cystatin C amyloid induces upregulation in all inflammation genes tested according to **Figures 15-16**. The magnitude of the response is gene dependent, however. In **Figure 15**, the transcription of *IL-8* and *IL-6* can be seen. *IL-8* is upregulated around four times compared to control, but the fold difference of *IL-6* is larger or around twenty times, with more variance, however. Another inflammation gene examined was *TNF-α*, which has previously been used to determine toxicity of different forms of Aβ (73). Since there was a large increase in the transcription of *TNF-α* using the initial 1µM concentration, 1/10 and 1/50 dilutions of the amyloid solution were also tested, see **Figure 16**. The transcription of *TNF-α* was upregulated with increasing amyloid concentration indicating the response to be dose dependent. Less than 0.1µM (1/10) soluble cystatin C amyloid is needed to initiate an inflammatory response in THP-1 monocytes. For comparison, Ajit *et al.* used 15µM of Aβ amyloid to induce an upregulation of *TNF-α* in the same cells (73).

This indicates that cystatin C amyloid in solution induces a pro-inflammatory response in the THP-1 cells. The response of *TNF-α* transcription was much greater than seen for *IL-6* and *IL-8* making *TNF-α* the marker of choice for further pro-inflammatory studies. Since the reaction was large to begin with, fluctuations and subtle changes could be more easily detected in further studies, using *TNF-α*. The exact mechanism of the activation of inflammation markers by amyloids has not been elucidated yet.

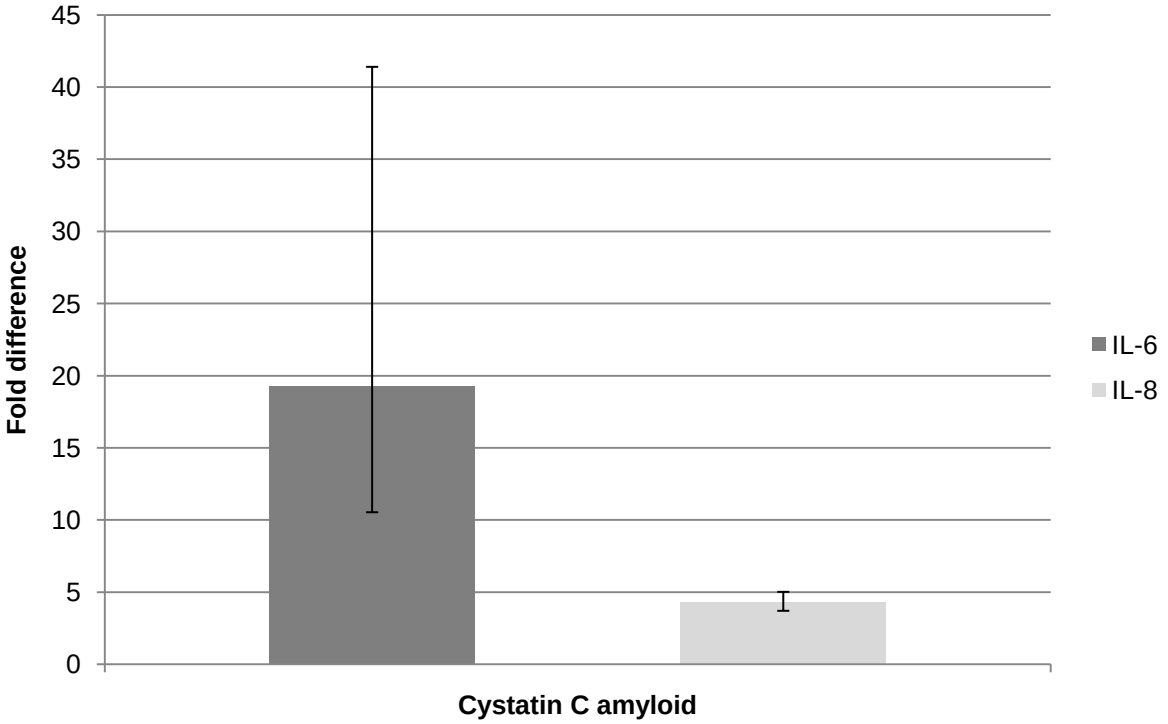

Figure 15. RealTime PCR analysis of the transcription of *IL-6* and *IL-8* in THP-1 cells after incubation with soluble cystatin C amyloid. The cells were incubated with 1µM cystatin C amyloid in solution for 4 hours. RealTime PCR was performed using SYBR green, *GAPDH* as control gene. Fold difference of the gene is relative to control cells incubated without amyloid (fold difference of control=1). Error bars show 95% confidence intervals (N=5).

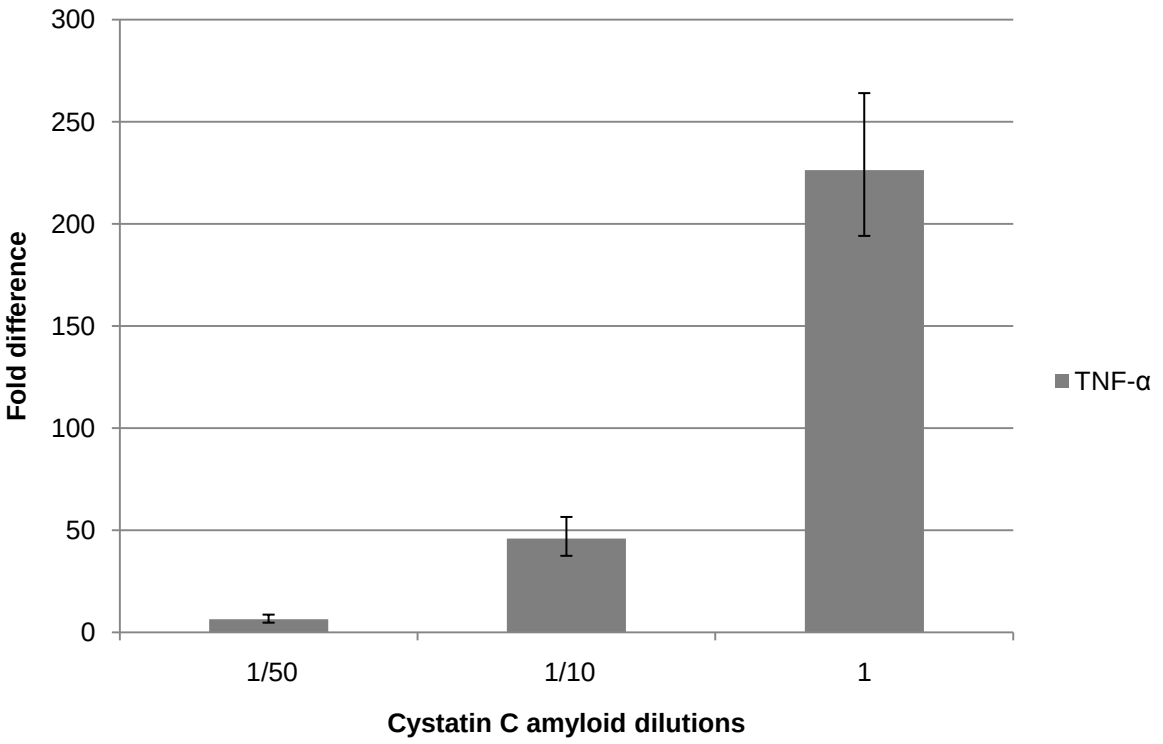

Figure 16. RealTime PCR analysis of the transcription of *TNF-α* in THP-1 cells after incubation with dilutions of soluble cystatin C amyloid. The cells were incubated with 1µM and 1/10 and 1/50 dilutions of cystatin C amyloid in solution for 4 hours. RealTime PCR was performed using SYBR green, *GAPDH* as control gene. Fold difference of the gene is relative to control cells incubated without amyloid (fold difference of control=1). Error bars show 95% confidence intervals (N=5).

The stability of the cystatin C amyloid solution in regard to fibrillar state had already been tested, see 4.2.2. There, the concentration of fibrils in solution seemed to diminish with increased temperature. It was of interest to determine whether heating the cystatin C amyloid had an effect on the transcription of *TNF-α* in THP-1 cells. Samples of the solution were heated at 37°C and 100°C for 15 min and treated with THP-1 cells for 4 hours and RNA isolated as described in 3.11, see **Figure 17**. Heating the amyloid solution to 37°C for 15 min did not have an effect on the transcription of *TNF-α*, data not shown. Heating the solution to 100°C did have an effect, however. *TNF-α* was upregulated 20-fold in THP-1 cells treated with boiled amyloid. This was unexpected since heating Aβ amyloid to high temperatures in a similar study, resulted in no induction of an inflammatory response (73). This could indicate that the structure of the cystatin C amyloid is more ordered than Aβ amyloid's seeing as it can still induce an inflammatory response even after being boiled. In a

related study, boiling the amyloid solution for 2 min abolished the cytotoxicity of cystatin C amyloid towards smooth muscle cells (Finnbogi Þormóðsson, personal communication). It has been shown that the fragmentation of fibrils enhances amyloid cytotoxicity (87). This suggests that the smooth muscle cells are either more sensitive towards larger species of cystatin C aggregates or that longer time is required in breaking down the amyloid into non-toxic species.

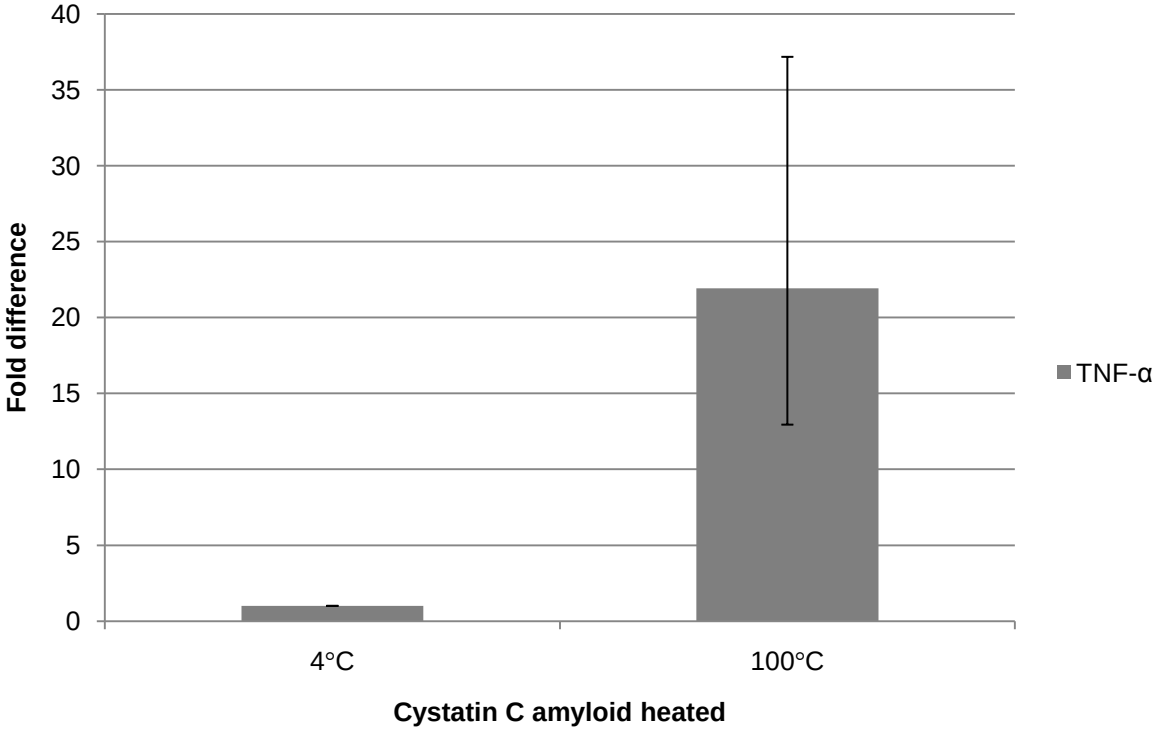

Figure 17. RealTime PCR analysis of the transcription of *TNF-α* in THP-1 cells after incubation with heated soluble cystatin C amyloid. The cells were incubated with 0.1μM cystatin C amyloid in solution for 4 hours. The amyloid solution had been boiled for 15 min prior to incubation. RealTime PCR was performed using SYBR green, *GAPDH* as control gene. Fold difference of the gene is relative to control cells incubated with cystatin C amyloid stored at 4°C (fold difference of control=1). Error bars show 95% confidence intervals (N=3).

In order to rule out the unlikely possibility of an endotoxin contamination in the cystatin C amyloid, the effect of LPS, an endotoxin (88), on the differentiation of THP-1 cells was studied. THP-1 cells were treated with LPS in a dose dependent manner for 5 days. The concentrations were: 1, 10, 100, 1000 and 10000ng/mL, the results can be seen in **Figure 18**. The cells did not differentiate when treated with the lower concentration samples; 1 (a) and 10ng (data not shown), but at the higher concentrations; 1000ng/mL (data not shown) and 10000ng/mL (c), the cells did

differentiate. The cells adhered to some extent when exposed to 100ng of LPS (b) indicating that to be the ideal concentration for studying the differentiation effect of LPS on THP-1 cells. The results in **Figure 18** indicate that high amounts of LPS are needed to initiate THP-1 cell differentiation. To test whether the cystatin C amyloid solution actually contained endotoxins, polymyxin B (Sigma), an endotoxin inhibitor (89) was added to the amyloid solution (0.1µg/mL) and incubated with THP-1 cells for 4 hours. THP-1 cells were also treated with LPS with/without polymyxin as a positive control for the inhibition of endotoxins, data not shown. Polymyxin B inhibited the effects LPS had on the upregulation of *TNF-α* in THP-1 cells demonstrating that the polymyxin B was inhibiting the endotoxins fully in the LPS sample. The effect that cystatin C amyloid had on the transcription of *TNF-α* is prominent as previously shown in **Figure 16**. Once polymyxin B was added to the amyloid solution, *TNF-α* transcription decreased slightly, or 30% (not significant). Compared to the 400 fold difference in the increase of *TNF-α* due to cystatin C amyloid. This shows that if there is any endotoxin contamination in the sample, it is insignificant to the end results.

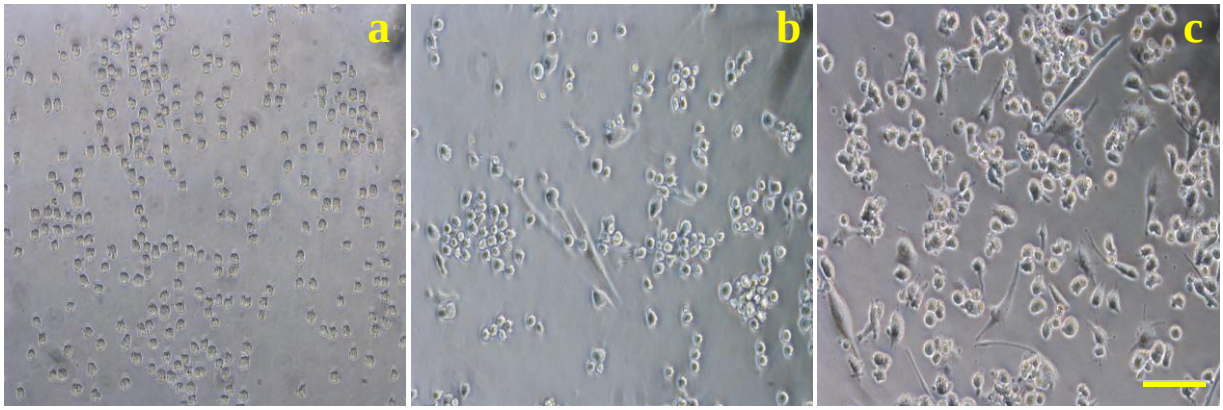

Figure 18. High concentrations of LPS differentiate THP-1 monocytes. LPS was added to a THP-1 cell culture in a dose dependent manner. The concentrations were: a: 1ng; b: 100ng; c: 10000ng. Images were obtained 5 days later from a phase contrast microscope. Magnification: a-c: 20x. Scale bar: 10µm.

In this part of the study, gelatin was determined to be an inducer for the adherence of THP-1 cells. Also, the cystatin C amyloid solution is made up of fibrils in dried and soluble form, determined with the fluorescence of Thioflavin T, and the amyloid aggregates taken up by the cells were visualized on a SDD-AGE blot. The amyloid solution contains monomers and multiples of monomers when denatured with SDS, visualized on a Western blot. THP-1 cells adhered to and likely phagocytosed dried cystatin C amyloid on cover slips supported by the disappearance of cystatin C

amyloid in a cell culture of THP-1 cells after 48 hours. Cystatin C amyloid in solution was found to stimulate the adherence and differentiation of THP-1 similar to PMA. This seemingly stimulatory effect of the amyloid is parallel to an induction in pro-inflammation markers.

4.3 T-ChOS

4.3.1 The effect of T-ChOS on THP-1 cells

The effect of T-ChOS on THP-1 cells was studied in a similar manner as the effects of cystatin C amyloid were studied. It was of interest to know the alleged role of T-ChOS in the inflammatory process, both due to its current usage as well as possible therapeutic uses in the future. T-ChOS is a well defined mixture of ChOS and was investigated by studying its effect on THP-1 cells, see 3.4.3.

T-ChOS was obtained from Genís ehf. and its effects on the adherence/differentiation of THP-1 cells was studied. The cells were incubated with three concentrations of T-ChOS for 7 days: 0, 50, 100 and 200µg/mL and the cells monitored for adherence/differentiation. The cells did not differentiate nor did they adhere to the underlying surface at any T-ChOS concentration, **Figure 19**. The images are of cells that were treated with 100µg/mL of T-ChOS, a concentration used for further studies. The cells were morphologically identical to control cells. Also, T-ChOS had no effect on THP-1 cell proliferation after 5-7 days as they proliferated at a similar rate as the control cells, receiving no T-ChOS, data not shown. Chitosan, the degradation product of chitin that ChOS is derived from, was also tested, but it also did not result in differentiation of the THP-1 cells. This indicates that monocytes do not respond to chitooligosaccharides with regards to differentiation.

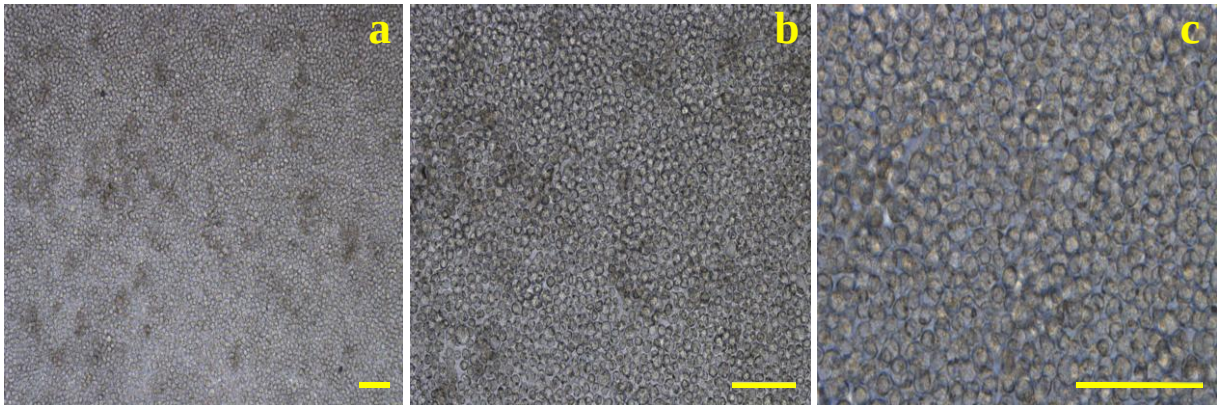

Figure 19. T-ChOS does not differentiate THP-1 monocytes. THP-1 cells were treated with 100µg/mL T-ChOS for 5 days. Images were obtained from a phase contrast microscope. Magnification: a: 10x; b: 20x; c: 40x. Scale bars: 10µm.

4.3.2 The effect of T-ChOS on chitinases and CLPs in THP-1 cells

Chitinases and CLPs are the proteins responsible for the breakdown of or binding to chitin and its derivatives. These proteins are thought to play a prominent role in inflammation. ChOS are believed to be possible inhibitors of these proteins, making ChOS a possible candidate for inflammation therapy (45). The hypothesis was that chitin derivatives might induce the expression of chitin binding proteins, which would either bind to the chitin derivatives, and in the case of CHIT-1, cleave it, or act as signaling proteins e.g. signaling that chitin was present in the cell surroundings. The changes in transcription of chitinases and CLPs in THP-1 monocytes and THP-1 derived macrophages when exposed to T-ChOS were measured with RealTime PCR. RNA was isolated from THP-1 monocytes treated with 100µg/mL T-ChOS for 4 and 24 hour and a RealTime PCR study performed according to 3.11. The transcription of the two chitinases: *CHIT-1* and *AMCase,* in addition to the two CLPs: *YKL-39* and *YKL-40,* was measured.

There was no difference in the transcription of *YKL-39* or *YKL-40* in THP-1 cells treated with T-ChOS for 4 and 24 hours and expression of the *AMCase* gene was not detected, data not shown. T-ChOS exposure caused the downregulation of *CHIT-1* after 4 and 24 hours, see **Figure 20**. *CHIT-1* is a marker for macrophage activation so the downregulatory effect of T-ChOS coincides with T-ChOS not inducing the differentiation of monocytes to macrophages, **Figure 19**.

The transcription of the same genes was studied in THP-1 derived macrophages. THP-1 cells were treated with 50ng/mL PMA for 2 days and kept in PMA free media

for 3 days. RNA was isolated from macrophages treated with 100µg/mL T-ChOS for 4 hours and a RealTime PCR study performed according to 3.11. There was no difference in the level of transcription of *YKL-39* and *AMCase* with T-ChOS compared to control, data not shown. T-ChOS did cause the upregulation of *CHIT-1* and *YKL-40*, as seen in **Figure 21**. The increase is large and this might indicate that T-ChOS is a powerful inducer of macrophage activation (52). The results coincide with an earlier study where chitosan caused an upregulation in *CHIT-1* but no difference in inflammation markers was observed (76). In our study, *TNF-α* was upregulated in THP-1 derived macrophages but not in monocytes.

Analyzing the protein levels of YKL-40 showed that after 24 hours of incubation with chitosan and T-ChOS, T-ChOS did induce higher levels of YKL-40 in the cell medium, but no YKL-40 was detected in the medium of the chitosan stimulated cells, data not shown. Further experiments are required to confirm this and to quantify the protein levels. The experiment from **Figure 21** was repeated for verification and to determine whether the upregulation of *CHIT-1* and *YKL-40* was dose dependent. When repeated, differences in the gene regulation of the chitinases and CLPs were only seen in the highest dose of T-ChOS, which was double the amount used in the previous experiment (**Figure 20**). Repeating the experiment the third time, also showed attenuated response. This variability in the effect of T-ChOS was surprising since the only known difference in the two studies was the passage number of the THP-1 cells. High passage numbers of THP-1 cells have been shown to affect their response to stimuli (90).

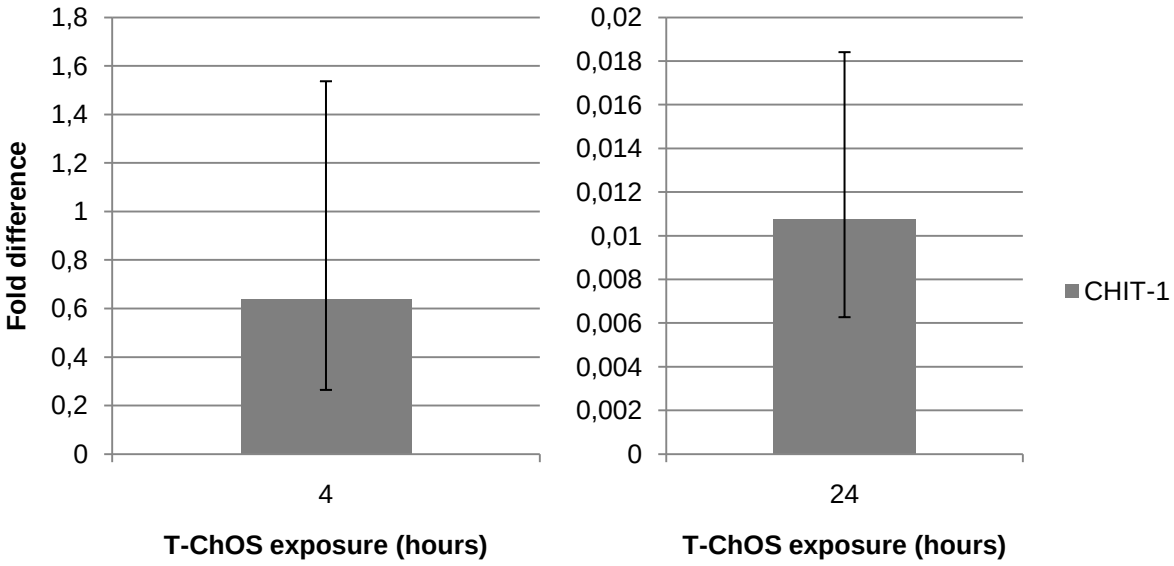

Figure 20. RealTime PCR analysis of the transcription of *CHIT-1* in THP-1 monocytes. The cells were incubated with 100µg/mL T-ChOS for 4 and 24 hours. RealTime PCR was performed using TaqMan, *18srRNA* as control gene. Fold difference of the gene is relative to control cells incubated without T-ChOS (fold difference of control=1). Error bars show 95% confidence intervals (N=5).

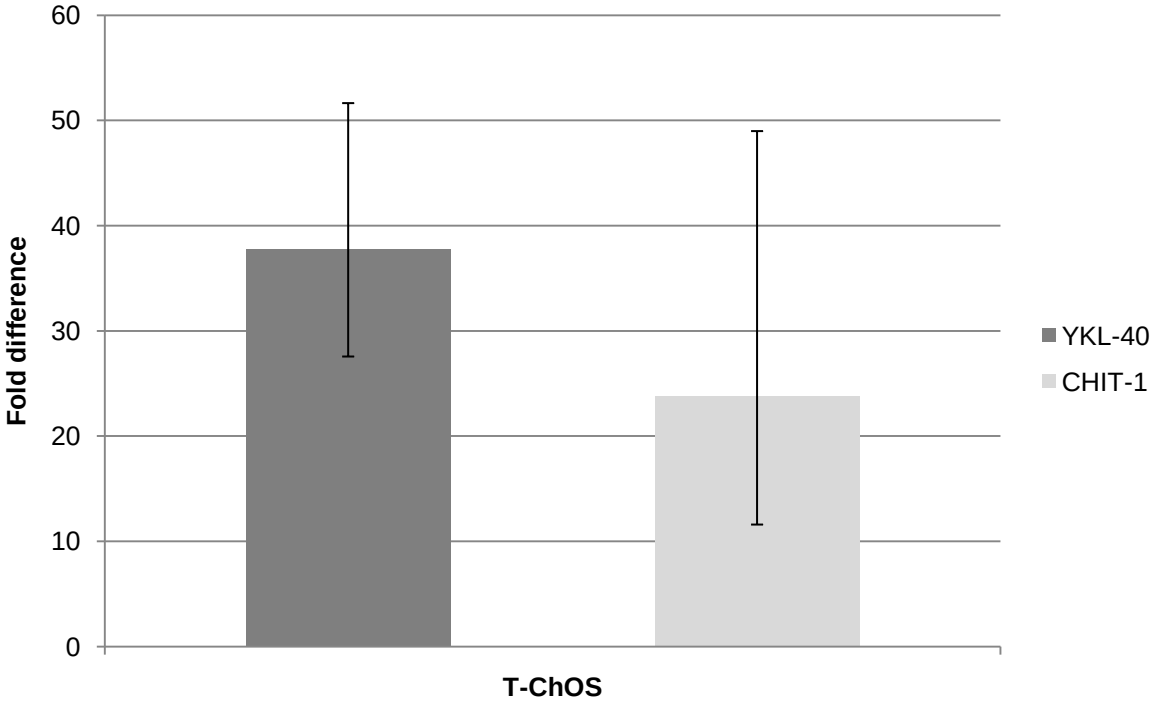

Figure 21. RealTime PCR analysis of the transcription of *YKL-40* and *CHIT-1* in THP-1 derived macrophages. The cells were incubated with 100µg/mL T-ChOS for 4 hours. The cells were in their 28th passage when differentiated. RealTime PCR was performed using TaqMan, *18srRNA* as control gene. Fold difference of the gene is relative to control cells incubated without T-ChOS (fold difference of control=1). Error bars show 95% confidence intervals (N=5).

Since T-ChOS might cause the upregulation of *CHIT-1* and *YKL-40* in THP-1 derived macrophages, it was of interest to know whether T-ChOS had an effect on the morphology of the cells in culture. *GAPDH* had been used as a control gene for determining ΔCt but it was noticed that this gene was upregulated in macrophages exposed to T-ChOS. The macrophages were therefore stained for GAPDH as well to examine whether there might be a change in the protein expression of GAPDH. THP-1 cells were differentiated onto cover slips with PMA for 2 days and in PMA free medium for 2 days, as described in 3.4.1, and incubated with or without 100µg/mL T-ChOS. The cells were subsequently fixed and stained blue for F-actin and red for GAPDH. The results can be seen in **Figure 22**. T-ChOS does not appear to be having an effect on the morphology of the macrophages in (a-b). GAPDH protein levels in macrophages exposed to T-ChOS or chitosan did not show an increase on a Western blot, data not shown. There have been reports of GAPDH having a function as a membrane protein in macrophages (91). If T-ChOS does have an effect on the upregulation of *GAPDH,* the effects are not seen at a protein level in the experiments performed in this study, but the results show that *GAPDH* is not suitable for use as an endogenous control in these or similar studies. Further experiments are needed to determine whether there is an actual increase in GAPDH protein levels in T-ChOS treated macrophages. If not, it is possible that a second mechanism is required to induce translation of the gene.

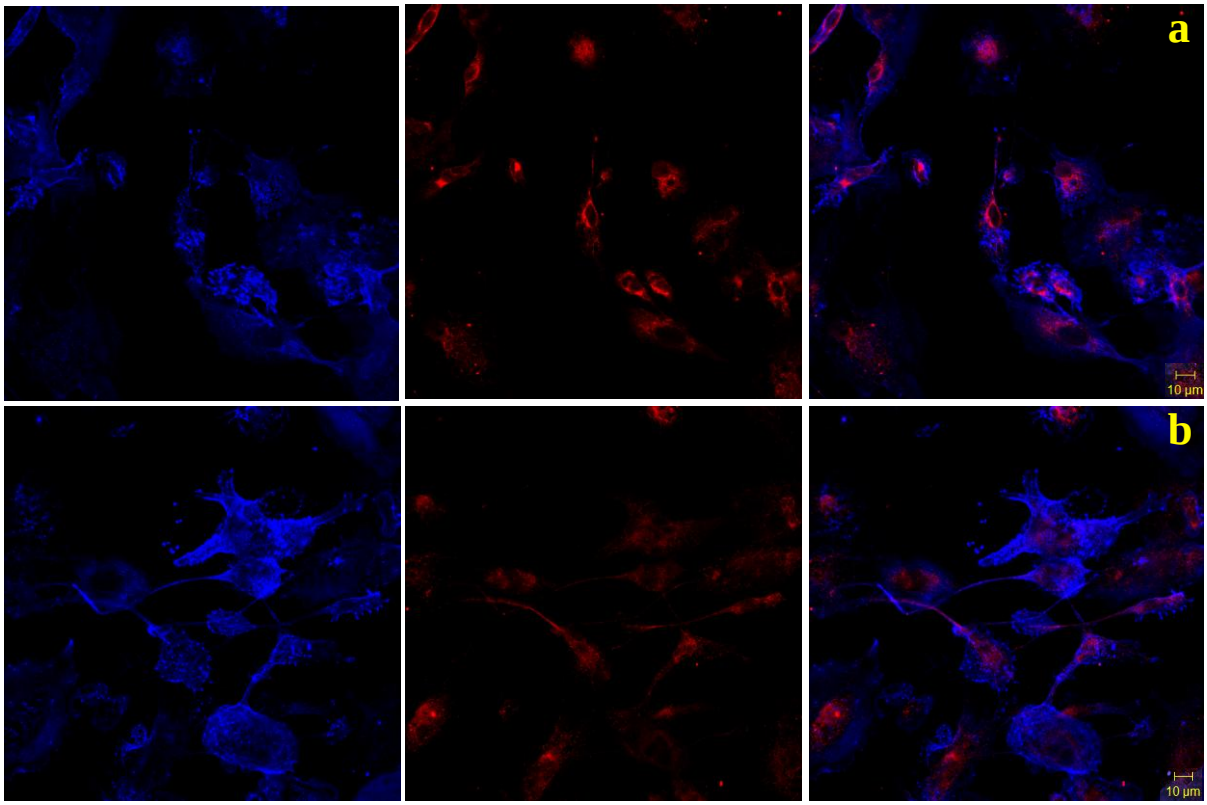

Figure 22. T-ChOS does not affect macrophage morphology. Cover slips were incubated with 50ng/mL PMA for 2 days in a THP-1 cell culture, fresh media for 2 days and 100µg/mL T-ChOS for 2 days (b). Control was macrophages with no addition of T-ChOS (a). Images were taken with a confocal microscope. Magnification: 40x. F-actin GAPDH

In this part of the study, T-ChOS did not differentiate THP-1 cells. It caused a downregulation of *CHIT-1* in THP-1 monocytes and variable results on *CHIT-1* and *YKL-40* in THP-1 derived macrophages. T-ChOS did not appear to have an effect on the morphology of THP-1 derived macrophages and did not induce increased protein levels of GAPDH even though *GAPDH* gene levels were induced in the macrophages according to RealTime PCR data.

4.3.3 The effect of T-ChOS and cystatin C amyloid on adhesion proteins

The interactions of monocytes and endothelial cells is a key step in the inflammatory process in the body as cells of the immune system need to migrate through tissues toward sites of damage or infection. This is a complicated process, but it involves surface proteins on the cells interacting with adhesion proteins, e.g. VCAM and ICAM (92). The upregulation of adhesion proteins, which attach immune cells to the endothelium, as an immune response in endothelial cells is well characterized in

HUVEC cells. ICAM and VCAM are examples of these adhesion proteins. HUVEC cells were obtained and cultured according to 3.1.3. The effect of T-ChOS on the transcription of adhesion proteins in HUVEC cells was examined. RNA was isolated from HUVEC cells in passage 3 treated with 100µg/mL T-ChOS for 4 and 24 hours and a RealTime PCR study performed according to 3.11. T-ChOS has a large effect on the transcription of *ICAM* and *VCAM* seen in **Figures 23-24**. When replicated with HUVEC cells of passage 7, the same effect was not seen, indicating that the HUVEC cells change in this regard with higher passages. These results indicate that when endothelial cells are exposed to T-ChOS, monocytes are more likely to adhere to them. The adhesion protein ICAM, on the surface of endothelial cells, has been found to extravasate leukocytes from the vasculature (93). The next step in this study could be to measure this tendency, e.g. by labelling THP-1 cells with a fluorescent marker and monitor their adherence, if any, to HUVEC cells, of different passages.

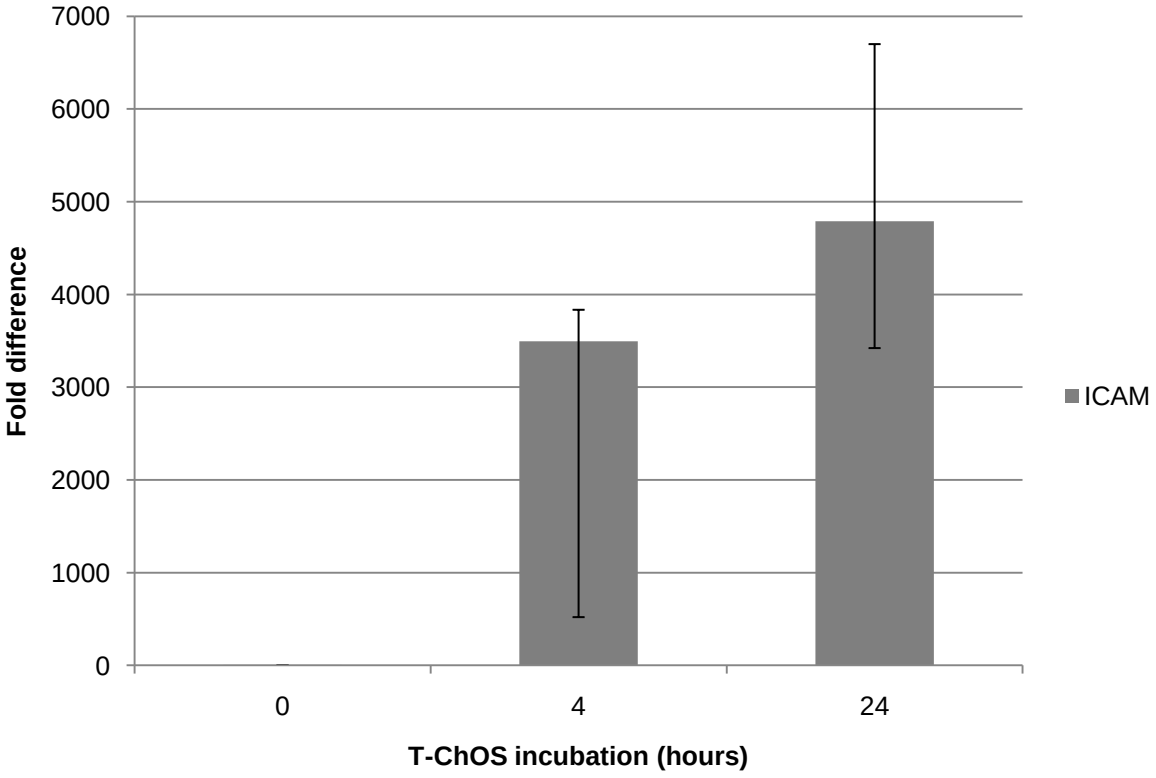

Figure 23. RealTime PCR analysis of the transcription of *ICAM* in HUVEC cells. The cells were incubated with 100µg/mL T-ChOS for 4 and 24 hours. RealTime PCR was performed using SYBRgreen, *GAPDH* as control gene. Fold difference of the gene is relative to control cells incubated without T-ChOS (fold difference of control=1). Error bars show 95% confidence intervals (N=4). Significance: 0-4 hours:***, 0-24 hours:***, 4-24 hours: -. Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05. ANOVA with a TukeyHSD test performed.

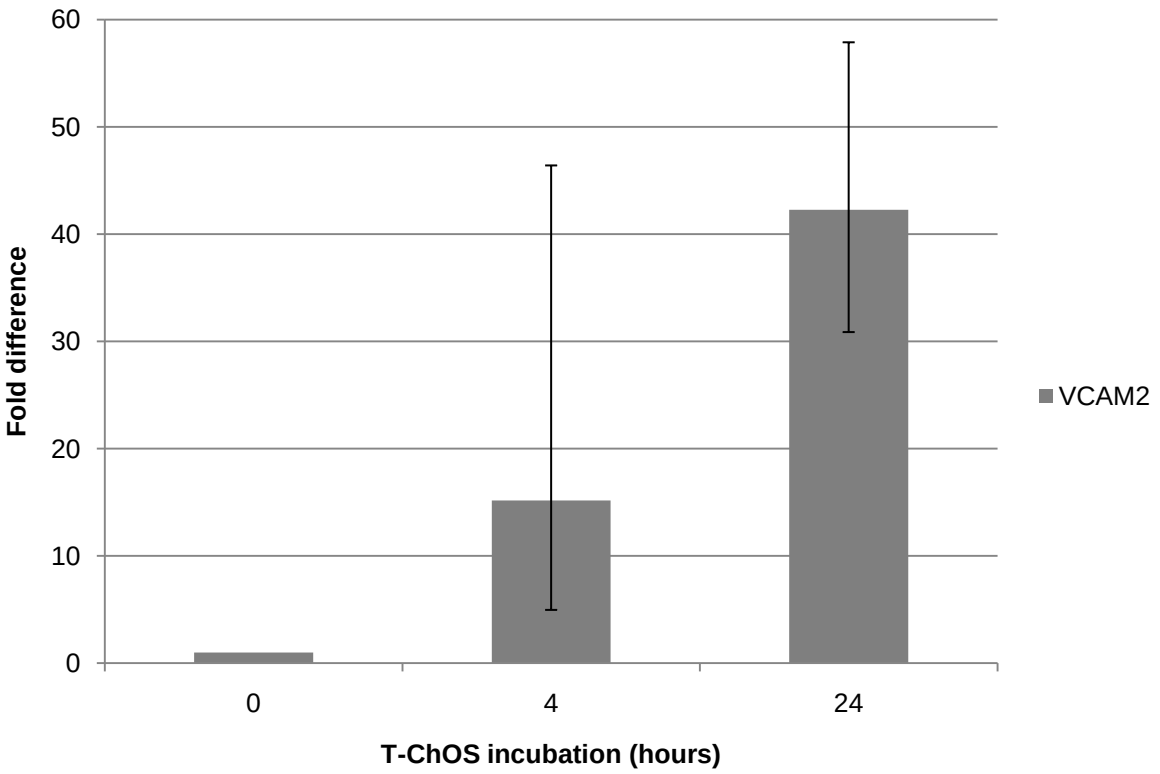

Figure 24. RealTime PCR analysis of the transcription of *VCAM* in HUVEC cells. The cells were incubated with 100µg/mL T-ChOS for 4 and 24 hours. RealTime PCR was performed using SYBRgreen, *GAPDH* as control gene. Fold difference of the gene is relative to control cells incubated without T-ChOS at 0 (fold difference of control=1). Error bars show 95% confidence intervals (N=4). Significance: 0-4 hours:**, 0-24 hours:***, 4-24 hours: -. Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05. ANOVA with a TukeyHSD test performed.

Cystatin C amyloid causes THP-1 monocytes to adhere and differentiate into macrophages, see **Figure 10**. When the cells are in an adhered state, there is presumably an increase in the expression of various adhesion proteins. RNA was isolated from THP-1 cells treated with 1µM soluble cystatin C amyloid for 4 hours and a RealTime PCR study performed according to 3.11. The transcription of the adhesion proteins *ICAM* and *VCAM* in THP-1 cells was examined, see **Figure 25**. There was a substantial increase in the transcription of *ICAM* as well as a significant increase in the transcription of *VCAM*. This indicates that the THP-1 cells are expressing adhesion proteins as well as being in an inflammatory state when exposed to soluble cystatin C amyloid as judged by gene expression data which coincides with the other inflammatory effects the cystatin C amyloid has been shown to cause.

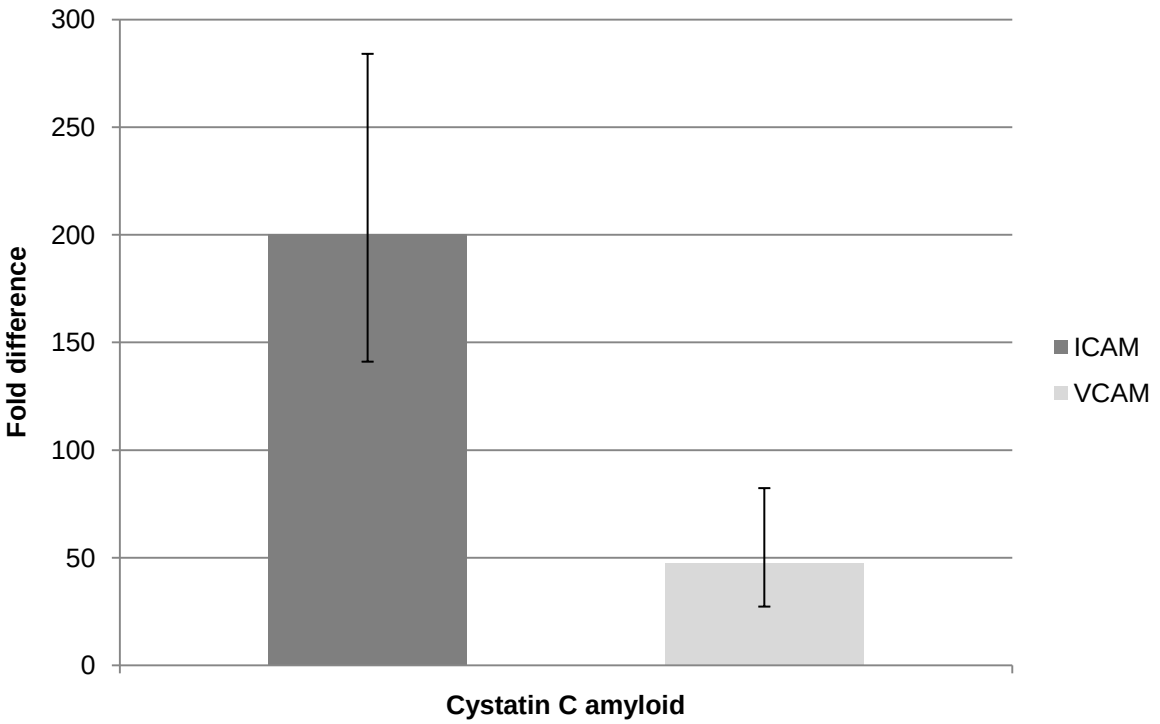

Figure 25. RealTime PCR analysis of the transcription of *ICAM* and *VCAM* in THP-1 cells after incubation with soluble cystatin C amyloid. The cells were incubated with 1µM cystatin C amyloid in solution for 4 hours. RealTime PCR was performed using SYBRgreen, *GAPDH* as control gene. Fold difference of the gene is relative to control cells without amyloid (fold difference of control=1). Error bars show 95% confidence intervals (N=4).

In this part of the study, the transcription of adhesion proteins in HUVEC and THP-1 cells when exposed to T-ChOS and soluble cystatin C amyloid, respectively, was studied. The adhesion proteins were upregulated in both cell types, but was passage dependent in HUVECs.

V DISCUSSION

The study of bioactive compounds and their effect under well defined conditions is of great importance whether to confirm their pro or anti stimulatory activity. In this study, two distinct bioactive compounds were analyzed, cystatin C amyloid, known to be cytotoxic, and T-ChOS, with no known cytotoxicity. The roles these substances play in the immune system are largely unknown. By using an immune model system such as the THP-1 monocytes used in this study, more consistent and, in theory, more reproducible results can be obtained, mimicking the interactions in the body without having to manage the unpredictability of primary cell cultures. Cystatin C amyloid has a clear effect on THP-1 cells. It stimulated the differentiation of monocytes and induced a pro-inflammatory response which was ruled out to be caused by an endotoxin contamination. Both non-adherent monocytes and adherent macrophages could degrade cystatin C amyloid. The effect cystatin C amyloid has on the immune model system is perhaps not surprising due to its known cytotoxicity to cells in culture, but of potentially great importance when it comes to clinical intervention. The results concerning T-ChOS were also clear. ChOS are currently used in a variety of ways in the body as e.g. scaffolds, drug carriers or as a food supplement. T-ChOS showed no effect on the differentiation of THP-1 cells, but showed variable effects on the gene expression of *CHIT-1* and *YKL-40.* Also, T-ChOS had large effects on the expression of cell adhesion proteins in both primary endothelial cells and in THP-1 cells. This strongly indicates that chitin derivatives have important pro-inflammatory effects, although the corresponding protein levels still need to be examined as well as changes in cellular behavior.

Hereditary Cerebral Hemorrhage with Amyloidosis, Icelandic type (HCHWA-I), is a devastating disease, causing premature death of patients due to cerebral hemorrhage (3, 6-9). HCHWA-I is a rare type of CAA, defined as amyloid deposition in brain vasculature (1). Other, more common forms of CAA exist where the amyloid is made up of Aβ amyloid (2). A mutated form of the protein cystatin C forms amyloid deposits in the vascular system (13, 18) shown to cause cytotoxicity to smooth muscle cells (3) although much remains unresolved regarding the exact pathological mechanisms behind the vascular defect. The amyloid can be isolated from brains *post mortem* (14), however, minimal amounts of it can be isolated each time making the

substance very valuable. By using *ex vivo* material, presumably *in vivo* situations can be mimicked better than with *in vitro* generated fibrils.

In this study, the effect of cystatin C amyloid on the differentiation of THP-1 cells, a model of human monocytes and macrophages (54), was studied. The cystatin C amyloid solution used in this study is a mixture of the protein in different aggregation states as monomers or multiples of monomers (**Figure 6**) compared to the CSF control sample, as determined under denaturing conditions using SDS-PAGE and Western blotting. The larger fibrils in the amyloid cannot be visualized on the SDS-PAGE gel due to their large size. A better visualization and a more sensitive way of detecting all the components of the amyloid solution might be accomplished with silver staining. During this study, different silver staining protocols were tried multiple times but did not show sufficient sensitivity. Another electrophoresis method was also used to visualize the protein aggregates, an SDD-AGE blot (**Figure 14**). This method allows larger protein aggregates to enter the gel. Further determination of protein composition and a more detailed description of the amyloid species in the solution is warranted and could be evaluated with e.g. Mass Spectometry analysis.

Cystatin C amyloid induces differentiation of THP-1 cells as effectively as PMA (**Figure 4-5**), from round suspended monocytes, to adhered, spindle-like macrophages (**Figure 10**). Aβ amyloid and amylin have also been shown to differentiate THP-1 cells (24, 73, 74). Holes that form in the dried cystatin C amyloid layer after incubation with THP-1 cells strongly indicates phagocytosis by the cells (**Figure 12**), also supported by cellular outgrowths around cells that have adhered to a cystatin C amyloid layer (**Figure 13**). Also, after 48 hours in culture, the intracellular protein aggregates are no longer visible on an SDD-AGE blot (**Figure 14**), showing that the majority of the cystatin C aggregate in solution has been removed and probably degraded. A similar study of the uptake of cystatin C amyloid, showing intracellular amyloid, has not been performed before, but microglial cells of the immune system, derived from monocytes, phagocytose amyloid deposits in the CNS (28) and take up Aβ *in vitro* (29, 30). Experiments showed that cell secretions did not degrade the amyloid, again supporting the notion that cells take up and degrade the protein aggregates. The reason why cystatin C amyloid accumulates in patients despite the cells phagocytosing it is still unknown. The current study shows that monocytes and macrophages are capable of removing it *in vitro.* Further studies are needed to determine whether the patient´s macrophages are defective in clearing the cystatin C

amyloid, as is the case for some AD patients (94) or whether the amyloid causes cytotoxicity to the monocytic cell lineage as has been shown for e.g. smooth muscle cells (3). It is also not known why the cystatin C amyloid deposits are not found in the parenchyma, i.e. in close proximity to neurons. Perhaps macrophages phagocytose the amyloid in the parenchyma and enter the vascular system resulting in the amyloid being deposited there by an unknown mechanism, perhaps due to apoptosis of the macrophages. This in turn would cause toxicity to the smooth muscle cells and the vascular system and brain hemorrhage in the long run.

The study of the relationship between inflammation and neurodegeneration is complex and the causal relationship not straightforward or simple (95). Activated immune cells have been found to be intimately associated with AD senile plaques, secreting a number of inflammatory and pro-inflammatory mediators (27, 96). These same pro-inflammatory proteins are found in AD brain tissue and in association with senile plaques. The induction of these bioactive species in the vicinity of senile plaques implicates that a chronic inflammatory process contributes to AD progression (96). TNF-α is an example of an inflammation marker and has been identified as having a special role as a pro-inflammatory cytokine. It has been named the „master regulator“ (97) of the immune response, since it is important in the regulation of other pro-inflammatory cytokines. TNF-α is the key initiator of inflammation in multiple organ systems including the brain (97). The effects of cystatin C amyloid have not been tested before in regards to TNF-α secretion, *in vitro* or *in vivo*. The large upregulation of *TNF-α* induced by the cystatin C amyloid is therefore of special interest (**Figure 16**). The increase in *TNF-α* transcription was much higher than that found for *IL-6* and *IL-8* (**Figure 15**), making *TNF-α* the marker of choice for further pro-inflammation studies, apart from it being the “master regulator” as already noted. Also, a *TNF-α* upregulation of this magnitude is likely to translate into increased protein levels of TNF-α*,* though it has not yet been resolved. Interestingly, it seems that very low levels of cystatin C amyloid are needed to initiate an inflammatory response in THP-1 monocytes, much lower than are needed of Aβ amyloid to induce an upregulation of *TNF-α* in the same cells (73), indicating that cystatin C amyloid is more toxic to immune cells than Aβ amyloid. However, the levels of cystatin C amyloid in patients are not known and therefore it cannot be stated that this would be seen in patients.

The upregulation of the pro-inflammatory marker *TNF-α* has been shown to cause defects in the vascular system (36), which is what ultimately causes the deaths

of patients with HCHWA-I. THP-1 cells are sensitive to low levels of cystatin C suggesting that at least a part of the HCHWA-I pathology, including the fibrosis, might be caused by direct effect of locally increased level of TNF-α on the integrity of vasculature. Future studies should include testing TNF-α levels of HCHWA-I patients, also prior to the onset of the disease. We predict, based on this study, that levels of TNF-α could be very high early in the progression of the disease and that inhibition of TNF-α, for example with the drug Etanercept, might be a step in the right direction in finding clinical treatment for the disease. Etanercept has already been tested on AD patients, a study suggested that the inhibition of TNF-α may hold promise as a potential approach to AD treatments (37), since increased CNS TNF-α levels is related to increased risk of developing AD (98). There are other diseases, which are characterized by amyloid depositions in the vascular system, such as Dutch HCHWA-D (4). It is of great interest whether the protein aggregates generated in these patients also increase levels of TNF-α locally and whether in a similar manner, as suggested above, inhibiting the detrimental effects of TNF-α might be a potential approach in treating the disease. Admittantly, this is speculative, but at the minimum any insights gained from other more common CAA or AD should be considered an entry point to the treatment of HCHWA-I patients.

Although the pathogenesis of amyloid diseases is not fully understood, a common pathogenic mechanism based on a common structure is most likely shared (99). Studying the nature of cytotoxicity and the mechanism of accumulation of amyloids, is important in order to be able to inhibit the progression of these diseases. Understanding the immune response is a part of this. Even though the HCHWA-I disease in question is extremely rare and localized, it is our hope that any knowledge gained about the nature of the disease, and other amyloid disorders, will lead to a common therapeutic solution for treating patients with different types of amyloid diseases. Also, as eluted to above, if inhibiting TNF-α in AD patients is successful, the same should be tried in HCHWA-I patients as soon as possible.

The second part of this study involved a similar analysis of an unrelated compound, T-ChOS, a derivative of chitosan, using the same cell system. Chitosan, the N-deacetylated derivative of chitin, is an important biomaterial due to its biocompatibility, non-toxicity, biodegradability and adsorption properties (100). ChOS are derivatives of chitosan and are regarded as being bioactive physiologically, since they possess various biological activities (39-41, 101). The molecular basis that the

proposed bioactivity of ChOS rests upon is unknown, but of importance for any proposed therapeutic use, especially long term. It has been suggested that ChOS function through their effect on chitin binding proteins (45), proteins that have important although ill-defined roles in the immune response. Inflammatory responses play an important role in most diseases and in this study the effect of T-ChOS on the THP-1 monocyte cell line was studied, using a well defined mixture of ChOS, T-ChOS. The THP-1 cells differentiate by a well defined process and can be studied both as monocytes and macrophages; both cell types have pivotal roles in immunity and tissue repair, the processes where ChOS has been suggested to have beneficial activity. It is very useful to know the response of immune cells towards ChOS, since the possible uses of ChOS include incorporation into biomaterials for various applications e.g. to be used in contact with living tissue.

The results show that T-ChOS does not induce THP-1 cells to differentiate (**Figure 19**) nor does it induce expression of any genes examined in monocytes, that have been related to inflammation (*AMCase*, *YKL-39, YKL-40*), indicating that T-ChOS does not activate the inflammatory response. The gene upregulation of *TNF-α* in monocytes was also tested, there was no change. The upregulation of CHIT-1 protein levels in macrophages in response to chitosan has been reported previously (76). ChOS have been shown to be possible inhibitors of chitinases or chitinase-like proteins (CLPs), and therefore possibly inhibitors of inflammation. In the current study, *CHIT-1* was downregulated in THP-1 monocytes exposed to T-ChOS (**Figure 20**). Large changes in gene expression were observed at first in macrophages treated with T-ChOS (**Figure 21**), but subsequent experiments showed little or no increase. The most plausible explanation for these variable results is the passage number of the cell line, but this needs to be determined. The cells went through less than 5 passages between experiments; and the highly variable results indicate that the changes must have been abrupt. Higher passage numbers of THP-1 cells have been shown to decrease the ability of the cells to be stimulated (90). Currently it can be stated that T-ChOS can induce expression of *CHIT-1* and *YKL-40* in PMA differentiated THP-1 cells. A previous unpublished study of the effect of ChOS on THP-1 cells was performed by Ólafur B. Einarsson, as a graduate student at the University of Iceland, under the supervision of Dr. Finnbogi Þormóðsson and Dr. Jón M. Einarsson at Genís ehf (Ó.B.E unpublished). That study showed downregulation of *YKL-40* in non-differentiated THP-1 cells and upregulation of *YKL-40* in PMA differentiated THP-1

cells. While the execution of these two studies differ in many significant ways (differentiation protocol different, higher serum levels, molecular composition of ChOS different, cell treated with ChOS for 24 hours, lack of contamination not proven, ChOS led to morphological changes in cells, apparent downregulation of *CHIT-1* in PMA treated cells) the overall conclusion on the effect on *YKL-40* in PMA treated THP-1 cells is the same as in the current study, strengthening the results shown here concerning *YKL-40* and PMA treated THP-1 cells. Given the multiple differences between the two studies, the response of *YKL-40* appears very robust.

CHIT-1 is of special interest for clinical reasons, its activity is several hundred-fold elevated in patients suffering from Gauchers disease (52). It has been hypothesized that *CHIT-1* is involved in anti-fungal immunity since its levels are elevated in plasma and urine of neonates with infections of e.g. *C.albicans* (102) and plasma levels are increased in children with acute malaria (103). The elevated activity in tissues might be due to increased macrophage differentiation due to stimulation from chitin containing pathogens. This could explain the upregulation seen in our differentiated THP-1 cells when exposed to T-ChOS since it is a chitin derivative. The next step would be to fully determine whether T-ChOS has an effect on the upregulation of *CHIT-1* and *YKL-40* in differentiated THP-1 cells.

The differentiation of monocytes to macrophages requires adherence of monocytes to the endothelial cell layer for the cells to enter tissues where damage or inflammation is occurring (63). The endothelium responds to inflammation by expressing adhesion proteins such as ICAM and VCAM that initiate the inflammatory response by attracting monocytes, and other cells, to adhere to them, as the first step to pass through the endothelial layer. In inflammatory diseases, endothelial cells can be over activated, expressing proteins such as ICAM in high levels along with VCAM (67). ICAM and VCAM have also been implicated in the recruitment of leukocytes to atherosclerotic plaques (104). T-ChOS upregulated the expression of both of these genes in HUVEC cells (**Figure 23-24**). This effect was not present in later passage HUVECs, demonstrating the importance of passage number for both cell lines, especially primary cells. This indicates that the T-ChOS could activate the first steps in the migration of monocytes towards sites of inflammation; ChOS could therefore increase the risk of forming atherosclerotic plaques (104). Adhesion proteins are also upregulated in THP-1 cells stimulated by cystatin C amyloid, which is in accordance with the differentiation effect it has on the THP-1 cells. The next step could be to apply

T-ChOS to HUVEC cells and incubate with labeled THP-1 cells and monitor their adherence, if any, to the endothelium layer. This would prove whether T-ChOS induces cell contact between these two cell types. Also, the migration of monocytes through the endothelium, represented by HUVEC cells, could be studied by both inducing adherence of the HUVEC cells and THP-1 cells, with T-ChOS and cystatin C amyloid, respectively.

The experiments performed in this study have started to explore the role of cystatin C amyloid in the immune response of monocytes and the effect that chitin derivatives might have on macrophages. Many new questions have arisen, but it is our hope that this study may lead in the direction of better clinical treatment of amyloid diseases in general and a deeper understanding of the biology of chitin derivatives. Both compounds affect immunity, showed in this study, making it possible that there are many ways in which these effects could be connected. For example IL-6 induced by amyloid might lead to the upregulation of YKL-40 (105). Also, in AD, increased activation of the hexosamine pathway is seen, resulting in high levels of glucosamine which may result in the synthesis of glucosamine polymers such as chitin. As a result, chitin derivatives might act as signaling proteins in amyloid diseases (106). In that context, the induction of genes for adhesion proteins in endothelial cells is of great interest. It is plausible that chitin derivatives generated at sites of protein aggregation, act as cytokines in attracting monocytes. Animal models have shown that chitin can stimulate mouse macrophages *in vivo* to phagocytose vascular Aβ amyloid (107). In fact, this discovery was one of the reasons for starting this project. It would be of interest whether the effect of T-ChOS would result in a similar outcome. It is important to get a better understanding of the bioactivity of T-ChOS and this study is an important step in that direction.

VI APPENDIX

Table 2. List of antibodies used in this study.

Antibody	Clone	Species	Isotype	Dilution (IF)	Dilution (WB)	Origin Cat nr. (Ref.)
Cystatin C	Polyclonal	Rabbit	IgG	1/1000	1/5000	Millipore 06-458
Lamp2	Monoclonal	Mouse	IgG1	1/500	1/2000	DSHB
Cystatin C	Polyclonal	Rabbit	IgG	-	1/10000	DAKO
F-actin – phalloidin 488	-	-	-	1/40	-	Invitrogen (A12379)
F-actin - phalloidin 647	-	-	-	1/40	-	Invitrogen (A22287)

Table 3. Primers for RealTime PCR.

Gene	Primer sequence (forward/reverse)	Size (bp)	Source
huGAPDH	F: TTGTTGCCATCAATGACCCC	837	Sigma-Aldrich
	R: TGACAAAGTGGTCGTTGAGG		
huTNF-α	F: AGCCC ATGTT GTAGC AAACC-	420	Sigma-Aldrich
	R: CCAAA GTAGA CCTGC CCAGA		
huIL-6	F: GTACATCCTCGACGGCATCT	80	Sigma-Aldrich
	R: GTGCCTCTTTGCTGCTTTCAC		
huIL-8	F: GGCACAAACTTTCAGAGACAG	153	Sigma-Aldrich
	R: ACACAGAGCTGCAGAAATCAGG		
huICAM	F: GCAGACAGTGACCATCTACAGCTT	68	Sigma-Aldrich
	R: CTTCTGAGACCTCTGGCTTCGT		
huVCAM2	F: GCAAGGTTCCTAGCGTGTAC	335	Sigma-Aldrich
	R: GGCTCAAGCTGTCATATTCAC		

Table 4. TaqMan assays for RealTime PCR.

Gene	Reporter	Quencher	Assay ID
huCHIT-1	FAM	NFQ-MGB	Hs001086753_m1
huAMCase	FAM	NFQ-MGB	Hs00757767_m1
huYKL-39	FAM	NFQ-MGB	Hs00970222_m1
huYKL-40	FAM	NFQ-MGB	Hs00609691_m1

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