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ESTABLISHING A PROTEOMICS-BASED MONOCYTE ASSAY TO ASSESS DIFFERENTIAL INNATE IMMUNE RESPONSES

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Establishing a Proteomics-Based Monocyte Assay to Assess Differential Innate Immune Responses

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

The innate immune system represents the first line of organism defense. Monocytes and their derivatives, the macrophages, play a central role in this response. The monocyte response to various stimuli is complex and includes simultaneous activation in a variety of ways. The common methods currently used to estimate this response usually focus on one of the modes of activation. The aim of this thesis was to assess the possibility of designing an assay based on unbiased proteome analysis that would be capable of predicting the monocyte response to various challenges. The underlying hypothesis of the project was that the cell proteome response to different activating molecules would reflect the immunogenicity of these molecules.

The assay is based on comparison between the proteomes of human monocytic THP-1 cells treated with a stimulus of interest and the 'reference' proteomes obtained from THP-1 cells treated with positive and negative controls. The study described in Paper I (Project 1) revealed two main types of monocyte responses and features of monocyte separation into adherent and non-adherent subpopulations. The first subpopulation differentiated into macrophages, while the second one was activated by cytokines according to the changes in their proteomes compared to the control cells. The Pam3CSK4 and PMA stimuli were concluded to be the best positive controls for pro-inflammatory activation and differentiation, respectively, among the tested nine treatments (Paper II, Project 2). Anti-cancer drugs with known mechanisms of action were chosen as positive controls for cell death (Paper III). The workflow of the assay was optimized and validated in Paper II (Project 2). The established assay was applied to identify the response of monocytes to peptide containing isoaspartate (Paper II, Project 3) and nanoparticles (Paper III, Project 3). The epidermal growth factor (EGF) peptide containing isoaspartate primed monocytes for differentiation but not for pro-inflammatory activation. The three studied nanoparticles induced distinct proteome signatures despite equivalent overall toxicity effect (induction of 50±10% cell death). The CdTe-NH_2 nanoparticles induced the strongest response characterized by down-regulation of topoisomerases and similar to the effect of Camptothecin. The response to CuO nanoparticles included up-regulation of proteins involved in heat response. The gold nanoparticles showed the weakest response among the studied three nanoparticles and induced up-regulation of NF-κB and a number of proteins related to energy metabolism consistent with pro-inflammatory activation.

The established and validated assay has several possibilities for extension as well as optimization for particular aims of the experiment. For instance, the identification of induced post- translational modifications upon stimulation can be performed (Paper IV, Project 4). Because of its versatility, robustness and specificity, this new assay is likely to find a niche among the more established immunological methods.

LIST OF SCIENTIFIC PAPERS

I. **Tarasova N. K.,** Ytterberg A. J., Lundberg K., Zhang X.-M., Harris R. A., and Zubarev R. A. Proteomics reveals a role for attachment in monocyte differentiation into efficient pro-inflammatory macrophages. *Journal of Proteome Research, 2015, 14 (9), pp. 3940-3947*

II. **Tarasova N. K.,** Ytterberg A. J., Lundberg K., Zhang X.-M., Harris R. A., and Zubarev R. A. Establishing a Proteomics-based Monocyte Assay to Assess Differential Innate Immune Activation Responses. *Manuscript submitted to Journal of Proteome Research*

III. **Tarasova N. K.,** Ytterberg A. J., Chernobrovkin A., Zubarev R. A. Toxicity Effect of Nanoparticles on THP-1 Monocytes Characterized by Combined Proteomics Approach. *Manuscript*

IV. Ytterberg A. J., Joshua V., Reynisdottir G., **Tarasova N. K.**, Rutishauser D., Ossipova E., Haj Hensvold A., Eklund A., Sköld C. M., Grunewald J., Malmström V., Jakobsson P. J., Rönnelid J., Padyukov L., Zubarev R. A., Klareskog L., Catrina A. I. Shared immunological targets in the lungs and joints of patients with rheumatoid arthritis: identification and validation. *Annals of the Rheumatic Diseases, 2015, 74 (9), pp. 1772-1777*

PUBLICATIONS NOT INCLUDED IN THE THESIS

I. Pyrkova DV, **Tarasova N. K.**, Krylov NA, Nolde DE, Pentkovsky VM, Efremov RG. Dynamic clustering of lipids in hydrated two-component membranes: results of computer modeling and putative biological impact. *J Biomol Struct Dyn., 2013, 31(1), pp. 87-95*

II. Heyder T., Kohler M., Haag S., **Tarasova N. K.**, Rutishauser D., Sandin C., Malmström V., Wheelock Å. M., Wahlström J., Holmdahl R., Eklund A., Zubarev R. A., Grunewald J., Ytterberg A. J. Sensitive approach for identification of HLA-DR peptides that facilitates characterization of individual subjects. *Manuscript submitted to Molecular and Cellular Proteomics.*

CONTENTS

LIST OF ABBREVIATIONS

ILC	innate lymphoid cells
MC	monocytes
PBMC	peripheral blood mononuclear cells
CD14	cluster of differentiation 14
PAMP	pathogen associated molecular patterns
DAMP	danger/damage associated molecular patterns
LPS	lipopolysaccharides
HSP	heat shock proteins
ROS	reactive oxygen species
PRR	pattern recognition receptors
TLR	Toll-like receptors
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
IFN	interferon
PTM	post- translational modifications
APC	antigen presenting cells
PCR	polymerase chain reaction
RT-PCR	reverse transcriptase–polymerase chain reaction
qPCR	quantitative polymerase chain reaction
ELISA	enzyme-linked immunosorbent assay
cDNA	complimentary DNA
FSC	forward scatter in flow cytometry
SSC	side scatter in flow cytometry
ELISPOT	enzyme-linked immunospot assay
2D PAGE	two-dimensional polyacrylamide gel electrophoresis
SDS	sodium dodecyl sulfate
pI	isoelectric point
MS	mass spectrometry
m/*z*	mass-to-charge ratio
ESI	electrospray ionization

MALDI	matrix-assisted laser desorption/ionization
TOF	time-of-flight
FT	Fourier-transform
PMF	peptide mass fingerprinting
MS/MS	tandem mass spectrometry
LC	liquid-chromatography
PCA	principle component analysis
PLS	partial least squares
OPLS	orthogonal projections on latent structures
PMA	12-acetate 13-myristate
PKC	protein kinase C
AML	acute myeloid leukemia
MAPK	mitogen-activated protein kinase
NP	nanoparticles
CdTe-NH_2	cadmium telluride coated with amine group
Au-20-NH_2	gold particles 20 nm in diameter coated with an amine group
CuO	cuprum oxide without coating
LN	LPS-treated Non-adherent cells
LA	LPS-treated Adherent cells
UN	Untreated Non-adherent cells
UA	Untreated Adherent cells
IsoD	isoaspartate
hEGF	human epidermal growth factor
RA	Rheumatoid Arthritis
ACPA	anti-citrulline protein/peptide antibodies

CHAPTER 1. BACKGROUND

1.1. INTRODUCTION

1.1.1. Innate immune system

The answer to the question: *'what would be an organism's response to a particular substance?'* would include analysis of the responses of different tissues, organs and cell types to this compound. The immune system is the defense system of the organism and most likely would be the first system detecting the compound and responding to it. Information about the response of the immune system would be therefore valuable for understanding the initial response of the organism to a substance.

The first line of immune response is the innate immune system. It triggers inflammation and the second line of the immune response, adaptive immunity. The Nobel Prize in Physiology was awarded to the strong opponents Paul Ehrlich and Ilya Metchnikoff in 1908 for their discovery of these two major parts of immune system, adaptive and innate immunity, respectively.[1,2] Historically the distinction between these types of immunity is based on (1) specificity of response, (2) phase or rapidness of the response, and (3) memory (Table 1). The emerging information about one group of innate immune cells, the innate lymphoid cells (ILC), however, makes this distinction less clear.

Table 1. Differences between the innate and adaptive immunity

Comparison	**Innate immune system**	**Adaptive immune system**
time scale	fast (hours)	slow first time (days) (fast to the secondary infection)
phase of immune defense	first	second
specificity	nonspecific	highly specific
memory	recent studies has indicated that it exists[3]	yes[4]
cells	monocytes, macrophages, granulocytes (neutrophils, basophils, eosinophils), dendritic cells, innate lymphoid cells (e.g., NK cells)	T-cells, B-cells
non-cellular components	acute phase proteins, cytokines, complement proteins	cytokines, antibodies
specific responses	molecular pattern recognition, antigen engulfment and presentation on cell surface	antibody production against particular antigen

The term ‘innate immunity’ in some cases includes physical, chemical, and microbiological barriers, but more usually encompasses the elements of the immune system which provide an immediate host defense. It is highly conserved and evolutionarily older than the adaptive immune defense strategy, and is present even in the simplest animals and plants.[5,6] The elements of the innate immune system include several types of cells (Figure 1), complement proteins, cytokines, and acute phase proteins (Table 1).[5] The innate immune system plays an important role in the initiation of inflammation and it also has been reported to be able to facilitate autoimmune diseases.[7,8]

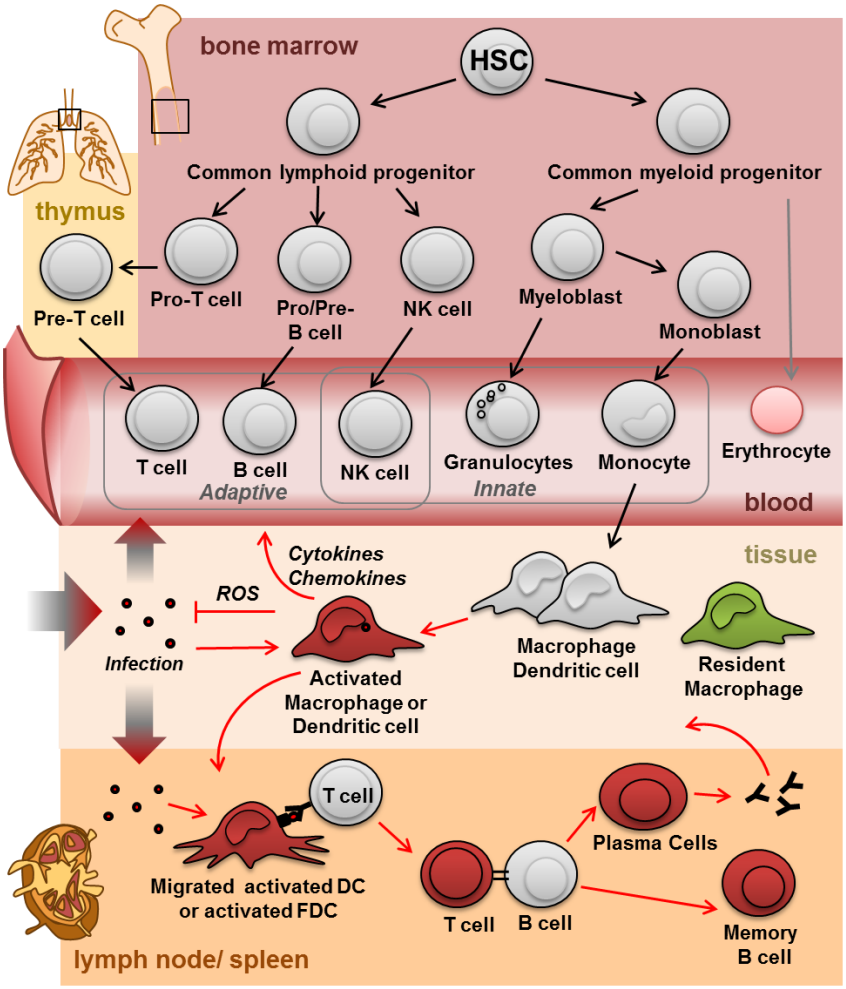


Figure 1. Simplified schematic diagram of immune cell development stages (black arrows), their interactions, and their response to infection (red arrows), focusing on monocytes/macrophages. All blood cells originate from a common progenitor, the hematopoietic stem cell (HSC). HSCs are located in the red bone marrow and give rise to myeloid lineages (monocytes, granulocytes, erythrocytes, megakaryocytes/platelets, and mast cells) and lymphoid lineages (T-cells, B-cells, and NK-cells). T-cells and B-cells subsequently need to pass positive and negative selection (in bone marrow for B-cells and in thymus for T-cells). Monoblasts migrate from the bone marrow to blood and become monocytes. Monocytes circulate in the blood, subsequently entering the tissue and differentiating into macrophages or dendritic cells. Macrophages and dendritic cells are the first responders to infection in tissues. They bind, engulf, digest and present parts of the foreign particles/bacteria to other cells. Activated T-cells transfer the signal to B-cells, which mature into plasma cells and start producing antibodies or develop into memory B-cells. Macrophages become activated by binding a danger associated molecular pattern and start producing cytokines and chemokines to activate and attract other cells. Macrophages also produce reactive oxygen species (ROS) to counteract the infection.

Upon an infection or damage of a tissue the first cells detecting and responding are the tissue resident macrophages.[9,10] The resident macrophage populations develop from monocytes in the yolk sac during embryonic development and self-replenish in the tissue.[11] These cells represent just an initial step of infection recognition and response. Their main function is to maintain homeostasis and in the steady-state they have anti-inflammatory functions.[12] In contrast, tissue clearance and inflammatory responses are mainly performed by macrophages derived from blood monocytes that upon stimulation leave the bloodstream, migrate into tissue and differentiate into macrophages or dendritic cells (Figure 1).[11] Monocytes thus act as 'immunological orchestrators',[13] and monocyte responses to different stimuli can in many cases reflect the innate immune responses to various compounds. Monocytes were therefore selected in this thesis as representatives of the innate immune system.

1.1.2. Monocytes and their responses

Monocytes are blood cells that can morphologically be distinguished by their kidney-shaped nucleus with mature chromatin, a low nuclear-to-cytoplasmic ratio and the presence of vacuoles. The average diameter of these cells is 14μm, which is about twice the size of erythrocytes. Monocytes belong to a mononuclear fraction of peripheral blood cells (peripheral blood mononuclear cells, PBMC). The PBMC can be isolated from the whole blood by density centrifugation. Monocytes can be purified from other PBMC by different approaches, one of the most common of which is based on high expression of the marker CD14 on their surface.

Isolated monocytes can be maintained in culture *in vitro* for several months, although changes in their morphology and activity were observed during such long-term culture.[14] The transition towards macrophages[14] correlates with the observation that *in vivo* monocytes stay in the bloodstream for 1-3 days and then migrate into tissues[3]. There are also examples of immortal monocytic cell lines derived from tumors (Table 2). In addition to their immortality the advantages of cell lines include independence from variability between the individual donors and easy maintenance in culture.

Table 2. Human monocytic cell lines derived from tumors

Cell line	Tumor	Reference
THP-1	acute monocytic leukemia	Tsuchiya *et al*., 1980[15]
U937	histiocytic lymphoma	Sundström *et al*., 1976[16]
HL60	acute promyelocytic leukaemia	Gallagher *et al*., 1979[17]
Mono Mac 6	Acute monocytic leukemia	Zeigler-Heitrock *et al*., 1988[18]

In the immortilised monocytic cell lines, the differentiation process from monoblast to monocyte is arrested. Several steps can be distinguished in the monocyte->monoblast differentiation: pro-monocyte, immature monocyte and mature monocyte. The differentiation

arrest in leukaemias occurs at relatively early stages of monoblast development due to the loss of proliferative potential in later stages of cell development.[19] The human monocytic THP-1 cell line (THP-1 cells) is derived from a young male diagnosed with acute monocytic leukaemia[15] and represents leukocytes stopped in their differentiation process between the promonocyte and immature monocyte stages. The earlier established cell lines employed for mimicking monocytes *in vitro*, such as U-937[16] and HL-60[17], require prior differentiation into monocytes.[20,21] Even though the later established Mono Mac 6 cell line is closer in its properties to mature human monocytes,[18] the THP-1 cell line is still employed in research more frequently. As such, THP-1 cells are considered to be one of the best models for monocyte-macrophage differentiation process, as well as for mimicking other monocyte responses.[22–25]

As with other cells of the innate immune system, monocytes are able to recognize and respond to various signals of danger, initiating inflammation (Figure 1, red arrows). Monocytes recognize a large number of molecular patterns, which can be generally divided into two groups (Figure 2). The first group, PAMP (pathogen associated molecular patterns), include conserved structures in pathogens (e.g. lipopolysaccharides (LPS), flagellin). The second group, DAMP (danger/damage associated molecular patterns), was discovered rather recently and includes signals originating from damaged tissue (e.g. HMGB1, HSP proteins, and ATP) and potential danger (e.g. silicon particles). PAMP and DAMP are recognized by pattern recognition receptors (PRR) on the surface of or inside the cells.[26] The PRR protein family includes Toll-like receptors (TLR), for the discovery of which Jules Hoffman and Bruce Beutler were awarded the Nobel Prize in Physiology or Medicine in 2011.

Table 3. Human toll-like receptors (TLR) and their ligands

TLR	**Ligand**
TLR1	triacyl lipopeptides
TLR2	peptidoglycan lipopeptides, lipoteichoic acid, lipoarabinomannan, GPI anchors, phenol-soluble modulin, zymosan, glycolipids
TLR3	dsRNA
TLR4	LPS, Taxol, RSV fusion protein, MMTV envelope protein, endogenous ligand (HSPs, fibronectin, hyaluronic acid)
TLR5	flagellin
TLR6	diacyl lipopeptides
TLR7	ssRNA, imidazoquinolines
TLR8	ssRNA, imidazoquinolines (only in humans)
TLR9	CpG DNA
TLR10	unknown

TLR detect molecular signatures which are conserved among a large group of pathogenic microorganisms.[27–29] Of the 13 currently known TLR members, 10 has been identified in humans (Table 3).[30] TLRs are located both on the cell surface (TLR1, TLR2, and TLR4-TLR6) and inside of cells. Each TLR recognizes a particular PAMP (Table 3). Ligand

binding to TLR initiates signaling cascades. The TLR signaling pathways consist of two major cascades depending on TIR domain–containing adaptors (MyD88 or TRIF). MyD88 mediates activation of NF-κB via all TLRs, except for TLR3, leading to the induction of inflammatory cytokine genes. TRIF mediates TLR3- and TLR4-dependent activation of IRF3 and NF-κB, inducing IFN-β production.[30] This leads to changes inside the cell (Figure 2), including post- translational modifications (PTM), increase/decrease in enzymatic activity, gene transcription, protein degradation and synthesis. These events in turn result in release of inflammatory mediators and the activation of the adaptive immune system, e.g. T-cells.

Apart from released inflammatory mediators, T-cells can be activated by antigens presented on the surface of monocytes bound to the MHC (major histocompatibility complex). The intracellular antigens are degraded in the proteasome, the degradation products are transferred to the endoplasmic reticulum and with aid of the TAP1/TAP2 complex, and loaded into the groove of MHC class I molecules. The antigen peptide bound to MHC class I is subsequently recognized by $CD8^+$ T-cells. MHC class I is ubiquitously expressed in all cells of an organism and is required for the cell to be recognized as a part of the organism. The external engulfed foreign molecular patterns (antigens) are digested in lysosomes and presented by the MHC class II to $CD4^+$ T-cells (Figure 2). The MHC class II is expressed at high levels in a subset of the immune cells that are hence referred to as antigen presenting cells (APC).

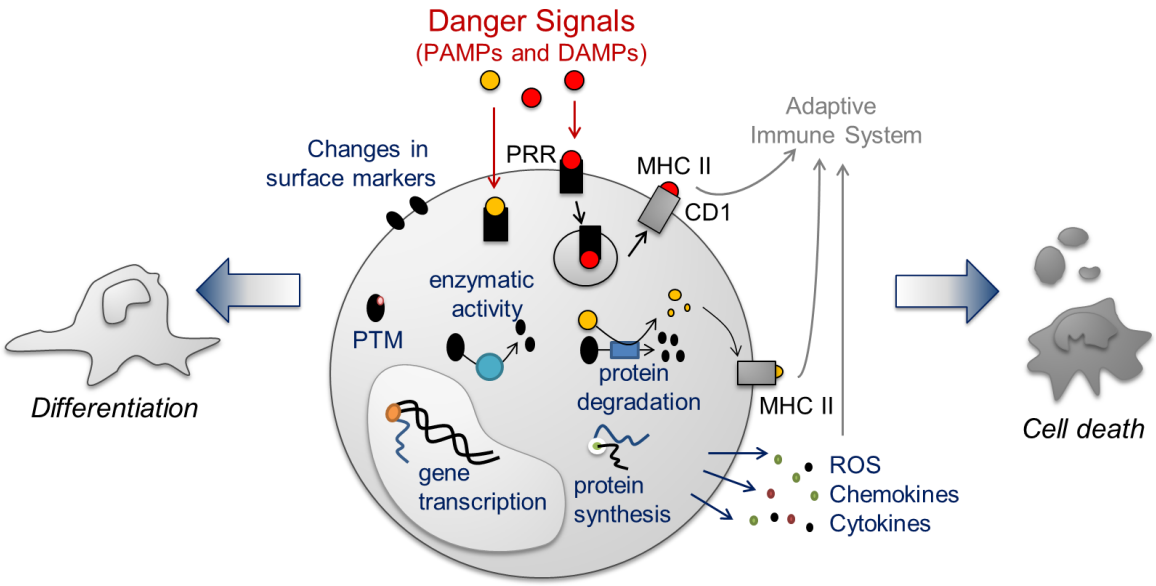


Figure 2. Simplified schematic diagram of molecular mechanisms of responses of antigen presenting cells with focus on events with proteins. Pathogen and damage/danger associated molecular patterns (PAMPs and DAMPs, respectively) are recognized by pattern recognition receptors (PRR; e.g. TLRs or NLRs) on the surface of or inside the antigen presenting cells. Extracellular antigens are engulfed, digested in endosomes and presented in complex with MHC class II (protein antigens) or CD1 (lipid antigens) to $CD4^+$ T-cells. Intracellular antigens are presented on the surface of the cell in a complex with MHC class I to $CD8^+$ T-cells. Antigen binding to PRR triggers an activation cascade in cells that transfers the signal to the nucleus. This leads to synthesis of new proteins, cytokines and ROS release, and activation of other signal cascades.

Monocyte responses include multiple processes and changes in the cells at all levels, from genome to proteome and secretome, and can eventually lead to cell differentiation or death (Figure 2). These responses and changes in the cells can be qualitatively characterized using a number of methods (e.g. RT-PCR, reverse transcriptase–polymerase chain reaction; immunohistochemistry). Monocyte responses can be also quantified and estimated using different methods, such as real-time polymerase chain reaction (qPCR or quantitative PCR), flow cytometry, enzyme-linked immunosorbent assay (ELISA), and kinetic studies.

1.1.3. Methods to study monocyte responses

The methods currently used to study monocyte responses can be divided into two large groups: focused and broad-based approaches (Table 4).

Table 4. Comparison between focused and broad-based approaches

	Focused approach	**Broad-based approach**
Goal	understanding the specific protein/gene regulation	understanding the system behavior (proteome, genome)
Pros	• less expensive, quick • can be more sensitive • precise, hypothesis-driven • does not require expensive equipment • many samples in parallel can be analysed	• information about a specific tissue/cell type under certain conditions • relationships between a large number of molecules • hypothesis-generating
Cons	• information about the target is required prior to analysis • hard to study global relationships between a large number of molecules	• more expensive and time-consuming • extensive planning is required in advance • more expensive equipment • fewer samples can be analysed

1.1.3.1. Focused Approaches

The focused approaches currently dominate in immunology (Table 5), potentially due to their low price and demands on instrumentation as well as availability and variety of this type of kits and assays. Their results are also quite easy to analyze and interpret. Depending on which type of cell response (e.g. cytokine release, surface marker expression) and cell compartment is studied, different approaches can be applied.

The cell responses include protein degradation as well as synthesis of new proteins. The changes synthesis of new proteins upon cell activation can be due to alterations on translational as well as transcriptional level. One of the ways to observe changes in gene expression is RT-PCR. The complimentary DNA (cDNA) molecules can be obtained from RNA template molecules with the reverse transcriptase enzyme. The cDNA molecules can then be amplified by PCR, the technology being based on DNA replication.[31] The typical cycle of PCR process includes three main steps: (1) denaturation of DNA, (2) annealing of the selected/designed primers, (3) elongation of new DNA strand. This cycle is repeated 20-40 times, resulting in thousands of copies of particular DNA molecule(s). If the PCR reaction

is monitored in real time, the number of RNA copies also can be determined, which is a basis of the qPCR method.

Table 5. Comparison between targeted approaches frequently used in immunology

Method	Cell response	Target type	Target identification	Quantification basis
RT-PCR	gene expression	RNA	primer	real time PCR monitoring (qPCR)
western blot	protein level regulation	protein/PTM	antibody	western blot signal
flow cytometry	surface markers	usually protein	antibody	particle counts
ELISA	cytokines	usually protein	antibody	light intensity
Luminex	cytokines	protein	antibody	bead counts and intensity of florescence

Proteins can be identified and quantified from complex mixtures using western blotting. In western blotting proteins are separated by gel electrophoresis, transferred to a solid support and the target protein is detected by primary and visualized by secondary antibodies conjugated to a fluorophore or enzyme.[32]

In flow cytometry, the cells of interest are first labeled with fluorescent markers, which are usually antibodies against specific cell surface proteins conjugated with fluorophores. In the cytometer the stained cell sample in a suspension buffer is hydrodynamically focused with the aid of a small nozzle, which allows cells to pass the laser light one at a time. Each suspended particle or cell passing through the beam scatters the light. The detectors can measure various parameters of light scattering by a single cell. One detector is placed in line with the light beam to measure forward scatter (FSC) and this correlates with the cell size. Several perpendicular detectors can measure side scatter (SSC) that depends on the inner complexity of the particle (i.e. shape of the nucleus, the amount and type of cytoplasmic granules or the membrane roughness). The data from the light scattering can be plotted using 2D plots (FSC vs SSC) to visualize different cell populations in the sample. The detection of fluorescent labels on the surface of the cells can be used to differentiate between cell types. The number or percentage of the cells expressing the marker of interest on its surface can be used to characterize a cell population. With some modifications to the procedure, flow cytometry can be also applied to detect intracellular proteins, including cytokines in cells.

Extracellular cytokines released from the cells are commonly analyzed using ELISA (enzyme-linked immunosorbent assay). The ELISA is a plate-based method that allows detection and quantification of various molecules. In a standard ELISA the antigens from the sample are attached to a surface. Specific antibody is applied over the surface so that it can bind to the antigen. This antibody is linked to an enzyme, which transforms a substrate into a detectable signal. Most commonly the signal is colorimetric. Indirect ELISA employs

labelled secondary antibody that binds to the primary antibody and increases the sensitivity of the method as well as improving immunoreactivity of the primary antibody. Sandwich ELISA is the most powerful assay format with the advantages of being sensitive and robust. It is called sandwich because the analyte is bound between two primary antibodies, the capture and the detection antibodies, respectively. The sandwich ELISA-like assay called ELISPOT (enzyme-linked immunospot assay) allows capturing the proteins locally as they are secreted by plated cells.

The Luminex technology is based both on ELISA and flow cytometry and allows the detection of multiple cytokines simultaneously. It is based on color- and size-coded polystyrene or superparamagnetic beads coated with analyte-specific antibodies. Beads recognizing different cytokines are mixed together and incubated with the sample. Captured analytes are subsequently detected using biotinylated secondary antibodies and a streptavidin-phycoerythrin (PE) conjugate. The polystyrene beads are usually analyzed using a dual-laser flow-based detection instrument. One laser classifies the bead and determines the analyte that is being detected. The second laser determines the magnitude of the PE-derived signal, which corresponds to the amount of the bound analyte. Luminex technology is a step towards multiplex analysis, but it is still based on detection of specific known analytes. Omics methods are able to solve this issue, representing unbiased, broad-based and hypothesis-generating methods.

1.1.3.2. Broad-based approaches

The broad-based approaches are also called omics methods, because their names end with '-omics': genomics, transcriptomics, proteomics, metabolomics. Depending on which part of the central dogma of biology is explored, different omics techniques are applied (Figure 3). Genomics studies entire DNA content of the cell and gives a possibility to predict what is going to happen in the cell. What cells intend to do is encoded in RNA molecules, studied by transcriptomics methods. Proteomics, the simultaneous analysis of the entire protein population, allows the identification of what is happening in a cell. Finally, metabolomics mainly studies what has happened inside a cell, i.e. the metabolic products o proteins' enzymatic activity. Our focus was on the proteomics approaches, which give information about the current situation inside a cell. We believe that this approach is the best for estimating the response of the cells, including monocytes.

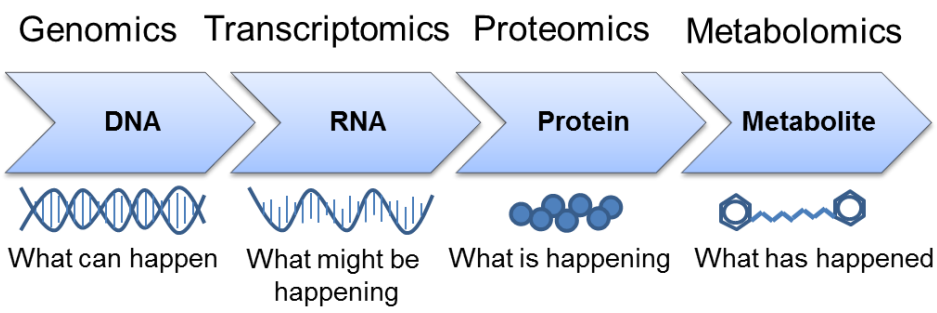


Figure 3. Central dogma of biology and 'Omics' methods to study each part of it.

Proteomics

The word ‘proteome’ was first used to describe the protein complement of an organism’s genome and later gave a rise to a new discipline ‘proteomics’.[33] Proteomics can largely be divided into functional, structural and differential proteomics.[33,34] *Functional proteomics* identifies protein-protein, protein-DNA or protein-RNA interactions affecting particular functions. *Structural proteomics* characterizes interactions of proteins with metal ions, toxins, drugs, etc. affecting the protein structure. *Differential*, or *protein expression, proteomics* determines the differences in concentration levels, which depend on protein expression and degradation. Historically, differential proteomic analyses have relied on two-dimensional polyacrylamide gel electrophoresis (2D PAGE).

In 2D PAGE proteins are separated by isoelectric focusing in the first dimension and SDS-polyacrylamide gel electrophoresis in the second one. While isoelectric focusing separates proteins according to their isoelectric points (pI), SDS-polyacrylamide gel electrophoresis separates them according to their molecular weights. The final result of the 2D PAGE is a map with proteins focused into spots, which can be subjected to further analysis using mass spectrometry (MS).[35] Proteins can be also directly analyzed with MS without prior gel separation.

The measurements in mass spectrometry are performed in the gas phase on ionized analytes. A mass spectrometer consists of an ion source, a mass analyzer that measures the mass-to-charge ratio (*m*/*z*) of the ionized analytes, and a detector that registers the number of ions at each *m*/*z* value. Electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) are most commonly used to volatize and ionize the proteins or peptides for mass spectrometric analysis.[36,37] ESI ionizes the analytes out of a solution and can therefore be coupled to liquid-based (chromatographic) separation devices. MALDI sublimates and ionizes the samples out of dry crystals with matrix via laser pulses. MALDI is usually used for the analysis of relatively simple protein/peptide mixtures, while integrated liquid-chromatography ESI systems (LC-MS) are more suitable for the analysis of complex samples.[38] The most commonly used mass analyzers for proteomics are: quadrupoles (Q), radiofrequency ion traps (quadrupole ion trap, QIT an linear ion trap, LIT or LTQ), time-of-flight (TOF) mass analyzers, and Fourier-transform (FT) mass analyzers, such as electronic FT Orbitrap or magnet-based FT ion cyclotron resonance (FTICR) mass analyzers.[39]

There are two mail approaches for protein identification and characterization.[40] In the first one, *top-down* approach, intact proteins or large protein fragments are analyzed. The alternative approach, *bottom-up*, is to analyze peptides generated by enzymatic cleavage of proteins. When bottom-up approach is applied to a mixture of the proteins it is called *shotgun proteomics* method (analogous to shotgun sequencing of DNA).[41] Trypsin, which cleaves on the C-terminal side of lysine and arginine residues, is a commonly used protease in bottom-up approach. Digestion with trypsin results in peptides of an appropriate length and generates spectra that are easy to interpret due to basic groups in both ends of the peptide: the free

amine group on N-terminus and either Lys or Arg on C-terminus. Peptides can be identified by peptide mass fingerprinting (PMF) or tandem mass spectrometry (MS/MS). In PMF, the masses of individual peptides in a mixture are measured in mass spectrum. In MS/MS, peptides are selected by a mass analyzer are fragmented into smaller pieces, with the masses of latter detected, which can be used to deduce the amino acid sequence of the peptide (Figure 4). This technique is more powerful and allows identification of the types and sites of peptide/protein modifications. The combination of LC with the bottom-up MS/MS approach is the most common incarnation of the shotgun proteomics.[42] Mass spectra derived from peptide fragmentation are compared to theoretical tandem mass spectra generated from *in silico* digestion of a protein database. The identified peptides are assigned to proteins.

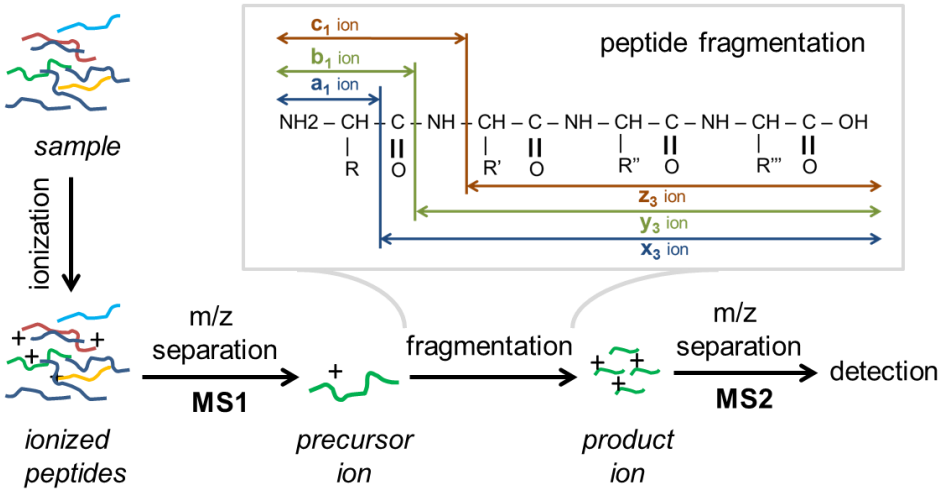


Figure 4. Scheme of tandem mass spectrometry and types of peptide fragmentation. Following ionization, precursor ions are selected at the mass selection stage (MS1) that are subsequently fragmented. The product ions are selected in the MS2 stage. Additional stages of selection and fragmentation can be performed before fragment ion detection. Peptide backbone fragmentation and nomenclature of formed fragments is presented in the insert in the right upper corner.

In addition to protein ID's the data can be used for quantification. Though there are number of MS-based methods for absolute quantification (e.g. AQUA), most techniques are based on relative quantification. Relative quantification can be performed with or without labelling. The labelling techniques employ metabolic labelling at cellular or tissue levels (SILAC), chemical (ICAT, iTRAQ, TMT) or enzymatic ($H_2{}^{18}O$) labelling at protein or peptide levels. Samples are merged after the labelling, which decreases the technically-induced variation between them.

The advancement in LC-MS equipment during the last decades together with the need to analyze larger and larger sample sets has increased the interest in label-free techniques. Label-free proteomics approaches can be divided into two groups by the way that the abundance of a peptide is measured. The first group includes methods that are based on ion counting. These methods compare either maximum abundance or volume of ion count for peptide peaks at specific retention times between different samples. The second group is

based on the identification of peptides by MS/MS and uses sampling statistics such as peptide counts, spectral counts, or sequence coverage to quantify the differences between samples.[43]

The studies in this thesis has relied on shotgun and label-free proteomics.[42] The common shotgun label-free proteomics workflow can be divided into a few general steps (Figure 5). The first step is common for all experiments, but is more important in the case of broad-based approaches compared to focused ones. This step includes the development of a hypothesis specific to the proteome being studied, selection of the most suitable technique among those described above and selection of comparable controls and samples. The following technical steps are (1) cell/organism treatment and sample collection, (2) sample preparation for MS analysis, (3) MS data acquisition, and (4) MS data *in silico* analysis.

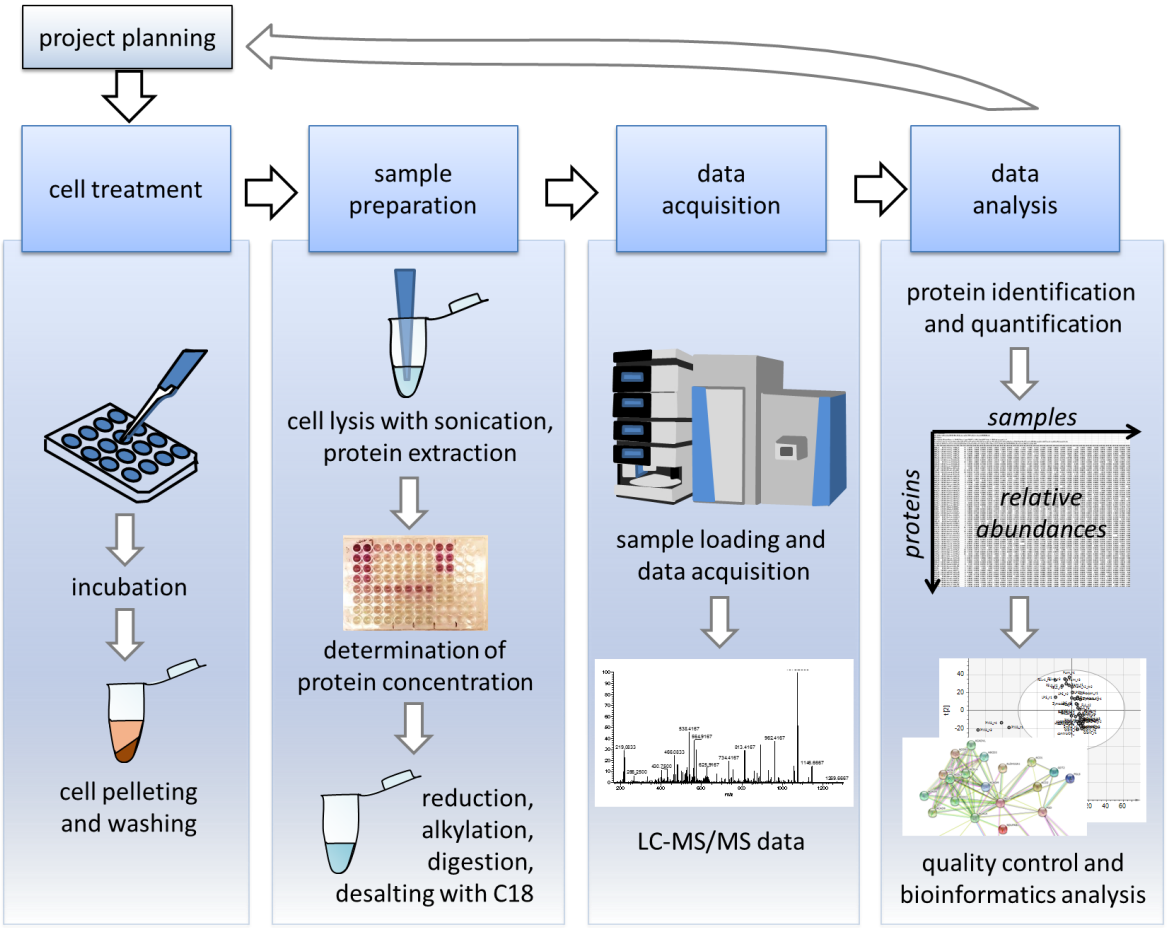


Figure 5. Common workflow for label-free shotgun proteomics to study the effect of cell treatment.

Proteomics data analysis requires quality control of the obtained and a correct choice and application of statistical analysis methods.[44] The number of proteins is normally greater than samples. Methods to analyze for data with multiple variables (multivariate analysis) are developed for such data sets.

These methods include principle component analysis (PCA), partial least squares (PLS), orthogonal projections on latent structures (OPLS) and various clustering methods.

The PCA analysis is a widely applied method for reduction of dimensions of complex data to reveal the potentially hidden, simplified dynamics that often underline it.[45] The PLS and OPLS methods are supervised versions of the PCA method, where the main variable or groups are defined, respectively. The position of sample on PCA plot represents a 'fingerprint' of the stimulus it was exposed to. Proteins affected by the treatment and therefore responsible for the position of the sample on PCA plot can be identified and further investigated as markers for the stimulation. The significance of up- or down-regulated proteins can be identified using T-test or other types of univariate analysis with correction for multiple testing (e.g. Bonferroni correction and false discovery rate) to avoid false positives due to the multiple testing. The proteins of interest identified in these analyses can be mapped to known cellular pathways or processes.

In summary, MS-based proteomics is a powerful unbiased approach to identify changes in cells occurring upon treatment.

1.2. PURPOSE AND HYPOTHESIS OF THE THESIS

The purpose of the thesis was to generate an unbiased approach based on proteomics able to assess complex monocyte responses. We aimed to design an assay based on proteomics of THP-1 cells that is able to estimate the biological effect of various challenges on monocytes. The hypothesis underlying the project was that different monocyte-activating compounds will trigger qualitatively different proteome responses that can be distinguished using label-free MS-based proteomics.

1.3. LITERATURE REVIEW

The current advances and knowledge of the project's topic are summarized in this chapter. The purpose of the thesis was to design a monocyte-based proteomics assay. The first part of the literature review is focused on aspects of assay development and different versions of biological assays. The second part of the review is focused on summarizing the results of proteomics studies of THP-1 cells.

1.3.1. Assays

An assay is a standardized procedure for qualitative assessment or quantitative measurement of the presence, amount or the functional activity of a target entity, the analyte. Originally in the 13th century the word 'assay' was used in the phrase *assay the coin* that meant to analyze the purity of a gold or silver coin. Later it was generalized to analytical procedures with other types of analytes. The analyte in biological assays for example can be a drug, biochemical substance or a cell. Currently there is a large variety of biological assays available, and their numbers are rapidly increasing. However, assays usually include a number of common steps, such as sample processing and manipulation, signal amplification, detection and discrimination from background.

The common procedure to establish an assay often also consists of several steps, starting with development and optimization, followed by validation and end with custom adjustment depending on the particular experiment. Establishment as well as application of each assay requires assessment of its quality. The number of assay characteristics can be employed as a basis to evaluate its quality, most important of which are sensitivity, specificity, reproducibility, area of applications and limitations.

A high reproducibility is required for a successful assay. This can be assessed through analysis of replicates within an experiment or using similar samples in different experiments. The specificity and sensitivity are interconnected and an increase in one often leads to a decrease in the other. The sensitivity (true positive rate) characterizes the assay's ability to detect an analyte in the sample, while specificity (true negative rate) relates to the assay's ability to distinguish it from other signals. The sensitivity depends on the detection limit. For an individual analytical procedure the detection limit is the lowest amount of analyte in a sample that can be detected. The limitations and area of application of each assay are defined by these characteristics as well as the type of assay.

There are different ways to classify assays:

- number of analytes studied (single analyte or multiplex assay)
- assay principle (e.g. response of cells, organisms etc)
- target being measured (protein, DNA, RNA, or other molecules)
- type of the result produced (quantitative, semi-quantitative, or qualitative)
- nature of assay process (end-point assays with detection at fixed time point or dynamic with detection on-line or at set of time points)
- detection methods

The assay that was established in this study is a semi-quantitative multiplex assay based on changes in protein abundances as a result of the cellular response (THP-1 cells) to stimulus. The assay can be applied as both an end-point and a kinetic assay. The MS-based label-free proteomics approach was selected as a detection method for the assay in the project.

1.3.2. Proteomics of THP-1 cells

The THP-1 cell line has been sucessfully employed for more than 30 years in hundreds of studies, including over 50 proteomics studies. Starting from the beginning of the century the number of the publications has been increasing exponentially (Figure 6). The years 2009 and 2015 are outliers, when the number of publications exeeded that predicted by the exponential curve, and this could be explained by rapid development of proteomics methods as well as increased interest in macrophages and monocytes within the immunological research community prior to these years.

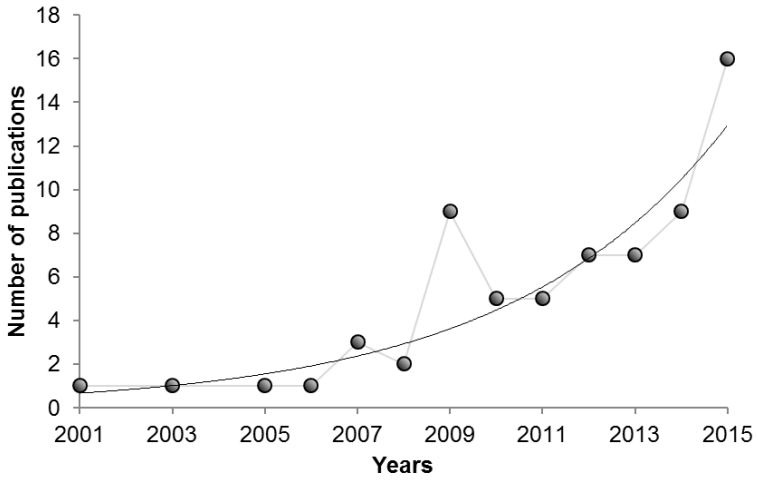


Figure 6. Number of publications per year that employ THP-1 cell line as a research tool and proteomics as a method. The studies where the THP-1 cell line was used at the confirmation step were also included.

Proteome changes upon THP-1 monocyte activation have been investigated in a number of studies. Monocytes can be activated by external (foreign) stimuli such as bacteria or their fragments (‘6’ in Figure 7).[46] Other sources of monocyte activation are internal signals, like immunogenic proteins/peptides (‘4’ in Figure 7),[47] hormones (‘5’ in Figure 7) or deviations in extracellular conditions from homeostasis (e.g. high glucose)[48]. Internal signals are a necessity for appropriate communication between cells and cooperative reactions to infections. The signals produced by monocytes, including the secreted ones (secretome), have also been studied using proteomics methods.[49,50]

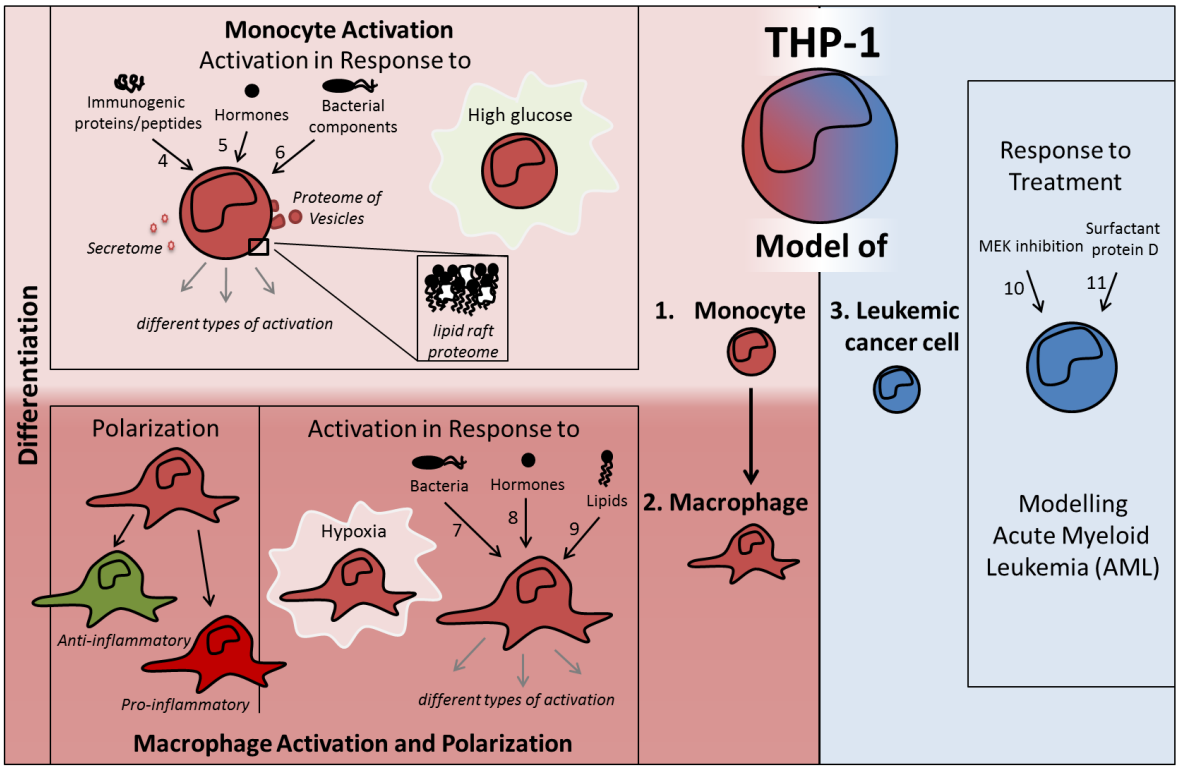


Figure 7. A summary of protemics studies of THP-1 cells is presented. THP-1 cells combine properties of monocytes and leukemic cancer cells. Differentiated monocytes are also often used as a model for macrophages. Both monocytes and macrophages can be activated by stimuli or extracellular conditions deviating from homeostasis (e.g. high glucose, hypoxia). Different sources of activation stimuli are presented. Macrophages can also be polarised into pro- and anti-inflammatory macrophages. THP-1 cells can model acute myeloid leukemia and were used to study the effect of treatments against leukemia.

THP-1 activation has been demonstrated to induce changes in the abundance of stress-associated proteins, including up-regulation of heat shock proteins and the proteasome.[51,52] Apart from the set of general stress-induced proteins, unique up- and down-regulated proteins related to the immune response were also identified for each stimulus. For example, cyclophilin is up-regulated in response to bacterial GpG DNA[51,] or HMGB1 and LCP1 are characteristic for cytokine activation (IL-1β and TNF-α)[52].

The THP-1 cell line has been successfully applied in proteomic studies, not only as a model of monocytes (section 1.1.2), but also for mimicking its derivatives, macrophages and

dendritic cells. There are also examples whereby THP-1 cells have been employed for modelling microglia[50] (resident myeloid cells in the central nervous system analogous in function to monocytes) or foam cells[53] (fat-laden macrophages which play a critical role in the occurrence and development of atherosclerosis).[54] About half of the proteomics studies employ macrophages derived from THP-1 cells, also termed 'THP-1 macrophages'. A trend can be observed over the years with the interest shift from THP-1 monocytes to THP-1 macrophages. THP-1 macrophages behave more like primary monocyte-derived macrophages compared to other human myeloid cell lines.[22] The standard method for THP-1 differentiation into macrophages is incubation with phorbol 12-acetate 13-myristate (PMA). Other inducers of THP-1 differentiation into macrophages include retinoic acid,[55] lipopolisacharide (LPS),[56] and 1,25-dihydroxyvitamin D3[57].

PMA is an artificial leukocyte differentiation signal and a substance originally purified from plants. PMA penetrates through the membrane and activates the enzyme protein kinase C (PKC) inducing signal transduction cascade. The effects of PMA on PKC result from its similarity to one of the natural activators of PKC, diacylglycerol.[58] The concentration of PMA used in the publications varied in the range 6-200 ng/mL, but the most frequently used concentration is about 50 ng/mL. The incubation time also varies between 12 and 120 h, with 48 h being the most common. In a number of investigations THP-1 macrophages after PMA stimulation were left resting in fresh media for 24 h prior to intended stimulation,[38,59–62] allowing cells to proceed further along the differentiation process[63]. THP-1 macrophages have been compared with macrophages derived from primary monocytes or with tissue macrophages [63], but not in proteomic studies.

The study perfomed by Kristensen and collegues[64] revealed the proteomics signatures of THP-1 differentiation induced by PMA. Differentiation is a process that requires structural and metabloic changes in cells that correlates with up-regulation of the proteins belonging to cytoskeleton, cell adhesion and junction processes, or cell membrane. The integrin family of the proteins is up-regulated during this process. Conversely, DNA replication and chromasomal processes are down-regulated upon differentiaiton. That fact correlates with the decrease of the proliferative ability of the cells and its almost complete loss at the terminal differentiation stage. Moreover, this study uncovered an interesting fact that during the differentiation the protein degradation rate remains constant while the synthesis rate increases. This observation reveals one feature of the differentiation mechanism that the less energy-efficient but better controlled way of changes in cell proteome is preferred.

THP-1 macrophages differentiated via PMA stimulation were involved in studies that revealed the macrophage responses to different stimuli, such as microorganisms (e.g. *Leishmania donovani,*[65], Campylobacter,[60] and *Porphyromonas gingivalis*[66]) or specific compounds and complexes (e.g. TLR ligands[51] or oxidized low density lipoproteins[24,67]). The stimulation effects include up-regulation of the proteins associated not only with immune function[60], but also with key metabolic pathways[60,65] and cytoskeleton[66]. The effects of conditions such as hypoxia have also been studied in THP-1 macrophages.

THP-1 cells have a dual functionality and can also be investigated as leukemic cells (‘3’ in Figure 7). The THP-1 cell line originates from leukocytes of a boy diagnosed with acute monocytic leukemia,[15] a type of acute myeloid leukemia (AML) and a cancer of the myeloid leukocyte lineage. Thus THP-1 cells combine properties of myeloid cells as well as cancer cells (Figure 7). A few proteomic studies employed the THP-1 cell line in order to model leukemia.[68–70] Aasebo and colleagues used super-SILAC mix and THP-1 cells together with a number of other AML-derived cell lines to create an internal standard for quantitative shotgun studies.[33] The internal standard was designed to approach the problem with strong heterogeneity of proteomic samples from patients with AML, which in turn makes their quantification challenging.

One of the potential approaches for treatment of AML patients was developed with the aid of THP-1 cells and a proteomics approach (‘10’ in Figure 7).[69] The mitogen-activated protein kinase (MAPK) pathway was identified to be aberrant in leukemic cells from AML patients.[69] The mechanism of MAPKK1/2 (MEK) inhibition, found to decrease AML cell survival, was studied in THP-1 cells using a proteomics approach. These findings gave the insight that MEK inhibition should be combined with VEGFR-2 inhibition to block the escape route of the leukemic cells.

Surfactant protein D (SP-D) was proteomically tested in leukemic cell lines, including THP-1, as a drug against AML (‘11’ in Figure 7).[70] This proteomic analysis elucidated the underlying mechanisms of rhSP-D-induced apoptosis, highlighting a decreased expression of proteins related to survival (such as HMGA1), upregulation of proteins protecting the cells from ROS, and a decrease in the mitochondrial antioxidant defense system.

In summary, THP-1 cells have mainly been employed in proteomics studies as models of monocytes or macrophages. However, a few investigations used them as models of other myeloid cells as well as cancer cells. The proteomics findings revealed features of THP-1 monocyte and THP-1 macrophage activation as well as of transition process between these cell types.

CHAPTER 2. PRESENT INVESTIGATIONS

2.1. AIM AND GOALS OF THE THESIS

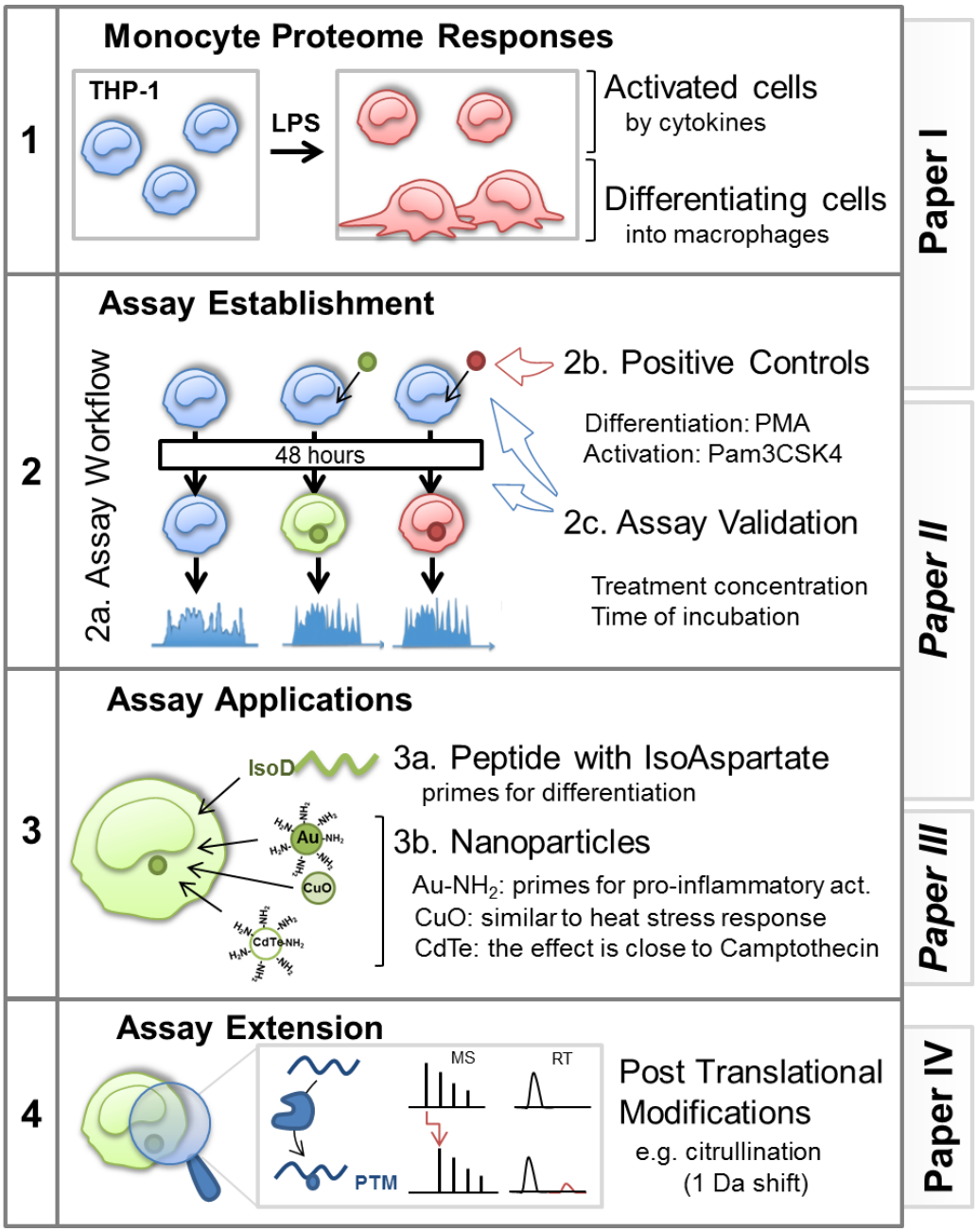
The aim of the thesis was to design an *in vitro* assay based on unbiased proteome analysis that would be capable of predicting the complex response of the innate immune system to various challenges.

The specific goals within the project were to:

1. Identify the proteome differences between the cell subpopulations following THP-1 cell treatment with LPS
2. Establish the assay:
    a. Establishing the assay workflow
    b. Finding the best positive controls
    c. Validating the assay: finding the best suitable treatment concentration and the time of inoculation
3. Apply the assay to assess the effects of the following substances on THP-1 cells:
    a. Isoaspartate-containing peptide
    b. Nanoparticles (CdTe-NH_2, Au-20-NH_2, CuO)
4. Investigate possible extensions of the assay and identify the citrullinated sites in the proteins using label-free proteomics

2.2. OVERVIEW OF THE STUDY AND CORRESPONDING PAPERS

The results of the thesis can be divided into four subprojects corresponding to the goals, which are summarized in Scheme 1. The subprojects will be referred to as *Project 1-4* and are described in more detail in the section *2.4 Results and Discussion* in this chapter. The results of the projects were included in the papers and the manuscripts listed above and are depicted on the right hand side of Scheme 1.



Scheme 1. The four goals of the thesis (section 2.1) and the main results of the project. The results are described in Papers I – IV included the thesis. The papers in the manuscript form are marked in Italics (Paper II is submitted to Journal of Proteome Research).

2.3. MATERIALS AND METHODS

This section summarizes the details of the proteomics experiments performed to establish and apply the assay (Scheme 1, Project 1-3). The details of the experiments in Project 4 (Scheme 1) are described in Paper IV. The numbers of the projects mentioned in this section are corresponding to Scheme 1.

Cell culture, harvesting and protein extraction

In all the experiments the human monocytic cell line THP-1 (ATCC) was employed. The cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Biochrome, Merck Millipore), 0.05 mM mercaptoethanol, 2 mM L-Glutamine and 100 U/mL penicillin-streptomycin mixture at 37 ºC. The experiments were performed in plates or flasks with 'traditional Tissue Culture surface' from Sarstedt. Nonadherent THP-1 cells were used at the initiation of all experiments. The cells in suspension were collected first. The cells remaining adherent were incubated for 5 min with 0.05% trypsin mixed with EDTA and phenol red (Gibco) before collection. Collected cells were counted using a TC10 cell counter (BioRad) with trypan blue staining. After centrifugation (6-7 min; 1,000 × *g*), the cell pellets were washed with PBS and lysed in a lysis buffer (8 M urea in 100 mM ammonium bicarbonate, with or without 50 mM NaCl) by sonication on ice. The sonication was performed in one of two ways: (1) 21 s, in three repeats with breaks in between (Projects 1, 2, 3a); or (2) 40 s with pulses (5 sec on/5 sec off), in two repeats with a break in between (Project 3b). The latter achieved almost twice the amount of protein per cell.

Protein isolation and digestion

Cell lysates were centrifuged (20 min; 20,800 × *g*; 8 ºC), and supernatants were collected. The protein concentration in the supernatants was determined using the BCA kit (Pierce). Following reduction (5 mM dithiothreitol, 37°C, 30 min) and alkylation (14 mM iodoacetamide, RT, 30 min), 10 µg of proteins were digested by serine protease trypsin (Promega) at a 1:30 trypsin:protein concentration ratio (37 ºC, overnight). Digestion was stopped with formic acid (FA; final concentration 5-7%). Digests were desalted with C18 StageTips (Thermo Scientific; following the manufacturers' instructions), dried, and resuspended in 0.1% FA with or without 2% ACN prior to analysis. In Project 3a, in which only adherent cells were analyzed, acetone precipitation was performed prior to digestion. The acetone precipitation was done at -20 ºC in 90% pre-chilled acetone solution (v/v) for 3 hours, followed by centrifugation (20 min; 20,800 × *g*; 8 ºC), acetone removal and evaporation of the remains of acetone and dissolving the pellet in the lysis buffer.

Mass Spectrometry

LC-MS/MS analyses were performed using a nanoflow HPLC (high-performance liquid chromatography) directly coupled online to a Q-Exactive mass spectrometer (both Thermo Scientific, Germany). For each sample, 1 or 2 µg were injected from a cooled autosampler onto an fused silica tip column (SilicaTips™, New Objective Inc., USA) packed in-house with C18-AQ ReproSil-Pur® (Dr. Maisch GmbH, Germany). Mass spectra were acquired with a resolution of 70,000. The data-dependent MS/MS spectra were taken using HCD with the collisional energy of 25. Samples were analyzed in a randomized order.

Protein Identification and Quantification

MS/MS data were searched against the concatenated version of the UniProtKB/Swiss-Prot or UniProt complete proteome database using the Mascot search engine (Matrix Science Ltd., UK; www.matrixscience.com). The following parameters were used: trypsin digestion with a maximum of two missed cleavages; carbamidomethylation (C) as a fixed modification; pyroglutamate (Q), oxidation (M) as variable modifications; a precursor mass tolerance of 10 ppm and a fragment mass tolerance of 0.25, 0.1 or 0.02 Da. The list of identified proteins was filtered using 1% false discovery rate (FDR) and at least two peptides per protein as limiting parameters. Label-free quantification was performed using the in-house developed program Quanti that compensates in silico for electrospray current fluctuations.[71] Further details and results of the analyses are provided in the papers. In total, between 1,595 and 3,289 proteins were quantified in each experiment with at least two unique peptides per protein.

Bioinformatics and Statistical Analysis

Protein abundances were normalized with the assumption that equal amounts of protein digests were injected for each sample. Proteins that likely originated from the media (serum albumin and hemoglobin) or sample handling (keratins) were excluded from the results. Log transformed abundance values were used for further analysis. Principal component analysis (PCA) and Orthogonal Projections to Latent Structures (OPLS) were performed using the Simca software (Umetrics). Unpaired Student's t-test with equal or unequal variance (depending on the result of Excel F-test) was applied to calculate the p-values. FDR (Benjamini-Hochberg)[72] adjusted or Bonferroni adjusted p-values (q- and E-values, respectively) with a threshold of 5 % were used to identify significantly up/down-regulated proteins and pathways. Pathway analysis was conducted using String (http://string-db.org/). The q-values for Gene Ontology (GO) terms enrichment were calculated by comparing to the dataset with all identified proteins. The fold change was calculated as a logarithm of ratio between the average relative abundance of a protein in treated cells and corresponding control cells.

The quality control of proteomics data was performed in several ways: (1) the retention times of peptides were compared for consistency among different samples within the same experiment and the samples not fulfilling the criterion were excluded from the experiment; (2) whether protein abundances correlated with the order of injection was assessed and the proteins fulfilling the criterion were excluded from an experiment; (3) PCA plots were analyzed for the presence of outliers. The strong outliers not grouping with other biological replicates were excluded from the experiment; (4) proteins quantified in less than 80% of experiments were excluded from the analysis.

Treatments

Table 6. Components applied to stimulate THP-1 cells

Treatment	Description/ structure (origin unless synthesized)	Diluent	Concentration
LPS	Lipopolysaccharide (*E. coli*)	PBS	20, 35, 100, 500 or 800 ng/mL
CpG DNA	5'-tcgtcgttttgtcgttttgtcgtt-3'	Water	0.01 μg/mL
HKLM	Heat Killed Listeria monocytogenes (*L. monocytogenes*)	Water	10^8 cells/mL
Flagellin	Principal component of bacterial flagella (*Salmonella typhimurium*)	Water	1 μg/mL
Zymosan	Glucan with repeating glucose units connected by β-1,3-glycosidic linkages	Ethanol	10 μg/mL
FSL-1	(Palmitoyl)2-CGDPKHPKSF	Water	1 μg/mL
Pam3CSK4	(Palmitoyl)3-CSK4	Water	0.1, 0.5 or 2.5 μg/mL
ssRNA	5'-GCCCGUCUGUUGUGUGACUC-3'	Water	1 μg/mL
PMA	Phorbol 12-myristate 13-acetate (12-O-tetradecanoylphorbol-13-acetate)	DMSO	0.01 μg/mL
N-cont.EGF peptide (N)	**N**SDSEGPLSHDGYGLHDGV	DMSO	0.15 μg/mL
D-cont. EGF peptide (D)	**D**SDSEGPLSHDGYGLHDGV	DMSO	0.15 μg/mL
IsoD-cont. EGF peptide (IsoD)	**isoD**SDSEGPLSHDGYGLHDGV	DMSO	0.15 μg/mL
CdTe-NH_2	cadmium telluride nanoparticles coated with amine group	PBS	5 μg/mL
CuO	cuprum oxide nanoparticles	PBS	22 μg/mL
Au-20- NH_2	gold nanoparticles 20 nm in diameter coated with an amine group	KCl 50 mM	15 μg/mL
Camptothecin	anti-cancer drug, alkaloid	DMSO	50 μM
Doxorubicin	anti-cancer drug, anthracycline	DMSO	30 μM

2.4. RESULTS

2.4.1. Project 1. Monocyte adhesion, activation and differentiation (Paper I)

The pilot experiment with pro-inflammatory stimuli led to the observation that THP-1 cells separate into two subpopulations after the treatment. One fraction of the cells adhered to the surface of the culture dish while the other remained in suspension. Adherent cells were detected in untreated cell population though were present at much lower fraction of the cells compared to treated cell population (Paper I, Figure 1C). The observations are consistent with the fact that *in vivo* monocytes are able to adhere to the surface of blood vessels and migrate into tissue, and that this process is enhanced during inflammation.

The separation of cells into subpopulations does not affect measurements in media (e.g. cytokines or ROS), but has a great influence on the results of the methods analyzing the cellular content, such as proteomics. The question about the difference between cell subpopulations is also important for macrophage-based immunotherapy. Monocytes can be differentiated and activated into pro-inflammatory macrophages that might be employed in tumor immune therapy. We hypothesized that proteomes of adherent and non-adherent monocytes following pro-inflammatory treatment are different and could be distinguished using label-free proteomics. We decided to focuse on one pro-inflammatory stimulus and chose LPS as a standard treatment to induce a pro-inflammatory macrophage state (‘1’ in Scheme 1).

Adherent and non-adherent THP-1 cells were collected and analyzed separately after incubation with or without LPS (Figure 8A, insert). The proteomes of four subpopulations could be distinguished by label-free proteomics and multivariate analysis (Figure 8A), supporting the main hypothesis of the study (section 1.2). The analysis of up- and down-regulated proteins revealed that the cells remaining in suspension upon LPS treatment were activated by cytokines and primed for rapid responsiveness to pathogens (Figure 8B). In terms of proteome change, the adhesion process was orthogonal to activation (Figure 8A). Adherent cells following LPS treatment exhibited signs of differentiation and enhanced innate immune responsivity, suggesting that they were closer to macrophages (Figure 8C). These findings indicate that adherent, LPS-treated cells would be more appropriate for use in tumor therapeutic applications. Potentially, the cells stimulated with LPS adhere to the surface and start producing cytokines, which in turn activates cells remaining in suspension, but further studies on primary monocytes are needed to confirm this mechanism.

Project 1 also addresses a more general question about the role of attachment in monocyte differentiation. According to our results, attachment seems to be required for differentiation, but on its own, attachment only primes cells, making them more sensitive to pro-inflammatory signals (Figure 8D). This conclusion implies that the population of adherent cells is a valuable part of the general cell population. It should be collected for

proteomics analysis and might be even analyzed separately depending on the focus of the investigation.

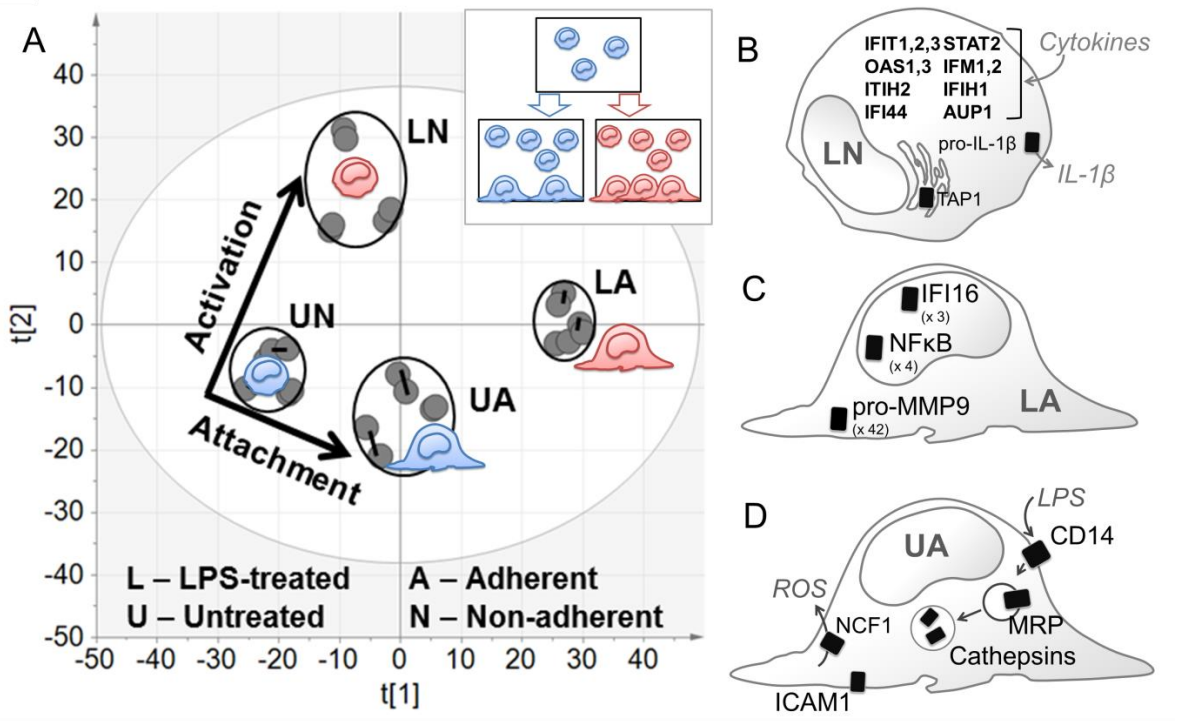


Figure 8. Summary of the findings in Project 1. **(A)** Principle component analysis (PCA; R^2=0.3; Q^2=0.2; four principle components) of proteomics data and schematic overview of the experimental design (insert). PCA revealed good reproducibility of biological (grouped by circles) and technical (connected by lines unless overlapping) replicates. Untreated Non-adherent cells were used as a control population because they stayed in the same morphological state after incubation. Vectors connecting UN to LPS-treated Non-adherent (LN) and Untreated Adherent (UA) are almost orthogonal suggesting that the proteome changes in these processes are largely independent. **(B)** Selected up-regulated proteins in LN cells (full list in Paper I, Table S5). About 70% of up-regulated proteins (listed in bold) are known to be up-regulated in response to cytokines. **(C)** Markers of macrophage differentiation up-regulated in LPS-treated Adherent (LA) cells with fold change values in parentheses (full list of up- and down- regulated proteins in LA in Paper I, Table S7). **(D)** Selected up-regulated proteins in UA cells suggested that the cells are primed for pro-inflammatory activation, which was also confirmed in additional proteomics experiment (Paper I, Figure 5).

2.4.2. Project 2. Assay establishment (Paper I and II)

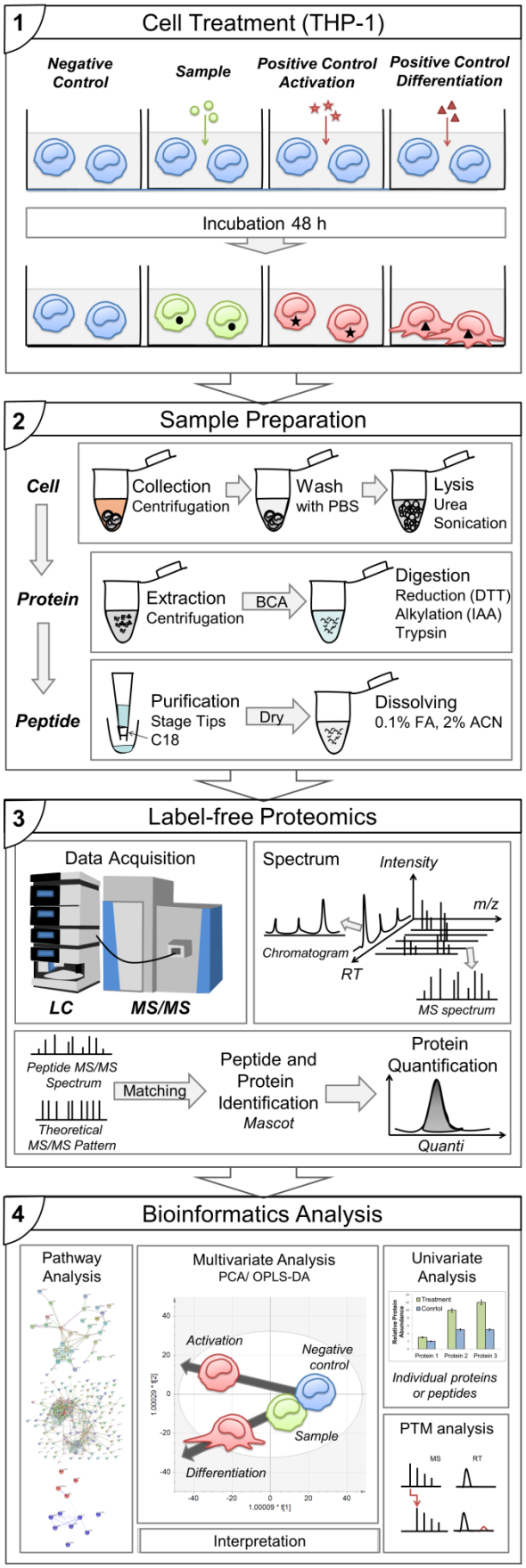


Figure 9. The workflow of the assay

The assay is based on comparisons between proteomes of monocytes treated with a compound of interest and the 'reference' proteomes derived from monocytes treated with positive and negative controls. The workflow of the assay consists of four major steps as depicted in Figure 9.

The first step is the cell treatment and incubation for 48 h with positive and negative control stimuli as well as the compound(s) of interest. The THP-1 cells line was chosen as a model of monocytes. Media without treatment was selected as negative control. The choice of positive controls can be optimized for the particular experiment. For establishing the assay we focused on two major types of monocyte responses we could identify in literature: activation (also referred as 'plasticity' or 'reversible changes') and differentiation (also referred as 'irreversible changes'). We searched for the best positive controls for 'pro-inflammatory activation' among TLR ligands and selected PMA as a control for 'differentiation'. The proteomics experiment supported by cytokine measurement in media and cell count results confirmed the hypothesis about two major types of monocyte responses (Figure 10). Based on the results of the experiment PMA was selected as a control for differentiation and Pam3CSK4 was chosen as the best control for pro-inflammatory activation. The number and set of the positive controls in the particular experiment can be changed and, for instance, control for cell death can be added in case of toxic compound (see section 2.4.3.2) or anti-inflammatory stimuli in case such an effect is expected.

The time of incubation is another parameter in the assay that can be optimized depending on the stimulus studied. We tested three time points (24, 48 and 72 hours of incubation) for selected pro-inflammatory treatments (Pam3CSK4 and LPS). The PCA analysis revealed that longer the incubation larger the distance between control and treated samples (Paper II, Figure 3). However, the total number of days needed for assay workflow increased with longer treatment. We chose 48 hours for general workflow of the assay, which seemed to be a reasonable compromise.

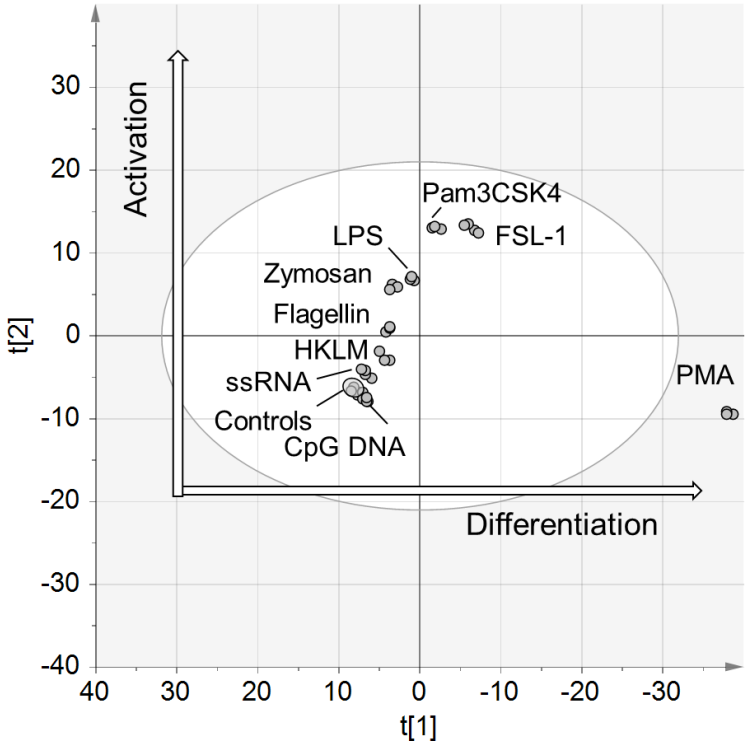


Figure 10. THP-1 cells were treated with eight TLR ligands and PMA for 48 h, harvested and analyzed using label-free proteomics. OPLS analysis (R^2=0.628, Q^2=0.266) revealed two major modes of monocyte response, activation and differentiation.

The second step of the assay workflow (‘2’ in Figure 9) starts with cell collection. As discussed in Project 1 and confirmed in experiment with TLR ligands and PMA, stimulation as well as the negative control results in a mixed population of adherent and non-adherent cells, with pro-inflammatory stimuli resulting in a much higher degree of adherence. Following the results in Project 1, there two possible approaches to identify the response of the monocytic cells. The first approach, in which both adherent and non-adherent cells are collected together following stimulation, evaluates the response of the whole cell population. The second approach, in which either only adherent or only non-adherent cells are collected, would assess the response of the potentially migrating and differentiating cells or activated cells, respectively (see section 2.4.3.1). The procedures in the second and third steps in the assay workflow (Figure 9, ‘2’ and ‘3’) correspond to preparation and analysis of cell pellets using a label-free proteomics approach (also discussed earlier in sections 1.3.2 and 2.3).

The final step of the workflow is the bioinformatics analysis (OPLS or PCA). Multivariate analysis allows assessing and visualizing the cellular responses to the stimuli of interest. The response can not only be characterized, but also estimated. An OPLS model can be linearly transformed with negative control set to zero (Paper II, Figure 2B). The distances from the origin along the ‘activation’ and the ‘differentiation’ axes could be used to estimate the response of THP-1 cells to various stimuli. These results can be also accomplished by PTM and pathways analyses to interpret the data and generate hypotheses about the mechanism of response.

2.4.3. Project 3. Assay applications (Paper II and III)

The established in Project 2 assay was applied in two studies described below. The first was aiming to test whether THP-1 monocytes can recognize presence of post-translational modification isoaspartate (IsoD) in peptide, while the second one was focused on identifying the effects of different nanoparticles on THP-1 monocytes.

2.4.3.1. Effect of IsoD-containing peptide

The peptide, with isoaspartate modification, selected as a test compound for the first application of the assay, originated from human epidermal growth factor (hEGF). The hEGF is a protein that influences cell growth, proliferation, and differentiation cell surface receptor EGFR.[73,74] Recombinant hEGF is used as a drug, in imaging methods and in targeted therapies against cancers with over-expression of EGFR.[75,76] The N-terminal sequence of hEGF starts with Asparagine-Serine- (N-S-), a sequence that is prone to rapid deamidation. The resultant hEGF with IsoD-S- at the N-terminus has a significantly lower affinity to EGFR. Moreover, IsoD in proteins has been reported to be immunogenic and to alter autoimmune responses.[77,78]

We chose the N-terminal hEGF peptide containing the first 19 amino acids of the protein with N-terminal asparagine (N) or IsoD to test with the assay. The approach with analyzing only adherent THP-1 cells following incubation was applied in this investigation. As a result the 'activation' and 'differentiation' axes were not orthogonal on the OPLS plot (Figure 11C, x1 and x2).

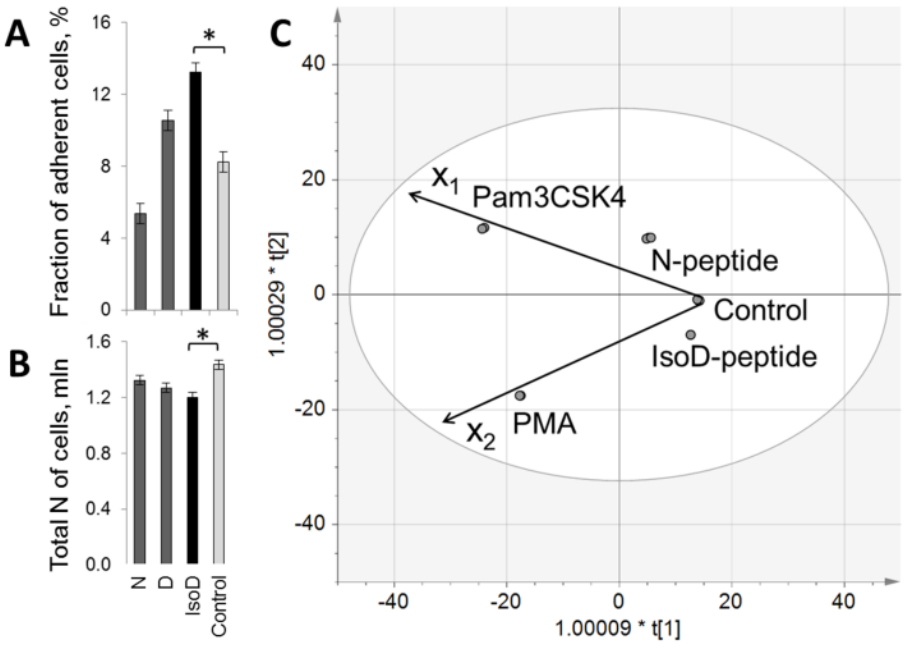


Figure 11. Effect of IsoD-containing hEGF peptide on THP-1 cells. The IsoD-peptide induced **(A)** an increase in percentage of adherent cells and **(B)** decrease in total cell number. **(C)** The proteomics assay was applied to characterize the effect of the IsoD-peptide on adherent THP-1 cells. The OPLS model revealed that the peptide primes cells for differentiation (x_1 axis), but not pro-inflammatory activation (x_2 axis). The control N-peptide primed cells for activation.

The results revealed that the IsoD-peptide primed cells for differentiation, while the natural form of the peptide primed cells towards activation (Figure 11C). This conclusion is consistent with the result of cell counting. The IsoD-peptide treatment resulted in an increase in the fraction of adherent cells and a decrease in the total number of the cells compared to control, while neither N- nor D-peptides induced such an effect (Figure 11A and 11B). The results of the study imply that the peptides differing in one amino acid can be distinguished by monocytes and it can be detected by the established assay.

2.4.3.2. Effect of Nanoparticles (Au-20-NH_2, CdTe-NH_2, CuO-core)

The second application of the assay was on nanoparticles, a rapidly developing group of materials as well as environmental contaminants. The estimation and characterization of the toxic effects of the nanoparticles is necessary for their safe application and handling. Three types of NP with different level of toxicity (different effective concentration, EC_{50}) were selected for the study. These NP were cadmium telluride coated with amine group (CdTe-NH2), gold NP 20 nm in diameter, also coated with an amine group (Au-20-NH_2), and cuprum oxide without coating (CuO-core). The effects of these NP on cells have been previously studied, but to best of our knowledge have never been combined and compared in one proteomics experiment before.

A positive control for cell death was added in this study. Two anticancer drugs (Camptothecin and Doxorubicin) with known mechanisms of action were selected as positive controls for cell death due the cancer origin of THP-1 cells (section 1.1.2). The anticancer drugs and NP were used at a concentration inducing 50±10% cell death in THP-1 (EC_{50}). The lipopolysaccharide (LPS) was used as the positive control for pro-inflammatory activation.

The strongest effect was induced by CdTe-NH_2 NP (Figure 12A). The THP-1 response to CdTe-NH_2 was similar to the Camptothecin effect and both induced down-regulation of topoisomerases (Figure 12B). The effect of CuO-core combined features of THP-1 responses to LPS and Camptothecin. Interestingly, CuO-core induced up-regulation of number of heat shock proteins. The response to gold NP could be characterized by up-regulation of inflammatory mediator NF-κB and a number of protein related to energy metabolism, which were also up-regulated in LPS-treated cells.

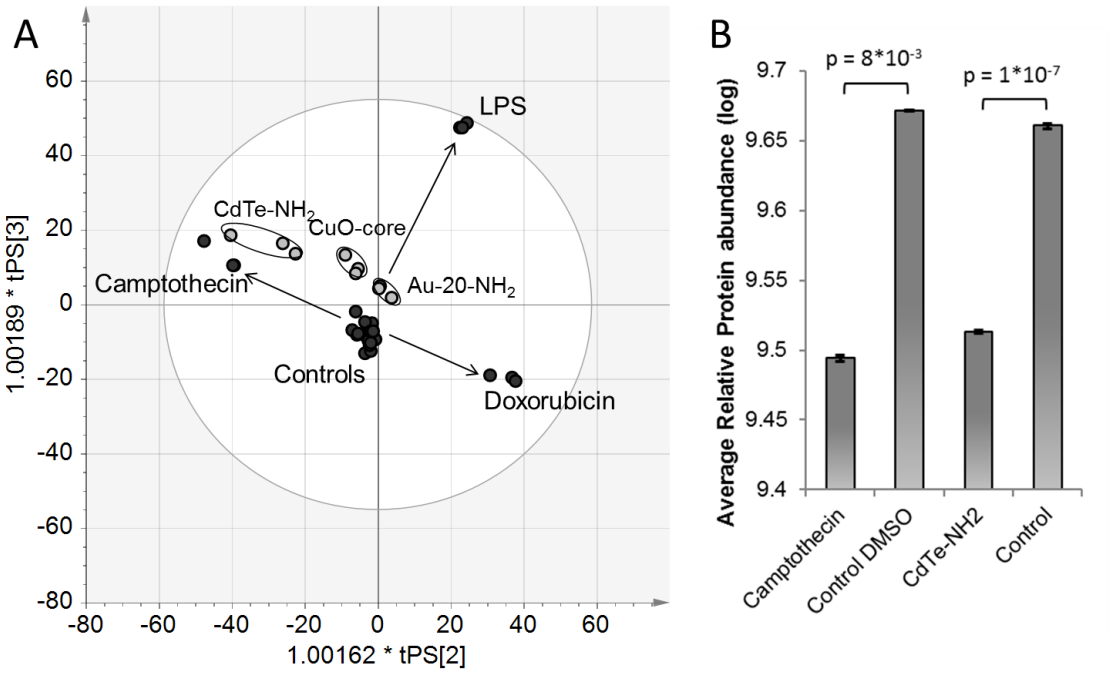


Figure 12. **(A)** Prediction of the positions of samples on the score plot based on the OPLS model with four groups (proteomes of LPS, Doxorubicin and Camptothecin treated cells as well as controls). **(B)** The average relative abundance (log transformed) of Topoisomerase 1 (TOP1) in cells treated with Camptothecin, CdTe-NH_2, and corresponding controls.

In summary, the applied assay appeared to be an applicable method to characterize the effect of NP. This method revealed that the nanoparticles inducing the same phenotypical effect (50% dead cells) have different impart on the THP-1 monocyte proteomes.

2.4.4. Project 4. Assay extensions and PTM analysis (Paper IV)

The data obtained in proteomics-based assay can be applied not only to identify 'fingerprints' of stimuli and compare expression of proteins in different treatments but also to 'look inside the proteins' and their changes induced by the stimuli. Project 4 was focused on one of the protein modifications occurring in a post-translational modification (PTM) process, citrullination.

Citrulline residues are formed from arginine. The conversion is catalyzed by a family of enzymes called peptidylarginine deiminases (Figure 13). Several proteins normally contain citrulline in their mature form (e.g., filaggrin, keratin and several histone proteins) while other proteins have been shown to be citrullinated during pathological processes, such as inflammation and cell death.

In Project 4 we applied and optimized a direct approach to identify citrullinated peptides from LC-MS/MS spectra without prior enrichment or fractionation. Trypsin cleaving after arginine or lysine but most likely not after citrulline would generate different peptides from citrullinated and unmodified version of a protein. The LysC enzyme was used instead of trypsin to be able to identify the modified peptides and compare the modified sites with the unmodified ones. Citrullinated peptides can be identified using a search engines such as Mascot, yet false positive identifications can be a large problem. Errors in precursor mass can lead to false positive results as well as presence of asparagine in the peptide sequence. Deamidation of asparagine results in the same mass shift. Manual analysis of spectra was used to validate and confirm the citrullinated sites. The markers of citrullination include shift in monoisotopic mass with ~1Da, shift in retention time, diagnostic mass shift(s) in MS/MS, difference in general MS/MS pattern (Figure 13). The citrullination results in loss of charge which leads to changes in hydrophobicity and shift in retention time towards elution of more hydrophobic peptides (Figure 13, 'RT'). Together with a shift in charge state, the distribution can validate the modification site in the peptide.

The approach described above was applied to find a link between lungs and joints in development of Rheumatoid Arthritis (RA). RA is an inflammatory autoimmune disease affecting joints. There is strong evidence linking RA and citrullination: a significant part of RA patients exhibit the presence of anti-citrulline protein/peptide antibodies (ACPAs).[79] The fact that these antibodies appear in the serum before any clinical signs of inflammation in the joints indicates that the initial triggering of ACPA production may take place somewhere other than the joints. The hypothesis was that the immunological events in the lungs might trigger production of ACPA during early RA. To test the hypothesis, the presence of shared immunological citrullinated targets in joint and lung biopsies of patients with RA was investigated.

In total 6,790 peptides (non-redundant on sequence level) were identified using the Mascot search engine in analyzed biopsies, including 385 potential citrullinated peptides. Only 10 candidates were confirmed to be citrullinated, meaning that about 97% of the

identified citrullinated peptides were false positive. More than half of the false positive peptides (60%) contained asparagine in their sequence. Eight citrullinated peptides were found in synovial biopsy specimens and seven were detected in bronchial ones (Paper IV). Five of these peptides were shared between the specimens. The identified peptides were synthesized and spiked into patient samples for absolute quantification by MS (AQUA method). One candidate peptide was synthesized and the presence of antibodies against this peptide in patients with RA was confirmed by ELISA.

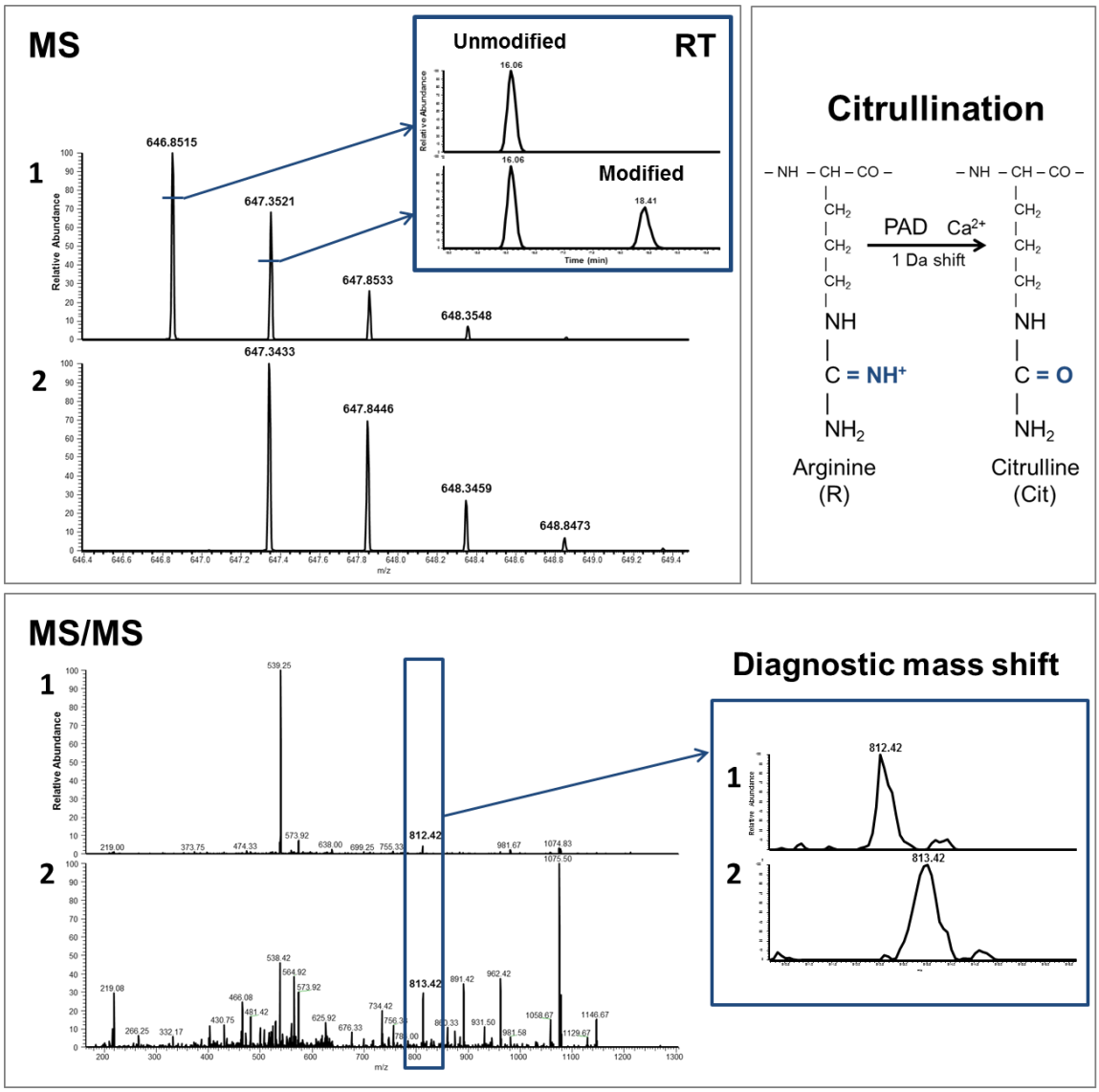


Figure 13. Example of citrullinated peptide verification and comparison to unmodified pepride with a scheme of arginine conversion to citrulline. (1) – unmodified peptide TNPPAGPVRAIAE (m/z = 646.8517, z = 2, mascot score = 47), (2) – citrullinated pepride TNPPAGPVRAIAE (m/z = 647.3441, z = 2, mascot score = 69). Peptides belong to protein PPAD digested by trypsin, GluC and pepsin B.

This study showed that the direct approach with validation can be used to identify citrullinated peptides in such complex systems like biopsies. It also gave support to the theory that lungs and joints have shared citrullinated epitopes and that RA inflammation might be initially starting in other organs than joints.

2.5. DISCUSSION

2.5.1. Assay evaluation

The main characteristics (section 1.3.1) of an assay, such as specificity, sensitivity, reproducibility, limitations and area of application, are discussed in this section in relation to the established procedure for identifying monocyte responses.

The selectivity of the assay refers to the main hypothesis of the study and depends on method of detection, monocyte capability to distinguish different substances and the uniqueness of the proteome responses. The experiments performed with the project confirmed the main hypothesis and showed that the different monocyte proteome responses can be detected depending on stimuli the cells were exposed to. The assay was capable to reveal that monocytes can detect and distinguish peptides with difference even in one amino acid (Paper II, Project 3).

The sensitivity of the assay similarly to selectivity also depends on the method of detection, monocyte sensitivity to compound and monocyte proteome responses to compound. Monocytes as part of innate immune system are sensitive and are capable of detecting a large variety of compounds. The high sensitivity of MS instruments allows the use of low amounts of samples (≈1 μg of protein per sample). Correspondingly, only a small number of cells are required (1-3 x 10^5 cells). The high sensitivity of the assay opens the perspective of applying it in clinical settings on human primary monocytes.

The monocyte proteome response to stimuli was observed to have saturation with increasing concentration of the compound and constant time of incubation (Figure 5 in Paper I and Figure 3 in Paper II). This observation is consistent with the fact that the protein abundances are well-regulated with positive and negative feed-back loops. The synthesis rate is also limited and regulated leading to restriction of protein production. On the other side of concertation range, low concentration of a treatment will lead to proteome changes induced in a few cells which might not be distinguished in the population. It implies that changes in proteomes can be detected within a certain concentration range of compounds depending on their potency.

The effects of the substances are investigated on the level of the cellar population instead of individual cells in proteomics. This can lead to a combination of signals from different cell subpopulations. However, there are methods to overcome this limitation. The separate collection of adherent and non-adherent cells and/or cell sorting by FACS separating subpopulations according to specific markers of interest can be used to narrow the cell population diversity. Another limitation of the assay is the throughput of approximately 2 hours/sample that is common for LC-MS-based proteomics techniques.

Using proteomics as a detection method require careful quality control to avoid false positive results. We suggest using several criteria for assessment of the quality of the

proteomics data which has been also described in section 2.3 *Materials and Methods* in this chapter. Comparison of the peptides' retention times for consistency among different samples within the same experiment and assessment of the correlation of the protein abundances with the order of injection evaluates the quality of LC. Proteins quantified in less than 80% of experiments are less reliable than others and can be excluded from analysis. The presence of outliers and position of the replicates on PCA plots shows the quality of the experiment in general as well as its reproducibility.

The reproducibility of the assay can be estimated from the performed experiments within the project and can be divided into two parts: reproducibility of the replicates within the experiment and reproducibility of the proteome response to a substance between the replicate experiments. The first can be evaluated based on the proximity of the replicates (biological and technical) on a PCA plot. The quality of technical replicates (same sample loaded on instrument twice) was assessed in Project 1 (Figure 8). Samples were loaded in random order. All the technical replicates were located close together or overlapped on the PCA plot, indicating the quality of the LC-MS/MS analysis was good. In all the performed experiments the biological replicates of the proteomes of cells treated with one treatment were grouped close together on the PCA plots (e.g. Figure 8, Figure 10) and separated from other samples. The reproducibility of the cell response to treatment in different experiments can be estimated based on the list of up- and down-regulated proteins found to be significant in cell response to a particular treatment. Such a comparison performed in Project 1 was concluded to be successful (Paper I). The lists of these proteins are not expected to be identical in different experiments, but the presence of key molecules in these lists and overlap between them indicate good reproducibility.

The total number of identified and quantified proteins in our experiments was in a range $1.5 - 3.3 \times 10^3$. This number was moderate compared to other current publications, especially taking into account that no fractionation or labeling was applied prior to LC-MS analysis. The goal of the study was not a deep proteomic analysis, but to provide enough specificity for reliable OPLS/ PCA separation. For reaching this goal, 3,000 proteins are more than enough. This number of proteins is even enough to make hypotheses about the mechanism of treatment action.

2.5.2. Assay applications

The assessment of the immunogenicity of various compounds is one of the main intended applications of the assay. Several modifications in proteins have been shown to induce immune response and play role in autoimmune disease (Project 4, Paper IV). In Project 3 (Paper II) we investigated the immunogenicity of the IsoD post translational modification. The IsoD formation is linked to protein damage and protein structure alterations.[80] IsoD in proteins has also been strongly correlated with development of autoimmune diseases[77] and Alzheimer's disease[81,82]. The established assay was applied to study the effect of a peptide from the human epidermal growth factor with the first amino

acid, N, replaced with IsoD. The cell counting and the assay revealed that the monocytes treated with the IsoD-containing peptide were primed for differentiation, but not a pro-inflammatory response like natural form of the peptide. The cell activation by the natural form of the peptide potentially can be explained by the presence of secondary structure in peptide and possibly partial binding to the EGF receptor. The priming for monocyte differentiation into macrophages by IsoD peptide supports the link between innate immunity and neurodegenerative disease, which could be important and intriguing to investigate further.

The estimation of immunogenicity as well as toxicity of newly created substances, such as nanoparticles, is an even more important issue. It is crucial for the protection of environment and people working with nanoparticles. The study described in Paper III (Project 3) was focused on three nanoparticles with different degrees of toxicity. The toxicity was measured in the concentration required to induce 50±10% cell death after 24 h (EC_{50}). The CdTe nanoparticles as well as their constituent heavy metal ion Cd^{2+} are known to be toxic and revealed response in THP-1 cells similar in magnitude to anticancer drugs. It affected topoisomerases, which might indicate that the toxicity mechanism of these nanoparticles involves effect on DNA. Indeed, Cd^{2+} ions which can be released from the nanoparticles were shown to inhibit the DNA repair mechanisms in the cells.[83]

CuO-core nanoparticles are less toxic than CdTe-NH_2 nanoparticles, and even at a higher dose, the effect on the proteome was significantly smaller. The proteins up-regulated in CuO-treated cells included four heat shock proteins as well as a number of molecules involved in response to unfolded proteins. Such effects could be due to Cu^{2+} being able to substitute in proteins other biologically important dications (Fe^{2+}, Mn^{+2}, and Mg^{2+}), causing structural changes in these proteins. Changes induced in extracellular matrix organization, similar to LPS treatment, could be due to the monocyte activation as well as in response to ROS induced by CuO.

As opposed to the CuO and CdTe nanoparticles, gold nanoparticles have been shown to induce cell death only in particular cases, and their cytotoxicity strongly depends on the size and the cover groups.[84,85] A positive charge on the NP surface (e.g. an amine group as in the current study) make them more accessible for the cells. In our study the Au-20-NH_2 nanoparticles induced cell death the in THP-1 cells, and the proteome response was the weakest among all tested treatments. It could be that the monocytes can recover after exposure to gold nanoparticles[15] but probably less-so after exposure to other nanoparticles. Among the proteins up-regulated in THP-1 after exposure to Au-20-NH_2 is the potent inflammatory mediator NF-κB, which is also up-regulated in LPS-treated cells. Therefore the monocyte response to this nanoparticles seems pro-inflammatory, which is consistent with earlier observations that gold nanoparticles induce cytokine release by immune cells.[86,87]

CHAPTER 3. CONCLUSIONS AND FUTURE PERSPECTIVES

We have designed a proteomics-based assay to characterize and estimate the monocyte response to various stimuli. The assay is based on comparisons between the proteome of cells treated with a compound of interest and reference proteomes.

The established and validated assay showed that an IsoD-containing peptide primes monocytes for differentiation, but does not induce pro-inflammatory activation. The assay also showed that the response to CdTe-NH2 is similar to Camptothecin and induced down-regulation of topoisomeras. The effect of CuO-core combined features of the THP-1 proteome responses to LPS and Camptothecin as well as induced up-regulation of proteins involved in the heat-shock response. The gold nanoparticles induced up-regulation of inflammatory mediator NF-κB and a number of proteins related to energy metabolism and also up-regulated in LPS-treated cells.

The developed assay has potential to be extended with analysis of PTMs, such as citrullination. There are more possibilities to develop further and extend the assay (Figure 14). It can be used for further in vitro testing of known as well as novel compounds involved in either disease or its treatment by predicting their effect on the human innate immune system. The developed approach could be extended and optimized to other innate immune cell types, as well as to the secretome of the immune cells instead of their proteome. If the hypothesis that there is a link between the sensitivity of monocytes to certain stimuli and the risk of certain disease is correct, the assay can be applied to predict patient response to treatment.

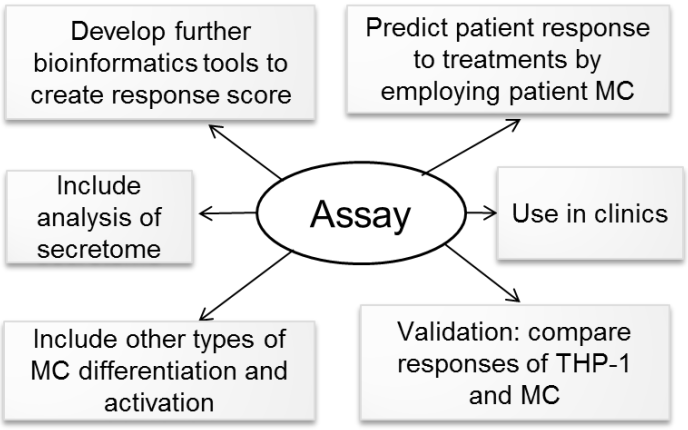


Figure 14. Future developments of the assay.

Validation of time of incubation and treatment concentration has been performed. The next step of validation could be a comparison between the responses of human primary cells and THP-1 cells. This will require samples from a large number of individuals for obtaining sufficient statistics to overcome the inevitable bias (e.g. sex, age, race, etc.) and interpersonal variability.

Another approach for further extension of the assay could be to increase the number of orthogonal positive controls, e.g. to include anti-inflammatory stimuli or damage-associated molecular patterns. This addition could increase the number of OPLS dimensions characterizing the type of response from two to three or four. The bioinformatics analysis can be also optimized and the standard procedure for obtaining a 'cell response score' can be developed.

The established assay is flexible and is based on one of the rapidly developing techniques, which implies that most likely even more perspectives for the developed approach will appear in the nearest future.

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