BENEATH THE SURFACE

CELLULAR RESPONSES IN THE VASCULATURE

Eelke Béguin

COLOFON

The studies described in this thesis were performed at the department of Molecular and Cellular Hemostasis, Sanquin Research, Amsterdam, the Netherlands.

Cover design: Eelke Béguin, Floris van Alphen and Carmen van der Zwaan

Layout: Eelke Béguin

Printed by Ridderprint

ISBN: 978-94-6375-852-9

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BENEATH THE SURFACE

CELLULAR RESPONSES IN THE VASCULATURE

ONDER HET OPPERVLAK

CELLULAIRE REACTIES IN HET VAATSTELSEL

(met een samenvatting in het Nederlands)

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de
Universiteit Utrecht
op gezag van de
rector magnificus, prof.dr. H.R.B.M. Kummeling,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op

woensdag 7 oktober 2020 des middags te 2.30 uur

door

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geboren op 24 januari 1988
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Financial support by the Dutch Heart Foundation and Sanquin Research for the publication of this thesis is gratefully acknowledged.

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CHAPTER 1

GENERAL INTRODUCTION

SCOPE OF THIS THESIS

Intercellular communication is a pivotal process in multicellular organisms to enable the concerted activity of specialized cells within different tissues and organs. To communicate with their environment, cells selectively express cell surface receptors that span the plasma membrane thereby linking the inside of the cell to the outside world. Interaction of these transmembrane receptors with extracellular signals triggers conformational changes that initiate intracellular signaling events. These receptor-mediated signal transduction pathways regulate a variety of biological processes, including cell differentiation, survival and migration.

To study the role of surface receptors in biological processes, most studies focus on single proteins or processes. However, in order to obtain a full understanding of the molecular mechanisms that underlie receptor-mediated biological processes, a system-wide approach is essential. Recent technological advances in mass spectrometry (MS) now provide the opportunity to dissect these receptor-mediated processes in an unbiased and quantitative fashion.

In this thesis, we dissect cellular responses resulting from receptor-ligand interactions in the vascular niche using MS-based proteomics. To focus on the interaction of cells with their environment, we developed a proteomics method to enrich for cell surface proteins. In addition, we conducted temporal profiling of cellular responses and we employed phosphoproteomics to explore signaling events that are independent of protein synthesis. We applied these proteomics strategies to 1) unravel the receptor-induced adaptive response of endothelial cells to inflammatory and mechanical cues, 2) dissect the molecular machinery involved in the cytokine-induced receptor-mediated terminal differentiation of monocytes, and 3) uncover macrophage receptors involved in the endocytosis of plasma proteins. In this first chapter we will provide background information on the topics covered in this thesis and we will provide an overview of the questions addressed in the experimental chapters of this thesis.

Cell surface receptors

Cell surface receptors are categorized in three major classes: ion-channel-coupled receptors, enzyme-coupled receptors and G-protein coupled receptors (Figure 1) [1]. Ion channel-coupled-receptors are pore-forming membrane proteins that transiently open or close upon activation by ligand-binding. This allows for the passage of selective ions from one side of the membrane to the other. As a result the membrane polarity is altered, which can generate action potentials and activation of electrically excitable cells, specifically muscle cells and neurons. The second class of cell surface receptors are enzyme-coupled receptors which either contain an intracellular enzymatic region or are linked to an intracellular enzyme. The binding of such receptors to ligands leads to the activation of the enzyme, which most often is a kinase. Kinases specifically phosphorylate protein sites and thereby modulate the function of the modified protein. The largest family of cell surface receptors are G-protein-coupled receptors (GPCRs), which are typically coupled to enzymes or ion channels via trimeric GTP-binding proteins (G-proteins) [2]. Activation of a GPCR induces the activation of G-proteins, which subsequently alters the activity of the enzyme or ion channel.

In addition to these three major classes of receptors, receptors have been described that display alternative modes of signal transduction. For example, the binding of cytokine tumor necrosis factor α (TNF α) to TNF receptor 1 (TNFR1) induces a conformational change in the cytoplasmic domain of TNFR1, which induces the formation of a signaling complex [3]. Another example is the integrin family of adhesion proteins. Integrin heterodimers typically exist in two conformations: an active form that displays high ligand-binding affinity

Figure 1. Schematic representation of signal-induced cellular responses. Biochemical or mechanical signals activate dedicated receptors on the cell surface. Via signal transduction pathways these can result in rapid responses by modifying proteins (post-translational modifications) and thereby modulating the function of those proteins. Alternatively, receptor activation can induce genetic programs thereby leading to gene expression and subsequent protein synthesis, which leads to a sustained yet delayed response.

and an inactive conformation that exhibits low ligand-binding affinity. Remarkably, a switch from the inactive to the active conformation can be induced by both intracellular signals (inside-out) and extracellular ligand binding (outside-in), resulting in a bidirectional mode of signaling [4]. Together, the different classes of cell surface receptors provide cells with the information they require for their respective functions.

The activation of cell surface receptors can trigger signal transduction pathways that initiate genetic programs via regulation of transcription factors in the nucleus, resulting in the synthesis of proteins (Figure 1). This leads to a delayed yet sustained response. In parallel, activation of signal transduction pathways can lead to the addition or removal of post-translational modifications (PTMs), including phosphorylation, methylation, acetylation, sulfation, sumoylation and ubiquitination, to pre-existing proteins, thereby rapidly altering their function or localization.

Receptors on the endothelium: the interface between blood and tissue

Blood vessels form an intricate network to supply tissues with oxygen and nutrients. This network removes waste products from tissues and transports the various blood components, including blood cells (erythrocytes, leukocytes and platelets), proteins, and signal molecules, throughout the body. The inner layer of blood vessels is formed by a monolayer of endothelial cells (ECs) that mediate the contact between blood and tissue. These cells maintain vascular homeostasis by highly adaptive responses induced by their cell surface receptors, which can respond to chemical and mechanical stimuli. Based on the information from these receptors, ECs can rapidly adjust their barrier function or switch from an anticoagulant to a procoagulant function by secreting the contents of Weibel-Palade bodies (WPBs), which mainly contain hemostatic protein Von Willebrand Factor (VWF). In addition, ECs can facilitate leukocyte extravasation, produce cytokines, present antigens, and regulate the vascular tone (Figure 2A). The adaptive response of the endothelium can be triggered by three major processes: inflammation, mechanical stress and hemostasis (Figure 2A). Failure of ECs to adequately mediate these processes has been associated with a variety of vascular disorders, including stroke, hypertension and atherosclerosis [5]. Understanding the molecular details that underlie the endothelial response to a changing environment may therefore contribute to the development of endothelial dysfunction-targeted therapies. Previously, our group has studied the endothelial signaling mechanisms involved in hemostasis [6,7]. We now aim to unravel the molecular mechanisms that underlie endothelial inflammation and mechanosensing.

Inflammation. Acute inflammation is the response of the immune system to the presence of pathogens or damaged tissue. In these conditions, innate immune cells can produce a range of cytokines which serve as signal molecules to locally activate other cell types, including ECs. ECs contain dedicated receptors for these pro-inflammatory cytokines, which include the well-studied tumor necrosis factor α (TNF α) and Interleukin 1 β (IL-1 β). These cytokines initialize signaling cascades leading to the activation of a set of MAPKKKs

Figure 2. Responses of endothelial cells to extra-cellular stimuli. (A) ECs regulate various processes to maintain vascular hemostasis and facilitate immune responses. WPB: Weibel Palade Body. (B) Schematic representation of the endothelial response to pro-inflammatory cytokines and (C) flow-induced shear stress. NO: nitric oxide.

(mitogen-activated protein kinase kinases) [8,9] resulting in the nuclear translocation of transcription factors NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) and AP1 (activator protein 1, Figure 2B). Ultimately, these signaling events induce a genetic program that results in the synthesis of a selection of inflammatory proteins, including cytokines and adhesion molecules ICAM1 (intercellular adhesion molecule 1) [10], VCAM1 (Vascular cell adhesion molecule 1) [11] and SELE (E-selectin) [10]. Eventually, the activation of ECs by pro-inflammatory cytokines results in cytoskeletal remodeling, leukocyte transmigration, and antigen presentation [12,13]. Cytoskeletal remodeling leads to leakage of protein-rich plasma into the tissue, which is necessary to deliver proteins, such as antibodies, complement proteins and hemostatic factors, to the site of infection or injury. Leukocyte transmigration is facilitated by the expression of cell adhesion molecules including ICAM1, VCAM1 and SELE, and allows for the migration of leukocytes to the site of infection or injury. The expression of endopeptidases, peptide transporters, and HLA molecules and B2M allows for the presentation of self-antigens to the immune system. Dysregulation of endothelial inflammation can contribute to vascular diseases and the vascular endothelium is therefore an attractive target to control inflammation. To fully understand the consequences of inflammation on vascular endothelium, it is necessary to study endothelial inflammation in a system-wide fashion. However, current proteomics-based studies do not provide information on the temporal development of the inflammatory response [14,15],

on the localization of effector molecules [16], or on the differences between the effects of the primary endothelial inflammatory cytokines TNF α and IL-1 β [12].

Flow. Besides inflammation, ECs are sensitive to flow-induced shear stress and adjust their morphology and behavior based on their exposure to flow. ECs appear elongated and aligned in regions of the vascular bed exposed to laminar flow, whereas a turbulent flow pattern or minimal flow induces a polygonal endothelial phenotype [17]. To be able to respond to the local flow characteristics, ECs contain multiple mechanosensors, including integrins, caveolae and a complex consisting of PECAM1 (platelet-endothelium cell adhesion molecule 1), VEGFR2 (vascular endothelial growth factor receptor 2, KDR) and VE-cadherin (vascular endothelial cadherin, CDH5) [18,19] (Figure 2C). Activation of these sensors induces the activation of transcription factors KLF2 (kruppel-like factor 2) [20] and KLF4 [21], as well as inhibition of YAP/TAZ [22] and NF- κ B [23]. In addition to the transition from a polygonal to an elongated phenotype, exposure of ECs to flow is accompanied by an increased production of the vasodilatory agent nitric oxide (NO), inhibition of cell proliferation [24], increased endothelial barrier function [25], and changes in the extracellular matrix (ECM) [26]. Recently, a systems biology analysis of the EC response to shear stress at a transcriptional level elucidated the dynamics of endothelial responses in several functional pathways, including cell cycle, oxidative stress, and inflammation [27]. However, it remains to be established which changes in protein levels accompany the morphological transition induced by flow exposure.

The interplay of biological processes. Inflammatory and hemodynamic cues are not the only factors that can affect the function of the endothelium. ECs are continuously exposed to hemostatic, angiogenic, and migratory signals as well. The information on these environmental parameters provided by cell surface receptors needs to be integrated to induce an adequate cellular response. Although this integration is generally understudied, some examples of interplay between biological processes have been reported. For instance protease activated receptor-1 (PAR1 or F2R), the main receptor for thrombin in an hemostatic event [7], has recently been reported to be involved in flow-induced responses [28]. In addition, the polygonal phenotype of ECs observed in regions that are not exposed to laminar flow has been associated with increased cellular turnover, leakiness to plasma macromolecules, and increased leukocyte adhesion [29,30]. It has therefore been suggested that a lack of flow exposure leads to an inflammatory phenotype [31]. This is supported by multiple studies that have shown that the absence of laminar flow is linked to an increased expression of typical inflammatory markers [32–34]. In addition, *in vitro* studies have reported a link between flow and inflammation [35–40], yet it remains to be established if inflammation-responsive proteins are directly affected by flow-exposure. Knowledge on the interplay of the multiple processes that affect EC behavior may improve our understanding of endothelial dysfunction and the development of vascular diseases.

Cytokine-induced differentiation of monocytes

In addition to the inflammatory effects on the endothelium, cytokines play an important role in cell fate decision of immune cells. The binding of specific cytokines to their respective cell surface receptors results in the specialization of these cells to perform dedicated functions, which include bone remodeling (osteoclasts), phagocytosis (M1 and M2 macrophages), and to orchestration of adaptive immune responses (dendritic cells (DCs)). Given their common ancestry, it is not surprising that these cells share certain features, including endocytic mechanisms and their ability to activate T-cells by antigen presentation [41]. Nevertheless, these monocyte-derived cells are highly dedicated to their specific function. DCs are responsible for the detection of pathogens and activation of T-cells, whereas osteoclasts are primarily known for their ability to absorb bone. Macrophages on the other hand are characterized by their ability to phagocytose large particles. In literature, two types of macrophages are often distinguished: classically activated pro-inflammatory macrophages (M1), that inhibit cell proliferation and induce tissue damage, and alternatively activated anti-inflammatory macrophages (M2) that induce tissue repair and promote cell proliferation [42,43].

The differentiation of monocytes into these functionally distinct cells can be induced *in vitro* by priming circulating monocytes with cytokines (Figure 3) [44–46]. These *in vivo* differentiated cells maintain most of their physiological characteristics, such as bone resorption for osteoclasts [47], and phagocytosis for macrophages [48]. Although cytokine-induced differentiation of monocytes has been widely used to study DCs, macrophages and osteoclasts, it remains to be established how this differentiation evolves at a protein level and how the specialized effector cells relate to each other. Can cytokine-induced monocyte-differentiation be characterized by a set of proteins that changes similarly in all cell types, or is the development for each of the effector cells completely distinct? Are there specific markers that define these specialized monocyte-derived cells? This knowledge will increase

Figure 3. Cytokine-induced monocyte differentiation. Circulating monocytes can be isolated and differentiated into dendritic cells with IL-4 and GM-CSF, M1 macrophages with GM-CSF, M2 macrophages with M-CSF and osteoclasts with M-CSF and, starting from day 3, RANKL. Abbreviations: interleukin 4 (IL-4), granulocyte macrophage colony stimulating factor (GM-CSF), macrophages colony stimulating factor (M-CSF), receptor activator of nuclear factor kappa-B ligand (RANKL).

our understanding of monocyte differentiation and may aid us to develop strategies to specifically modulate immune responses.

Ligand-induced receptor-mediated endocytosis by macrophages

In addition to cell surface receptors that primarily mediate signal transduction, cell express a variety of surface receptors that are involved in other processes, including maintaining structural integrity and endocytosis. Ligand endocytosis can serve to provide cells with nutrients, for instance by endocytosis of cholesterol via the LDL (low-density lipoprotein) receptor, and iron via the transferrin receptor. Alternatively, receptor-mediated endocytosis may serve to specifically remove redundant or unwanted proteins from the extracellular milieu. Clearance by receptor-mediated endocytosis plays an important role in hemostasis. Two important proteins involved in hemostasis, Von Willebrand Factor (VWF) and coagulation factor VIII (FVIII) are continuously secreted into, and cleared from the circulation. VWF facilitates primary hemostasis, *i.e.* the formation of a platelet plug upon vessel damage. FVIII on the other hand is involved in secondary hemostasis and thereby contributes to the generation of fibrin, which strengthens the platelet plug. The functional absence of these proteins leads to severe bleeding disorders [49–51], termed Von Willebrand disease (VWD) and Hemophilia A, for VWF and FVIII, respectively. For a subset of VWD patients and mild hemophilia A patients clinical treatment can consist of treatment with desmopressin (DDAVP) which triggers the release of VWF from ECs [52]. However, often replacement therapy is necessary, which comprises the intravenous infusion of recombinant or blood plasma-derived proteins. However, due to the short half-life of the infused proteins, frequent infusions are required to maintain effective VWF/FVIII levels. The half-life of these proteins is determined by their clearance mechanism from the circulation. In order to develop strategies to prolong the half-life of infused VWF and FVIII, and thereby reduce their clinical infusion frequency, it is essential to unravel their clearance mechanisms at a molecular level.

The serum half-life of FVIII largely depends on the plasma levels of VWF, as FVIII associates with VWF in plasma. The interaction with VWF shields FVIII from clearance, which prolongs its half-life from 3–4 hours [53] to 12–16 hours [54,55]. The VWF clearance mechanism is therefore also highly relevant for the treatment of hemophilia A. Although it is known that VWF is primarily produced by ECs, it is unclear how its clearance is mediated. In mice, infused radiolabeled VWF was primarily targeted towards the liver and the spleen [56,57], indicating a role for these organs in VWF clearance. Chemical inactivation of macrophages resulted in drastic increased plasma levels of VWF [56,58,59], indicating that these cells are involved in VWF clearance. However, not one, but multiple receptors have been implicated in VWF endocytosis, including LDL receptor-related protein-1 (LRP-1) [60–62], Macrophage scavenger receptor-1 (MSR-1, SR-AI or CD204) [63], Macrophage Galactose-type lectin (MGL) [64], Siglec-5 [65], Stabilin-2 (STAB2) [66], C-Type Lectin Domain Family 4 Member M (CLEC4M) [67], and the Asialoglycoprotein receptor (ASGPR) [59,68]. Previously, our group

has demonstrated that monocyte-derived macrophages (MDMs) can internalize VWF in flow conditions [62]. However, it is unclear which receptors are present on MDMs and what their relative contribution to VWF clearance is. Dissecting the molecular mechanisms of VWF clearance in macrophages may provide targets for extending the half-life of VWF and thereby FVIII in plasma.

Mass spectrometry-based proteomics

In order to study signal-induced cellular responses, a wide range of techniques can be employed, including biochemical techniques, (fluorescence) microscopy, and flow cytometry. During the last decades, large-scale screening methods have been developed to assess the cellular state on an unprecedented level. These include microarrays and RNA-Seq analysis to assess the transcriptome of cells. However, RNA transcript levels have limited value to predict protein levels [69]. The recent technological advancements in liquid chromatography-coupled mass spectrometry (LC-MS) have made it possible to directly assess the cellular protein composition in a quantitative manner. LC-MS is therefore particularly suitable to study receptor-induced cellular responses in a system-wide fashion.

Mass spectrometry-based proteomics allows for the identification and quantification of proteins. Common work-flows are based on the analysis of peptides as a proxy for proteins. In a typical workflow proteins are extracted from cells and subjected to enzymatic digestion, usually with trypsin (Figure 4A). The resulting peptides are subsequently separated by high-performance liquid chromatography (HPLC) to reduce sample complexity prior to MS analysis. The HPLC column is coupled on line to a tandem mass spectrometer (LC-MS/MS) via a nano-electrospray ionization source to ionize and vaporize peptides [70]. In the first MS analysis, the mass-over-charge (m/z) ratios of precursor ions are determined. From this spectrum, peptide precursors are selected and subjected for fragmentation. Multiple fragmentation methods can be applied, including electron transfer dissociation (ETD), collision-induced dissociation (CID), higher-energy collisional dissociation (HCD), and combinations of multiple techniques. Fragmentation products are subjected to MS2 analysis, which provides information on their amino acid sequences. Technological advancements, including the development the orbitrap technology, have greatly enhanced the performance of LC-MS and thereby its application in cell biology [71–73].

Various strategies have been developed to allow for quantification of proteins through a mass spectrometry-based read-out [74,75]. These include label-free approaches, such as ion intensity-based quantification, as well as stable isotope label-based methods (Figure 4B), including metabolic labeling using SILAC (Stable Isotope Labeling with Amino acids in Cell culture) [76,77], and chemical labeling employing DML (DiMethyl Labeling) [78] and TMT (Tandem-Mass Tags) [79]. The main advantages of label-free quantification are that the sample processing is straightforward and relatively inexpensive. However, label-free quantification does require longer instrument times as each sample must be analyzed separately. In contrast, stable isotope-labeling strategies are more laborious and expensive,

but allow for multiplexing, the mixing of distinctly-labeled samples prior to MS-analysis. Therefore, multiple samples can be analyzed in a single MS-run, resulting in significantly decreased instrument times. In contrast to the strategies that require covalent linkage of a mass tag, SILAC-based quantification does not entail chemical modification of peptides,

Figure 4. Mass spectrometry approaches applied in this thesis. (A) General workflow for LC-MS/MS analysis of cell samples. Cells are lysed and extracted proteins are enzymatically digested into peptides. These are subsequently separated via liquid chromatography and subjected to MS1 analysis via nano-electrospray ionization (ESI). Selected precursors are then fragmented and subjected to MS2 analysis. (B) Quantification strategies in MS-analysis. For label-free quantification samples are analyzed in distinct MS-analyses with the same chromatography. Based on the retention time, peptides are then matched between runs. For metabolic labeling, differently isotope-labeled amino acids are incorporated into cells. Cell lysates are mixed and processed together. For chemical labeling, isotope-containing tags are covalently linked to sample peptides prior to mixing and MS-analysis. The relative quantification in these MS1 quantification-based methods relies on the areas under the elution profiles. For isobaric labeling, chemical tags are used composed of mass reporter regions and balance regions. Upon fragmentation, these regions are separated and the mass reporters can therefore be compared in the MS2 or MS3 analysis. Adapted from [75].

but is based on the metabolic incorporation of isotope-labeled amino acids in proteins. Samples can therefore already be mixed on a cell lysate level, in contrast to the peptide level in the chemical labeling strategies. This difference makes SILAC-based quantification particularly suitable for protocols that require additional processing steps, such as subcellular fractionation or affinity-based purification, as these steps can induce additional processing bias. However, SILAC-based quantification depends on sufficient incorporation of the isotope-labeled amino acids in proteins. This can be achieved by passaging cells for multiple generations in SILAC medium. This requirement excludes the possibility for SILAC-based quantification for non-proliferating cells such as terminally differentiated cells. In this thesis, we have therefore used two quantification strategies, SILAC for endothelial cells that could be maintained in culture for multiple passages, and label-free quantification for primary cells that did not proliferate *in vitro*.

An important aspect of cellular behavior is its communication with the environment. As this contact is mainly mediated through cell surface proteins, we were particularly interested in the surface region. To specifically enrich for proteins originating from the cell surface, multiple MS-compatible strategies have been employed, including ultracentrifugation-, glycan capture-, and biotinylation-based methods [80]. Biotinylation-based methods employ biotin-esters that are covalently linked to primary amines, which are present in lysine residues and at N-termini of proteins. The labeling efficiency of a particular lysine residue is dependent on its availability for the biotin label. Lysine residues on the outside of a proteins may therefore be more efficiently labeled compared to a residue within a highly

Figure 5. Biotin labeling and pull-down of surface proteins. (A) Workflow for cell surface proteomics. SILAC-labeled cells are subjected to different experimental conditions and their surface proteins are labeled with a non-permeable biotin label. Subsequently, cells are lysed, proteins digested and, in parallel to whole proteome analysis, biotinylated peptides are enriched via biotin pull-down and subjected to MS-analysis. (B) Anticipated protein changes that can be detected using cell surface proteomics include protein-protein interactions, conformational changes and proteolytic processing. Stars indicate biotin labels, blue stars are affected in the depicted situations.

structured region inside the protein. The addition of a sulfo-group to these biotin-esters prevents their diffusion through membranes [81] and therefore allows for highly specific labeling of extracellular proteins. Biotin pull-down/MS approaches have been employed in combination with SILAC labeling [82,83], or with other quantification methods [84–88]. In this thesis, we applied a biotin pull-down combined with SILAC quantification to increase the proteomic depth at the cell surface, as well as at the detection of intramolecular changes. By performing the biotin pull-down on peptide-level rather than protein-level, we enriches for biotin-labeled peptides, and thereby increase the coverage of the cell surface biotin footprint (Figure 5A). Previously, a similar method has been used to extract structural information from proteins using TMT labels [89–92]. Now, we set out to extract comparable information on a cellular level. This method can potentially be used to detect functional changes such as protein-protein interactions, intramolecular changes, and proteolytic processing (Figure 5B).

QUESTION ADDRESSED IN THIS THESIS

In this thesis we explore receptor-mediated biological processes in the vascular niche using quantitative mass spectrometry approaches (summarized in Figure 6). In addition to established quantitative proteomics approaches, we developed a peptide-based cell surface proteomics method to focus on surface proteins and probe for site-specific changes within these proteins.

In Chapter 2 we employ these techniques to study the adaptive endothelial responses to receptor-activation by two pro-inflammatory cytokines: tumor necrosis factor α (TNF α) and interleukin 1 β (IL-1 β). To investigate the long-term converging and diverging effects of these cytokines, we assess the proteome and phosphoproteome of ECs after 24h of cytokine stimulation. Do these cytokines, in line with the similar effect they elicit on endothelial barrier function and leukocyte transmigration, induce a similar proteomic profile? And how does the proteomic profile of ECs change throughout the development of the inflammatory response?

In Chapter 3 we study the EC adaptive response to activation of mechanoreceptors by flow. How does the endothelial proteome change in flow conditions, and what are the functional consequences of these changes? In addition, to examine putative flow-induced intramolecular changes, we probe the site-specific labeling efficiency by cell surface proteomics. As our MS-data indicate that flow-exposure induces drastic changes in ECM proteins and proteins involved cell-ECM adhesion, does this also result in altered localizations of these proteins? Furthermore, it has been suggested that vascular regions deprived of blood flow exhibit a mild inflammatory signature. What is the interplay between flow-exposure and the inflammatory response?

Chapter 4 focuses on the receptor-induced terminal differentiation of circulating monocytes into distinct specialized effector cells. We compare the transition of monocytes to dendritic cells, macrophages and osteoclasts by employing proteomics. Do these cells display a common differentiation core, and how is the specialization of monocytes into the distinct subtypes characterized at a molecular level? Can we define additional marker proteins for these specialized cell types based on their proteomes?

In Chapter 5, we evaluate the putative interplay of receptors in the uptake of VWF by macrophages. Which VWF-clearance receptors are present on the macrophage surface, and how do these receptors contribute to VWF internalization?

Finally, in Chapter 6 we summarize the results reported in this thesis and discuss their implications.

Figure 6. Schematic representation of the research described in this thesis. MS-based approaches were applied to dissect the adaptive response of endothelial cells to a changing environment, to unravel the cytokine-induced terminal differentiation of monocytes, and to identify putative clearance receptors on monocyte-derived macrophages.

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CHAPTER 2

INTEGRATED PROTEOMIC ANALYSIS OF TUMOR NECROSIS FACTOR α AND INTERLEUKIN 1 β - INDUCED ENDOTHELIAL INFLAMMATION

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J Proteomics. 192 (2019) 89-101

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ABSTRACT

The vascular endothelium provides a unique interaction plane for plasma proteins and leukocytes in inflammation. The pro-inflammatory cytokines Tumor Necrosis Factor α (TNF α) and Interleukin 1 β (IL-1 β) have a profound effect on endothelial cells, which includes increased levels of adhesion molecules and a disrupted barrier function. To assess the endothelial response to these cytokines at the protein level, we evaluated changes in the whole proteome, cell surface proteome and phosphoproteome after 24 hours of cytokine treatment. The effects of TNF α and IL-1 β on endothelial cells were strikingly similar and included changes in proteins not previously associated with endothelial inflammation. Temporal profiling revealed time-dependent proteomic changes, including a limited number of early responsive proteins such as adhesion receptors ICAM1 and SELE. In addition, this approach uncovered a greater number of late responsive proteins, including proteins related to self-antigen peptide presentation, and a transient increase in ferritin. Peptide-based cell surface proteomics revealed extensive changes at the cell surface, which were in agreement with the whole proteome. In addition, site-specific changes within ITGA5 and ICAM1 were detected. Combined, our integrated proteomic data provide detailed information on endothelial inflammation, emphasize the role of the extracellular matrix therein, and include potential targets for therapeutic intervention.

SIGNIFICANCE

Pro-inflammatory cytokines induce the expression of cell adhesion molecules in vascular endothelial cells. These molecules mediate the adhesion and migration of immune cells across the vessel wall, which is a key process to resolve infections in the underlying tissue. Dysregulation of endothelial inflammation can contribute to vascular diseases and the vascular endothelium is therefore an attractive target to control inflammation. Current strategies targeting endothelial adhesion molecules, including PECAM, CD99, ICAM1 and VCAM1 do not completely prevent transmigration. To identify additional therapeutic targets, we mapped the endothelial proteome after pro-inflammatory cytokine treatment. In addition to the whole proteome, we assessed the surface proteome to focus on cell adhesion molecules, and the phosphoproteome to uncover protein activation states. Here, we present an integrated overview of affected processes which further improves our understanding of endothelial inflammation and may eventually aid in therapeutic intervention of imbalanced inflammation.

INTRODUCTION

Acute inflammation is part of the body's immune response aimed to protect against damage caused by for instance pathogens, trauma or toxins. Activation of innate and adaptive immune cells, by recognition of pathogen-associated molecular patterns or specific antigens, induces the release of a variety of biochemical factors, including chemokines and cytokines. These affect the local environment, ultimately resulting in an increased blood flow, promotion of leakage of plasma-protein-rich fluid and localized recruitment and activation of circulating leukocytes [1]. Under normal conditions, the acute inflammatory response is quickly dampened upon removal of the stimulus. However, failure to regulate or resolve the inflammatory process can result in chronic inflammation, which can contribute to various diseases, including inflammatory bowel disease and rheumatoid arthritis [2–4]. Most research in the field of inflammation has focused on the role of innate and adaptive immune cells. However, endothelial cells (ECs) also play an important role in inflammation due to their strategic position lining the vasculature. This position on the border of blood and tissue allows ECs to facilitate the extravasation of circulating immune cells into the inflamed tissue [1,5] and may therefore represent an attractive target for therapeutic intervention. However, our insight in the molecular wiring of inflamed ECs is currently limited.

The endothelium responds to a variety of inflammatory cytokines and chemokines, produced by leukocytes, macrophages, platelets, vascular smooth muscle cells and by ECs themselves [6]. Most studies have focused on two pro-inflammatory cytokines: tumor necrosis factor (TNF) α and interleukin (IL)-1 β . Using classical biochemical techniques, it has been well established that inflammatory activation of ECs results in an increased expression of adhesion molecules such as E-selectin (SELE) [7], vascular cell adhesion molecule-1 (VCAM1) [8], and intercellular adhesion molecule-1 (ICAM1) [7]. More recently, mass spectrometry-based approaches have been employed to study cytokine-induced signaling pathways in numerous cell types [9], including ECs [10,11]. These have facilitated the simultaneous quantification of thousands of proteins, and thereby considerably improved our understanding of the complex pathways of pro-inflammatory cytokine activation. It is now well established that TNF α and IL-1 β binding to their respective receptors, TNF Receptor 1 (TNFR1) and IL-1 Receptor 1 (IL-1R1), results in recruitment of a signaling complex that activates a set of mitogen-activated protein kinase kinase kinases (MAPKKKs) [12,13]. These subsequently initiate signaling cascades that lead to the activation of the transcription factors NF κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) and Activator protein 1 (AP1, also known as JUN) resulting in increased expression of pro-inflammatory molecules, including adhesion molecules ICAM1, VCAM1 and SELE, chemokines and enzymes, as well as the reorganization of the actin cytoskeleton [14,15].

However, despite this progress, proteomic studies generally lack spatial and temporal resolution [9]. This is highly relevant, since continuous treatment with TNF α and IL-1 β results in an apparent time-dependent protein expression profile. This is illustrated by protein levels of SELE, VCAM1 and ICAM1 [16,17]. At first SELE expression is drastically increased,

which is followed by expression of VCAM1 and ICAM1 [16,17]. Yet, despite the continuous presence of cytokines, the increased proteins levels of SELE do not persist: within 24 hours, the abundance of SELE starts to decline [16,17]. This suggests that regulatory mechanisms are in place that control protein levels in a time-dependent fashion, and raises the question how levels of other proteins develop over time. In addition, blocking antibodies against the adhesion molecules ICAM1, VCAM1, PECAM1 or CD99 do not completely prevent leukocyte extravasation [18–23], spurring a renewed interest in the quantification of other cell surface receptors under inflammatory conditions. Lastly, the similarities and differences between the effects of TNF α and IL-1 β -treatment on endothelial cells are unclear [24].

Here, we addressed these questions by combining global proteome profiling with quantitative peptide-based cell surface proteomics and phosphoproteomics of prolonged TNF α and IL-1 β -induced inflammation. Furthermore, we assessed protein levels over time in order to evaluate their kinetic profiles. Our integrated analysis identifies various novel cytokine-responsive proteins and provides insight into regulation of cytokine-responsive processes and pathways.

MATERIALS AND METHODS

Cell culture

Blood outgrowth endothelial cells (BOECs) were isolated and cultured as previously described [25]. BOECs derived from 3 healthy donors were pooled and used for ECIS, immunofluorescence, and mass spectrometry experiments.

Electric Cell-substrate Impedance Sensing (ECIS)

BOECs were grown to confluency on ECIS Cultureware 8W10E PC 8 well clusters (Applied Biophysics) treated for 15 minutes with 10 μ M L-cysteine (Sigma-Aldrich) and coated with 50 μ g/ml collagen type I (BD Biosciences). Experiments were performed in three independent replicates on an ECIS Z θ Array station at 32000 Hz. Cells were stimulated by adding IL-1 β or TNF α (Peprotech) to a final concentration of 10 ng/ml, or thrombin at a final concentration of 10 nM. Resistance values were normalized to the time point before stimulation.

Immunofluorescence imaging

BOECs were grown on collagen-coated coverslips until confluent. Cells were stimulated with fresh pre-warmed EBM medium (Lonza), supplemented with SingleQuots (Lonza) and containing 18% FCS (Bodinco), and supplemented with IL-1 β , TNF α or mock treated as described above. Cells were washed three times with wash buffer (10 mM HEPES, 135 mM NaCl, 10 mM KCl, 5 mM CaCl₂·2 H₂O, 2 mM MgSO₄·7 H₂O, pH 7.5), fixed in 4% (v/v) paraformaldehyde (Electron Microscopy Sciences) in wash buffer for 15 minutes and quenched by incubating 15 minutes in 50 mM NH₄Cl in wash buffer. Samples were washed three times with phosphate buffered saline (PBS) (Fresenius Kabi), incubated for 10 minutes in blocking/permeabilization buffer (1% (w/v) bovine serum albumin (Merck), 0.1% (v/v) saponin in PBS), incubated 1 hour with primary antibodies (mouse α hICAM1 BBA3 (R&D Systems), goat α hVE-Cadherin sc-6458 (Santa Cruz Biotechnology)) in blocking buffer, washed three times with PBS, incubated 1 hour with secondary antibodies (chicken anti mouse IgG AlexaFluor (AF)488, Donkey anti Goat IgG AF568 (both ThermoFisher Scientific), CF633-labeled phalloidin (Biotium)) in blocking buffer, washed 3 times with PBS and once with demineralized water, and mounted in Prolong Gold Antifade Mountant (ThermoFisher Scientific). Samples were imaged on a LSM510/meta confocal scanning microscope system equipped with a 63x Plan-Apochromat 1.40 oil immersion objective (Carl Zeiss MicroImaging).

SILAC cell culture

For mass spectrometry analysis, BOECs were grown in SILAC (Stable Isotope Labeling with Amino acids in Cell culture) EBM-2 medium (without Arginine and Lysine, custom made, Lonza), EGM-2 bulletkit (Lonza) and supplemented with 20% FCS (Bodinco) during passage 1-3 and 20% 1 kDa MWCO-filter in-house dialyzed FCS for passage 4 and 5. The cultures were SILAC-labeled by supplying isotope-labeled Arginine and Lysine residues

in a 'light' (Arg0, Lys0) (Sigma-Aldrich), 'medium' (Arg6, Lys4) and 'heavy' (Arg10, Lys8) (Cambridge Isotopes) variant [26]. Cultures were passaged 5x to ensure >95% incorporation of labeled amino acids in the proteomes. Samples were prepared in triplicate with SILAC label switching. SILAC BOECs were grown to confluency and mock-, IL-1 β or TNF α (10 ng/ml) treated for 24 hours.

Cell surface labeling

SILAC-labeled cells were washed with wash buffer (10 mM HEPES, 135 mM NaCl, 10 mM KCl, 5 mM CaCl₂·2 H₂O, 2 mM MgSO₄·7 H₂O, pH 7.5) and labeled with 3 mM EZ-link Sulfo-NHS-LC-Biotin (Thermo Scientific) in wash buffer, for 30 minutes at 4 °C. The label was quenched by washing four times with 100 mM Glycine in wash buffer.

Mass spectrometry sample preparation

Cells were lysed in 4% SDS, 100 mM DTT, 100 mM Tris, pH 7.5, supplemented with phosphatase and protease inhibitor cocktail (Thermo Scientific). Light, medium and heavy SILAC-labeled samples were combined in a 1:1:1 protein ratio and subjected to Filter-Aided Sample Preparation (FASP) as described by Wiśniewski *et al.* [27]. For whole proteome analysis tryptic peptides were desalted using C18 StageTips [28]. In-depth whole proteomes were obtained by fractionation of 50 μ g tryptic peptides using Strong Anion eXchange (SAX) using Empore Anion and Cation Exchange-SR Extraction Disks (3M) as described by Wisniewski *et al.* [27], with elution buffers of pH 11, 8, 6, 5, 4 and 3. The flow-through was acidified with TFA and loaded onto a separate C18 StageTip. For cell surface proteome analysis, the remaining tryptic digests were vacuum dried, supplemented with NaCl to a final concentration of 150 mM with a volume of 450 μ l. The samples were divided over 3 wells of a SigmaScreen Streptavidin coated 96-wells cluster (Sigma-Aldrich), which was prewashed three times with wash buffer (50 mM NH₄HCO₃, 150 mM NaCl, pH 8.3). After overnight incubation at 4 °C, the unbound fraction was transferred and subjected to another round of streptavidin pull-down and wells were washed 5 times with wash buffer. Biotinylated peptides were subsequently eluted with 50 μ l 5% formic acid, 70% acetonitrile (ACN) per well for 2 hours at 4 °C followed by a second elution for 5 minutes at 4 °C with the same elution buffer. Eluates from the same sample and the same round of pull-down (in total 300 μ l) were pooled, vacuum dried to 20 μ l, diluted to 100 μ l with 0.5% acetic acid, acidified to pH <2.5 with trifluoroacetic acid (TFA) and desalted using C18 StageTips. For phosphoproteomic analysis, samples were processed as previously described [29]. Briefly, phosphopeptides were enriched using Phos-TiO beads (GL Sciences), after which the eluted peptides were desalted using C18 StageTips.

Mass spectrometry analysis

Tryptic peptides were separated by nanoscale C18 reverse chromatography coupled on line to an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific) via a nanoelectrospray

ion source (Nanospray Flex Ion Source, Thermo Scientific). Peptides were loaded on a 20 cm 75–360 μm inner-outer diameter fused silica emitter (New Objective) packed in-house with ReproSil-Pur C18-AQ, 1.9 μm resin (Dr Maisch GmbH). The column was installed on a Dionex Ultimate3000 RSLC nanoSystem (Thermo Scientific) using a MicroTee union formatted for 360 μm outer diameter columns (IDEX) and a liquid junction. The spray voltage was set to 2.15 kV. Buffer A was composed of 0.5% acetic acid and buffer B of 0.5% acetic acid, 80% acetonitrile. Peptides were loaded for 17 min at 300 nl/min at 5% buffer B, equilibrated for 5 minutes at 5% buffer B (17–22 min) and eluted by increasing buffer B from 5–15% (22–87 min) and 15–38% (87–147 min), followed by a 10 minute wash to 90% and a 5 min regeneration to 5%. For whole proteome and cell surface proteome samples, analysis was performed as described before [30]. Briefly, survey scans of peptide precursors from 400 to 1500 m/z were performed at 120K resolution (at 200 m/z) with a 1.5×10^5 ion count target. Tandem mass spectrometry was performed by isolation with the quadrupole with isolation window 1.6, HCD fragmentation with normalized collision energy of 30, and rapid scan mass spectrometry analysis in the ion trap. The MS2 ion count target was set to 10^4 and the max injection time was 35 ms. Only those precursors with charge state 2–7 were sampled for MS2. The dynamic exclusion duration was set to 60 s with a 10 ppm tolerance around the selected precursor and its isotopes. Monoisotopic precursor selection was turned on. The instrument was run in top speed mode with 3 s cycles. For phosphoproteome samples a slightly adjusted protocol was used: the resolution of the survey scan was set to 240K with a 2×10^5 ion count target. Tandem mass spectrometry was performed on the 10 most intense ions by isolation using the quadrupole and analysis in the Orbitrap at a resolution of 30K. The MS2 ion count target was set to 5×10^4 with a maximum injection time of 60 ms. All data were acquired with Xcalibur software.

Proteome data processing and analysis

Raw files were analyzed with the MaxQuant (1.5.3.8) computational platform [31]. Proteins and peptides were identified using the Andromeda search engine by querying the human Uniprot database (release 2-2015, 89,796 entries) using standard settings. Relative quantification was based on SILAC (Arg0Lys0, Arg6Lys4 and Arg10Lys8) using unique peptides for quantification only and enabling the ‘re-quantify’ and ‘match between runs’ options. To prevent bias from SILAC labeling, labels were switched for replicates. Log₂ SILAC ratios are reported. For the cell surface proteome the addition of a biotin group (339.16166 Da) was used as a variable modification and for the phosphoproteome ‘Phospho (STY)’ was used as a variable modification. Two subsequent pull-downs of biotin- and phosphopeptides of the same sample were treated as fractions of the same sample. MaxQuant output tables were loaded and analyzed using Rstudio 1.0.44 using R version 3.3.2. [32]. In brief, ‘reverse’, ‘potential contaminant’, ‘only identified by site’ and peptides quantified in less than 3 replicates in 1 condition were filtered out. For phosphoproteomic analysis only class I phosphopeptides [33] were selected (localization probability >0.75, score difference >5).

For cell surface proteome analysis biotinylated peptides were filtered according to the same criteria. Statistical analysis was performed using a linear model without intercept and applying contrasts for each comparison in cases with multiple comparisons. For data containing a single experimental group, one-sample t-tests were performed. P values were adjusted for multiple testing using Benjamini-Hochberg and $p < 0.05$ and a $\log_2$ fold change ≥ 1 was considered significant and relevant. Results were imported in Perseus (1.5.3.2) software to generate heat-plots. For Gene Ontology-term enrichment analysis, gene lists of significantly affected proteins/peptides were generated. For cell surface proteome enrichment, GO-term enrichment of biotin-modified peptides compared to the whole peptide pool was assessed using Cytoscape 3.4.0. [34] with the BiNGO plug-in [35]. Ontology and annotation datasets were downloaded on 3-3-2016 from the Gene Ontology Consortium website (www.geneontology.org). Enrichment analysis of stimulus-affected proteins was performed using the hypergeometric method from goseq [36] (1.24.0), FDR adjusted p-values < 0.05 were considered significant. The significantly enriched GO-terms were grouped and visualized with gogadget [37] (2.0), using hierarchical clustering of the overlap indexes (with ward.D agglomeration method and Euclidean distance). Similarity between the GO-terms were further visualized in networks with Cytoscape's Enrichment Map [38] plugin. The PhosphoPath plug-in [39] was employed to visualize affected proteins. The .raw MS files and search/identification files obtained with MaxQuant have been deposited in the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org/cgi/GetDataset>) via the PRIDE partner repository [40] with the dataset identifier PXD009174.

RESULTS

TNF α and IL-1 β induce ICAM1 expression and decrease barrier function in primary endothelial cells

Our mass spectrometry-based protein quantification relies on the metabolic incorporation of stable isotope-labeled amino acids in cell culture (SILAC). For accurate protein quantification, this incorporation should exceed 95%, which is typically achieved after 5 cell passages. Blood outgrowth endothelial cells (BOECs) were used in this study for their superior propagation capacity [25]. To study endothelial inflammation, BOECs were treated with TNF α or IL-1 β . Immunofluorescence imaging confirmed that ICAM1 expression was drastically increased in cytokine-treated cells, yet a high degree of heterogeneity between cells was observed (Figure 1A). Pro-inflammatory cytokine treatment reduces the endothelial barrier function [41]. To determine the duration of the cellular response, we assessed the endothelial barrier function in time using the transendothelial electrical resistance (TEER). In contrast to the rapid decrease (<30 min) of TEER induced by thrombin, an enzyme of crucial importance in hemostasis [42], TNF α and IL-1 β induced a delayed decrease in TEER similar in both extent and duration (Figure 1B). After 2 hours of cytokine treatment, the TEER gradually decreased to a steady-state, which was reached after about 7.5 hours and continued for the duration of the stimulus. Combined, these two hallmarks of endothelial inflammation, increased expression levels of ICAM1 at the cell surface and the decreased endothelial barrier function, confirm the effectiveness of our cytokine-treatment.

TNF α and IL-1 β provoke a comparable proteomic profile in ECs with specific fine-tuning

To gain insight into the effect of cytokine-induced signaling on protein levels, we performed proteomic analyses on cytokine- and mock-treated cells. Four experiments were performed, each consisting of a SILAC triplicate: 1) whole proteome (fractionated to improve depth), 2) whole proteome time-course, 3) cell surface proteome, 4) phosphoproteome (Figure 1C). For the cell surface proteome and the phosphoproteome, the whole proteome was assessed as well. In these four whole proteome datasets, 4596 proteins were quantified of which 136 were significantly affected after 24 hours of TNF α or IL-1 β treatment (Figure 2A). The majority of the affected proteins (106) showed an increased protein profile. Interestingly, TNF α and IL-1 β induced a highly similar response. Our quantitative mass spectrometry-based approach enabled us to estimate the relative protein quantities. Although this revealed subtle differences in protein levels, TNF α and IL-1 β treatment resulted in very similar protein profiles. Only five proteins were significantly different between these cytokines: CLDN11 (claudin 11), MX1 (Interferon-induced GTP-binding protein Mx1), CPD (Carboxypeptidase D), COL8A1 (collagen alpha-1(VIII) chain) and SLC12A2 (solute carrier family 12 member 2) (Figure 2B). As expected, for both cytokines, the most prominently affected proteins were adhesion receptors ICAM1, VCAM1 and SELE [7,8], which were on average 20 to 32 fold increased in TNF α -treated cells, followed by

other known TNF α -responsive proteins including SOD2 (superoxide dismutase 2), TRAF1 (TNF receptor associated factor 1) and MX1 [43–45], which were on average between 12 and 22 fold increased (Figure 2A, Table S1). In addition to these well-known proteins [7,8], we identified upregulation of a variety of proteins that have not been previously associated with endothelial inflammation, such as TFPI2 (tissue factor pathway inhibitor 2), BPGM (bisphosphoglycerate mutase) and FNDC3B (fibronectin type III domain containing 3B) (Figure 2A). In contrast, a number of proteins showed a decreased expression profile (Figure 2A). These data are in agreement with previous observations that expression of NOS3 (nitric oxide synthase 3) is decreased under inflammatory conditions [46] and that secretion of VWF (von Willebrand Factor) is increased [47]. In addition to these, the abundance of other unknown cytokine-responsive proteins were lower in treatment conditions, including the extracellular matrix protein LTBP2 (latent transforming growth factor beta binding protein 2), membrane proteins ENG (endoglin), ESAM (endothelial cell adhesion molecule) and

Figure 1. Endothelial cells respond strongly to pro-inflammatory cytokines and proteomic workflow. (A) Immunofluorescence micrographs of BOECs treated with 10 ng/ml IL-1 β or TNF α for 24 h. Green represents ICAM1, red VE-cadherin and blue is DAPI. The scale bar is 20 μ m. (B) Transendothelial electrical resistance of BOECs treated with 10 nM thrombin, TNF α or IL-1 β (both 10 ng/ml), assessed by ECIS. Depicted are mean values of 6 replicates. The arrow indicates the moment of treatment, the arrowhead the time-point of proteomics read-out. (C) Proteomics workflow. SILAC-labeled BOECs are treated with 10 ng/ml TNF α or IL-1 β for 24 hours, either or not labeled with a non-membrane-permeable biotin label and combined in a 1:1:1 protein ratio. Proteins are digested into peptides by trypsin using FASP and tryptic digests are directly subjected to high resolution LC-MS/MS. In parallel, biotinylated peptides are pulled-down via streptavidin-coated plates or phosphopeptides are pulled-down using phospho-TiO beads. Subsequently, samples are subjected to LC-MS/MS analysis.

Figure 2. TNFα and IL-1β provoke a similar time-dependent proteomic signature with specific fine-tuning. Heat maps of proteins affected by treatment with either TNFα or IL-1β (10 ng/ml). Color coding represents log₂ SILAC ratios, grey values were not quantified. (A) Of the 4596 quantified proteins, 136 were significantly affected in either of the treatment conditions. The heat map represents data after 24 h cytokine treatment obtained from 4 independent experiments: a fractionated whole proteome (set 1), cell surface labeling experiments (set 2, see also Figure 3), the time-course experiment (set 3, see also panel c) and the phosphoproteome data (set 4, see also Figure 4). (B) Proteins differentially affected by TNFα- and IL-1β treatment, and as a reference ICAM1. (C) Affected proteins identified in a time course experiment, which were also significant in the whole proteomes after 24 h. (D) Affected proteins identified in a time course experiment which did not correspond with the whole proteomes. (E) Time curves of selected proteins. Mean values and ranges of individual values are plotted for each condition. See also Table S-1.

ITGB4 (integrin beta 4), and secreted proteins MMRN1 (multimerin 1) and NTN4 (netrin 4) (Figure 2B). Interestingly, PLVAP (plasmalemma vesicle associated protein), which forms a physical sieve that regulates lymphocytes transmigration and plasma protein leakage [48,49], was also decreased (Figure 2B).

Pro-inflammatory cytokines induce early and late-responsive proteins

To understand the temporal regulation of the induced response and to gain further insight in the dynamics of regulated proteins, we performed a longitudinal time-series by assessing protein levels after 4, 8, 16 and 24 hours of cytokine-treatment. In agreement with a rapid onset of action, already after 4 hours of treatment several proteins showed increased expression, including the leukocyte-interactors SELE and ICAM1. Consistent with previous reports [16,17], ICAM1 levels were still increasing after 24 hours, whereas SELE levels were peaked well before 24 hours of stimulation (Figure 2C,E). Likewise, other proteins, including serine protease inhibitor B2 (SERPINB2), and sequestosome 1 (SQSTM1), a scaffold protein involved in NF κ B signaling, were rapidly elevated within the first 4 hours of treatment (Figure 2C). In contrast, the abundance of other proteins, including PDCD4 (programmed cell death 4) and IGFBP7 (insulin-like growth factor binding protein 7), were decreased after 4 hours of treatment (Figures 2C and D). However, most regulated proteins were stable before 8 or 16 hours of treatment, indicating the presence of a limited initial protein response, supporting leukocyte interaction, and a later extensive inflammatory response. Interestingly, a subset of proteins was transiently affected (Figure 2E): a transient peak in ferritin levels (FTL [ferritin light chain] and FTH1 [ferritin heavy chain 1]) and a transient dip in CTGF (connective tissue growth factor) and IGFBP7 levels was observed after 8 hours, after which these proteins returned to basal levels (Figure 2C and E).

Cytokine-induced inflammation affects the endothelial cell surface

The interaction of ECs with leukocytes and inflammatory mediators is primarily mediated via cell surface proteins. To study proteins specifically localized at the cell surface, we expanded the analysis with quantitative peptide-based cell surface proteomics data. Therefore, ECs, which were cytokine- or mock-treated for 24 hours, were incubated with a non-membrane permeable biotin label that chemically modifies lysine residues. After lysis of the cells and enzymatic digestion of the proteins into peptides, biotinylated peptides were pulled-down, and subjected to mass spectrometry analysis. In total, 1260 labeled sites were quantified, representing 359 proteins, of which 70 peptides (originating from 31 proteins) were affected upon cytokine treatment (Figure 3A, Table S2). A Gene Ontology (GO)-term enrichment analysis was performed in which we compared these 359 proteins to all proteins identified in any of the proteomes. The group of proteins which were biotin-labeled was highly enriched for proteins associated to the extracellular region (Figure 3B, Table S3), indicating that biotin-labeled peptides originated from the cell surface. In agreement with our proteomic data, TNF α and IL-1 β treatment resulted in a highly similar effect on the cell surface proteome, with a slightly stronger response of TNF α for most regulated proteins. These data correlated well with the whole proteomes (Figure 3A). For most proteins, we identified multiple biotinylated lysine residues, of which the SILAC ratios were consistent throughout the protein (for instance ITGA2 (integrin alpha 2) [non-affected] and LIPG

Figure 3. Peptide-based cell surface proteomics allows for quantification of additional cell surface proteins and detects differential labeling within ITGA5. (A) Heat maps of affected cytokine-induced changes of labeled cell surface peptides ('Cell surface') and corresponding median protein values ('Prot'). Color coding represents log₂ SILAC ratios, grey values are not quantified. '+' represents significance. (B) GO term enrichment analysis of labeled proteins compared to all identified proteins. Top 10 enriched GO-terms are depicted in the table. The full analysis is available in Table S-2. (C) Scatter plots of peptides quantified in the cell surface proteome. Values on the X-axis represent the labeled lysine residue in the cell surface proteome. For reference, quantifications present in only 1 or 2 replicates are also depicted in this plot, in contrast other figures and analyses. See also Table S-2 and S-3.

(endothelial lipase) [increased], Figure 3C). However, our chemical labeling approach also revealed specific changes within proteins, including ITGA5 (integrin alpha 5) position 893 and ICAM1 position 433, although the latter was quantified in only 2 replicates (Figure 3C). These changes indicate that these sites are more (ITGA5) or less (ICAM1) available for the labeling agent.

Prolonged TNF α -treatment affects protein activation states

Independent of protein expression levels, the activity of proteins can be regulated by post-translational modifications, most notably through phosphorylation [50]. The activation of kinases and phosphatases is a powerful regulatory mechanism to rapidly induce a response, without the need for de novo gene expression and protein production. In order

Figure 4. Phosphoproteomic analysis of endothelial inflammation provokes mostly phosphorylation events. (A) Heat map of the quantification of regulated phosphosites. Color coding represents log₂ SILAC ratios. Phosphosites are depicted with corresponding whole proteome SILAC ratios ('Prot'). Grey values are not quantified. (B) SILAC ratios of phosphopeptides (grey dots), lines represent the mean values. Red lines represent median whole proteome values. For reference, modified sites that were not quantified in all replicates have been displayed here as well. See also Table S-4.

to screen for modified phosphosites after prolonged treatment with TNF α , we assessed the steady-state phosphoproteome by a pull-down approach using Phos-TiO₂ beads. 1728 phosphosites were quantified, which originated from 1045 proteins. Of 67 of these sites, the protein activation state was changed (Figure 4A and Table S4). The majority of these showed increased phosphorylation levels whereas only about a quarter of the peptides was dephosphorylated (Figure 4A). For 441 phosphopeptides, originating from 337 proteins, no corresponding whole proteome ratios were available, possibly indicating the low abundance of these proteins. For the remaining phosphosites, the correlation between the whole proteome and the phosphoproteome was poor, in contrast to the cell surface-whole proteome correlation. For a limited number of phosphosites, the phosphosite SILAC ratio reflected the whole proteome SILAC ratio, indicating that the increased phosphorylation reflects increased protein levels (for instance DTX3L [deltex E3 ubiquitin ligase 3L], Figure 4C). However, for most affected phosphosites, we found no evidence for changes at the protein expression level, suggesting that these phosphosites were regulated by kinase and/or phosphatase activity (e.g. PKN [paxillin], NES [nestin] and AHNAK [neuroblast differentiation-associated protein AHNAK]). For the majority of the phosphorylated proteins, we detected only one or a few regulated phosphosites, suggesting that these may function as on/off switches for function and/or interaction. However, a few proteins appeared to be decorated with differentially regulated phosphosites, indicating that their function may be regulated in a highly complex manner (Figure 4C: AHNAK and NES). For example, for the scaffold protein AHNAK, we quantified 31 phosphosites in all replicates, of which

Figure 5. TNF α affects immune- and cardiovascular-specific processes in endothelial cells. Graphic representation of enriched GO terms in either of the analyses clustered in gogadget. (A) Based on the overlap of gene sets of a GO-term, GO-terms were clustered. Keywords represent the most notable GO-terms in a group. (B and C) Enrichment map representations of 2 clusters of panel A (indicated by asterisks). The node size represents the term size and green connections the overlap between terms. See also Table S-5.

multiple were regulated, creating a large variety of possibilities to regulate interactions with other proteins. Interestingly, we found one regulated phosphorylation site in ICAM1 at position T530. Although this particular phosphosite was increased compared to control, the protein ratio far exceeded the phosphosite ratio, which indicates that the phosphorylation machinery is not sufficient to modify all available proteins. Alternatively, this could be the result of specific regulation in which the activity of a kinase or phosphatase is affected.

Integrated analysis of endothelial inflammation

To obtain an overview of all affected processes in endothelial inflammation, we combined data from the whole proteome, cell surface proteome and phosphoproteome and performed a gene ontology (GO)-term enrichment analysis comparing the regulated proteins to all quantified proteins. The enriched GO-terms are partially redundant: a single protein can be present in multiple GO-terms. Based on proteins shared between GO-terms, the 199 enriched terms were clustered to gain insight in their biological implications (Figure 5A). Ten subsets were defined and the largest two were visualized as separate networks (Figures 5B and C). The most prominent GO-terms were related to inflammation, including the immune response and leukocyte migration (Figure 5A and B). In addition, specific pathways related to increased adhesion molecule expression, self-antigen presentation, cytokine-production and cell migration were enriched as well as generic processes, including signaling, response to stimulus and biological regulation (Table S5). Notably, this analysis emphasizes that many of the affected proteins are part of the extracellular matrix (ECM) or involved in the cell-ECM interaction. Although it has been reported that the ECM plays a role in endothelial inflammation [51], and that TNF α can affect the expression levels of ECM

proteins such as laminin $\alpha 4$ and $\alpha 5$ [52,53], it has not been described that $\text{TNF}\alpha$ or $\text{IL-1}\beta$ can affect the protein levels of for instance laminin $\beta 3$ or $\gamma 2$ (LAMB3 and LAMC2), and integrins $\alpha 6$ and $\beta 4$ (ITGA6, ITGB4). Combined, this analysis provides an overview of $\text{TNF}\alpha$ -affected endothelial processes.

DISCUSSION

Chronic inflammation can contribute to various diseases, including inflammatory bowel disease and rheumatoid arthritis [2–4]. In order to successfully intervene in pro-inflammatory cytokine activation, a thorough understanding of endothelial inflammation is necessary. Here, we studied the effects of two well-known pro-inflammatory cytokines, TNF α and IL-1 β using an integrated proteomics approach.

In line with the hypothesis that TNF α - and IL-1 β -induced signaling complexes activate the same set of MAPKKs, our mass spectrometry-based study revealed a highly similar effect of TNF α and IL-1 β on protein expression in ECs (Figure 2A). Although we expected a partially analogous protein signature, since both cytokines provoke the translocation of transcription factor complex NF κ B to the nucleus, the resemblance of the proteomes induced by both cytokines was remarkable. Apparently, though via different adaptor molecules, TNFR1 and IL1-R1 elicit largely the same changes in protein profiles. Interestingly, in addition to the well-established adhesion receptors ICAM1, VCAM1 and SELE, expression of a large number of cell surface proteins was upregulated, which may contribute to interactions with leukocytes.

Biotinylation of cell surface proteins is a well-known labeling technique that has been widely used in biochemical assays and has been applied in mass spectrometry-based studies [54,55]. Commonly, labeled surface proteins are pulled-down via an immobilized streptavidin surface. In our set-up however, the pull-down was performed on tryptic peptides, which allows for detection of the labeling efficiency of each modified residue separately. Similar SILAC ratios were detected for biotin-labeled lysine residues within the same protein with two exceptions. For integrin α 5 (ITGA5) we identified that lysine 833 was more exposed upon TNF α and IL-1 β treatment compared to the other lysine residues (Figure 3C). Since this particular lysine is located at the N-terminus of the heavy chain, we speculate that the increased exposure may be indicative of integrin α 5 activation, which is in line with the role of integrin α 5 activation in leukocyte adhesion in TNF α -treated ECs [56]. In addition, we observed that ICAM1 lysine 433 in domain D5 was less increased compared to 6 other labeled sites in ICAM1. ICAM1 exists as both a monomeric and dimeric form on the cell surface [57]. Based on crystallography, the D4 and D5 domains have been suggested to form a stem-like structure through which dimers are formed [58]. Furthermore, the D5 domain contains an epitope to which the CA7 antibody can bind, but only in the monomeric and not in the dimeric form [59]. Combined, these data suggest that TNF α and IL-1 β treatment may induce dimerization of ICAM1, possibly due to the higher cell surface concentration. Dimerized ICAM1 displays a higher affinity for its ligand integrin α M β 2 (ITGAM-ITGB2) [59], which is present on leukocyte cell surfaces, and thereby aids in leukocyte extravasation. Furthermore, we observed that whole proteome and surface proteome levels correlate well, supporting the cell surface proteomics quantification. This suggests that changes in surface protein levels can be accurately detected in whole proteomics.

To obtain an impression of the cellular processes affected by endothelial inflammation,

we integrated whole proteome, cell surface proteome and phosphoproteomic data of TNF α -treated ECs. The complex network was visualized by classifying regulated proteins according to specific processes and general terms (Figure 6). Although many of these proteins have been shown to be involved in inflammatory processes, others are relatively poorly studied or have not been associated to inflammation. Our integrated approach describes not only leukocyte adhesion, but a full array of affected processes, including self-antigen presentation, cytokine production, nitric oxide production and ECM-interaction. Proteins involved in leukocyte adhesion and extravasation include early TNF α responsive proteins such as ICAM1 and SELE, but also many other TNF α responsive proteins, including VCAM1, CD44, ITGA5, PLVAP, CD47 [60] and LIPG [61]. In agreement with an important role for NF κ B-related signaling, expression of a wide range of immunity related proteins was affected, including the NF κ B complex itself, type I interferon pathway and proteins involved in cytokine synthesis, which may reflect TNF α -induced production of interleukin 6 [62] and 8 [63,64]. In addition, proteins involved in nitric oxide production, including (NOS3)

Figure 6. Summary of changed proteins and model of affected processes in TNF α -treated endothelial cells. Schematic overview of affected proteins (large circles), biotin-labels (triangles) and phosphosites (small circles). If a protein is affected in either of the analyses, the protein and all quantified modifications are depicted. The color coding of the proteins and the modifications represent the respective median $\log_2$ SILAC ratios. Non-quantified proteins are depicted grey. Proteins are clustered according to the enrichment analysis of Figure 5 / Table S-5, with minor manual curation. Localization of proteins within the cell is based on the presence of biotin-modifications and minor manual curation.

and the arginine transporter (SLC7A2 [solute carrier family 7 member 2]) are decreased as reported earlier [46,65], likely leading to a decreased nitric oxide production. Furthermore, an important category of proteins which showed an increased expression level relates to endogenous antigen presentation. Expression of the large majority of proteins belonging to peptide-presentation machinery was upregulated after 16 hours of TNF α treatment, ranging from the MHC class I complex (B2M [beta-2-microglobulin], HLA [HLA class I histocompatibility antigen]-A/B/C) to peptide transport (TAP [antigen peptide transporter] 1/2, TAPBP [tapasin]) and trimming (ERAP [endoplasmic reticulum aminopeptidase] 1/2). In addition to the endothelium itself, it has been suggested that the ECM also plays a role in endothelial inflammation [51]. In agreement with this hypothesis, we identified regulated expression of a variety of proteins associated with ECM organization. Among the most affected proteins are two laminin subunits: β 3 (LAMB3) and γ 2 (LAMC2), whereas in resting conditions the basement membrane mainly consists of laminin 511 (α 5 β 1 γ 1) and 411 [66]. In contrast to these laminins, two laminin receptors (integrin α 6 β 4 [51,67,68] and BCAM [basal cell adhesion molecule]) are decreased, which indicates that the increased laminins do not simply strengthen the cell-ECM interaction. Instead, these may have another function, such as leukocyte interaction, or, after proteolysis, as mediators of inflammation [69]. In addition to proteins that have previously been associated with endothelial inflammation or TNF α , we found regulated expression of proteins that have remained under the radar so far, including the cell surface proteins ANTXR2 (anthrax toxin receptor 2), EFNB1 (ephrin b1) and CLEC1A (c-type lectin domain family 1 member A).

Endothelial activation is categorized into a rapid transient response (type I activation) and a slower but more sustained response (type II activation) [1]. Type I activation is mediated by (mostly) chemical factors, such as bradykinin, histamine and prostaglandins, that bind to G-protein coupled receptors (GPCRs). GPCRs are typically desensitized within 10-20 min [70], which limits the potential of inflammation and neutrophil extravasation induced by type I activation. In contrast, type II activation is mediated by biochemical factors, such as pro-inflammatory cytokines, binding to their cognate receptors. Since this induces new gene transcription and protein translation, it requires a longer initiation time, however, it also results in a more effective and long-lasting increase in vascular permeability and neutrophil recruitment [1]. Timing of this response in ECs is crucial to prevent excessive leukocyte extravasation and other adverse effects. So far, it has remained unclear how protein expression is regulated over time and if and which feedback mechanisms are in place that shut off inflammatory gene and protein expression. Transcriptomic data from TNF α -treated fibroblasts indicate a crude classification in 3 kinetic profiles with early, intermediate and late genes, peaking at respectively 0.5, 2 and >12 hours [71] corresponding with the biological processes of leukocyte recruitment, tissue remodeling and wound repair. Our temporal protein data in ECs demonstrate similar profiles, although only two phases can be distinguished. Within the first 4 hours a limited number of proteins is synthesized, including the adhesion receptors ICAM1 and SELE. Although protein levels of ICAM1 steadily

increase until the 24 hour time point, the increase in SELE stops and even decreases, despite continuous presence of the cytokine. This is in agreement with the reported peak of ICAM1 after 48 hours [72] and the temporal pattern of SELE expression [16,72,73]. The increased expression of adhesion molecules is followed by a transient decrease in the expression of CTGF and IGFBP7 and a temporal increase in the expression of the 2 subunits of the ferritin complex, FTH1 and FTL. Although TNF α has previously been implicated in the regulation of ferritin levels, albeit only for the heavy and not for the light chain subunit [74], the role of ferritin in TNF α - and IL-1 β -treatment of ECs remains unclear. The ferritin complex stores Fe²⁺ ions in the non-toxic Fe³⁺ form to prevent oxidative damage. It has been described that treatment of ECs with TNF α induces intracellular generation of reactive oxygen species (ROS), a process which requires iron as a catalyst and has been directly associated with destabilization of endothelial junctions [75]. Interestingly, temporal changes in ferritin occur within the same time frame as changes in transendothelial resistance (Figure 1B), which starts to decrease after 4 hours and stabilizes after 8 hours. Therefore, we speculate that initially iron is sequestered and utilized to increase ROS production. This results in destabilization of endothelial junctions and degradation of the ferritin complex to replenish available iron levels. Once ROS production is normalized, intracellular iron levels rise and result in ferritin production to levels above the initial concentration, that will eventually normalize. Temporal upregulation of the ferritin complex is followed by a second burst of protein expression, which includes proteins that belong to the self-antigen peptide presentation pathway (HLAs, B2M, ERAP1, TAP1&2 and TAPBP) and proteins involved in conquering viral infections (GBP1 [guanylate binding protein 1], OAS3 [2'-5'-oligoadenylate synthase 3], MX1 and SLFN11 [schlafen family member 11]). Taken together, our results hint towards a two-step process of endothelial inflammation, comprising an initial wave of expression of proteins aimed to attract innate immune cells and mediate their transmigration, followed by a second burst of expression aimed to equip ECs to fight viral infection and present hostile peptides to the immune system, thereby allowing adaptive immune cells to scan the endothelium for possible infections.

The data presented in this study provide novel insight in the effect of TNF α -treatment on ECs, but several questions remain. Our data indicate that TNF α and IL-1 β provoke a largely identical response in ECs. Does that imply redundancy, or can more differences be expected in a physiological setting? *In vivo*, ECs are confronted with many other cytokines, such as IFN- γ , which induces MHC class II expression [76], and interleukin 6, which exerts both pro- and anti-inflammatory effects through trans-signaling [77]. Would a combination of any of these cytokines affect additional processes, or evoke a more severe response compared to the individual cytokines? Furthermore, the question remains what the function is of the newly identified cytokine-induced surface proteins. Do these have a role in leukocyte interaction or other processes and why are these processes affected during inflammation? Answers to these questions may further improve our understanding of endothelial inflammation and may eventually aid us in therapeutic intervention of imbalanced inflammation.

AUTHOR CONTRIBUTIONS

E.P.B. and B.L.v.d.E. performed experiments, E.P.B., K.M., A.B.M. and M.v.d.B. designed the research, E.P.B., A.J.H. and B.N. analyzed the data and E.P.B. and M.v.d.B. wrote the manuscript.

CONFLICT OF INTEREST DISCLOSURE

The authors have no conflicts of interest to declare.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Jaap van Buul for his valuable input to improve this manuscript.

FUNDING SOURCES

This work was supported by Sanquin Blood Supply Foundation [grant PPOP 13-002]; and the Landsteiner Foundation for Blood Transfusion Research [LSBR fellowship 1517 to M. van den Biggelaar].

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SUPPLEMENTAL TABLES

The following supplemental tables are available online or upon request.

Table S1. SILAC ratios of Proteomes after 24 hours cytokine stimulation and time courses.

Log₂ SILAC ratios of TNF α or IL-1 β stimulated BOECs. This table contains data from in-depth whole proteomes (ID), Surface Proteome (SP), PhosphoProteome (PP) and time course experiments (eg. TC_1 represents the first replicate of 4 hours cytokine stimulated BOECs). For each experimental condition separate columns indicate whether the sample meets p-value (_p_) and log fold change (_LFC_) requirements and thereby meet our statistical criteria.

Table S2. SILAC ratios of the Cell Surface Proteome after 24 hours of cytokine stimulation.

Log₂ SILAC ratios of biotin-labeled pulled-down peptides. Indicated are whether these peptides passed our p-value (_p_) and log-fold change (_LFC_) criteria. Position refers to the position within the protein of the modified lysine residue.

Table S3. GO-term enrichment analysis of proteins identified in the Cell Surface Proteome.

Enriched cellular component GO-terms of proteins identified in the Cell Surface Proteome compared to all identified proteins in the proteomic datasets. 'x' and 'n' refer to the number of proteins which is annotated for the GO-term in the subset and reference set, respectively, whereas 'X' and 'N' refer to the size of these datasets.

Table S4. SILAC ratios of the Phosphoproteome after 24 hours of cytokine stimulation.

Log₂ SILAC ratios of phosphopeptides. Indicated are whether these peptides passed our p-value (_p_) and log-fold change (_LFC_) criteria. The modified residues are 'S' (serine), 'T' (threonine) or 'Y' (tyrosine). Position refers to the position of the modified residue within the protein.

Table S5. GO term enrichment analysis of TNF α responsive proteins.

GO term enrichment analysis of protein affected by TNF α in either of the proteomic datasets, compared to all identified proteins. 'x' represents the number of affected proteins within a GO-term, whereas 'n' represents the number of proteins in the reference set in the GO-term.

CHAPTER 3

FLOW-INDUCED REORGANIZATION OF LAMININ-INTEGRIN NETWORKS WITHIN THE ENDOTHELIAL BASEMENT MEMBRANE UNCOVERED BY PROTEOMICS

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Mol Cell Proteomics. 19 (2020) 1179-1192 (revised form)

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ABSTRACT

The vessel wall is continuously exposed to hemodynamic forces generated by blood flow. Endothelial mechanosensors perceive and translate mechanical signals via cellular signaling pathways into biological processes that control endothelial development, phenotype and function. To assess the hemodynamic effects on the endothelium on a system-wide level, we applied a quantitative mass spectrometry approach combined with cell surface chemical footprinting. SILAC-labeled endothelial cells were subjected to flow-induced shear stress for 0, 24 or 48 hours, followed by chemical labeling of surface proteins using a non-membrane permeable biotin label, and analysis of the whole proteome and the cell surface proteome by LC-MS/MS analysis. These studies revealed that of the >5000 quantified proteins 104 were altered, which were highly enriched for extracellular matrix proteins and proteins involved in cell-matrix adhesion. Cell surface proteomics indicated that LAMA4 was proteolytically processed upon flow-exposure, which corresponded to the decreased LAMA4 mass observed on immunoblot. Immunofluorescence microscopy studies highlighted that the endothelial basement membrane was drastically remodeled upon flow exposure. We observed a network-like pattern of LAMA4 and LAMA5, which corresponded to the localization of laminin-adhesion molecules ITGA6 and ITGB4. Furthermore, the adaptation to flow-exposure did not affect the inflammatory response to tumor necrosis factor α , indicating that inflammation and flow trigger fundamentally distinct endothelial signaling pathways with limited reciprocity and synergy. Taken together, this study uncovers the blood flow-induced remodeling of the basement membrane and stresses the importance of the subendothelial basement membrane in vascular homeostasis.

INTRODUCTION

The vascular network is crucial to supply tissues with oxygen and nutrients and remove waste products. The inner lining of this network is formed by a monolayer of endothelial cells (ECs), which provide a dynamic interface between blood and underlying tissues and actively mediate various physiological processes, including regulation of the vasomotor tone, vessel wall permeability and inflammation [1]. The EC phenotype and function are modulated by hemodynamic forces in the circulation, including blood flow-induced shear stress. Depending on the local flow type and its strength, EC morphology ranges from polygonal, in which the cells are randomly orientated, to spindle-like, in which cells are aligned in the direction of the flow. Aligned cells are characterized by enhanced cell survival, an effective barrier function, and limited capacity to support leukocyte transmigration. In contrast, the non-aligned phenotype is associated with enhanced cellular turnover, leakiness to plasma macromolecules, and increased leukocyte adhesion [2,3]. It has therefore been proposed that absence of flow elicits a pro-inflammatory effect [4]. However, the molecular mechanisms underlying the endothelial hemodynamic response are incompletely understood. In addition, multiple studies have demonstrated a link between flow and inflammation [5–10], yet the associated proteomic alterations remain to be explored.

Functional studies have established that ECs respond to changes in hemodynamics through a set of mechanosensitive receptors, which include PECAM1 (platelet and endothelial cell adhesion molecule 1), VEGFR2 (vascular endothelial growth factor receptor 2, KDR), VE-Cadherin (vascular endothelium cadherin, CDH5) [11,12], PAR1 (proteinase-activated receptor 1, F2R) [13], ion channels [14] and integrins [15,16]. Sensing of flow by the endothelial mechanosome initiates signaling cascades that activate a variety of transcription factors, including KLF2 (kruppel-like factor 2) [17] and KLF4 [18], and inhibit others, such as YAP/TAZ (Yes-associated protein, tafazzin) [19] and proteins of the NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) complex [20]. These signaling events eventually lead to a transient increase in endothelial barrier function [21], an increased production of the vasodilatory agent nitric oxide via upregulation of NOS3 (nitric oxide synthase 3, eNOS) and a transient inhibition of cell proliferation to prioritize flow adaptation [22]. In addition, flow-induced mechanosensory signaling initiates remodeling of the actin cytoskeleton and subendothelial extracellular matrix (ECM), resulting in alterations in cell anchorage to the underlying sheet-like specialized ECM: the basement membrane (BM) [23,24]. Recently, a systems biology analysis of the longitudinal response of ECs to shear stress at a transcriptional level elucidated the dynamics of several functional pathways, including cell cycle, oxidative stress, and inflammation [25]. However, how these transcriptomic changes translate into molecular mechanisms that guide the flow-induced phenotypical and functional transition, specifically at the cell surface and extracellular region, has remained unclear.

To address this issue, we performed a global quantitative proteomic analysis on the effects of flow on ECs and particularly focused on cell surface proteins by employing

a quantitative chemical footprinting approach. Combined, our data show that flow most prominently induced changes in proteins involved in cell adhesion and ECM organization, most notably in the laminin and integrin protein families. By employing immunofluorescence microscopy, we subsequently uncovered a drastic remodeling of the ECM and cellular adhesion molecules, including laminin α 4 (LAMA4), α 5 (LAMA5), fibulin-2 (FBLN2), EGF Containing Fibulin Extracellular Matrix Protein 1 (EFEMP1 or fibulin-3), integrin α 6 (ITGA6) and integrin β 4 (ITGB4). Furthermore, colocalization of integrin α 6 β 4, LAMA4 and LAMA5 suggests that integrin α 6 β 4 may provide a cellular link to the laminin ECM to withstand flow-induced mechanical forces. In addition, we showed that the response of ECs to tumor necrosis factor α (TNF α) was independent on flow-adaptation, indicating that flow and inflammation trigger distinct signaling pathways. Combined, these data provide molecular details on the flow-induced remodeling of the endothelial BM and expose a role for integrin α 6 β 4 in cell-matrix adhesion in flow-conditions.

MATERIALS AND METHODS

Primary cell culture

Blood Outgrowth Endothelial Cells (BOECs) [26] were obtained from blood from healthy volunteers in accordance with Dutch regulations and the Declaration of Helsinki after approval from the Sanquin Ethical Advisory Board. Written informed consent was given by all participants. BOECs originating from 3 healthy volunteers (mixed sexes) were pooled and used in the described experiments. Culture flasks and dishes were coated with 50 µg/ml collagen type I (BD biosciences) prior to use. BOECs were grown in Endothelial Cell Growth Medium (Promocell) supplemented with 18% fetal bovine serum (FBS) (Bodinco) and maintained in a humidified 5% CO₂, 37 °C incubator. For SILAC labeling, BOECs were maintained for 5 passages as previously described [27] using custom-made EBM-2 medium (Lonza), containing 18% 1 kDa dialyzed FBS (Bodinco) and isotope labeled amino acids (light: Arg0 & Lys0, medium: Arg6 & Lys4, heavy: Arg10 & Lys8, Cambridge Isotopes).

Flow-exposure of endothelial cells

BOECs were seeded in a ring-shaped culture dish, formed by placing a Ø 54 mm (outer diameter) petri dish bottom in the middle of a Ø 86 mm (inner diameter) petri dish. In the middle of the inner dish a magnet was placed, underneath the outer dish a T-shaped metal strip. The formed ring-shaped dish was then coated with 50 µg/ml collagen type I (BD biosciences), seeded with BOECs in 20 ml medium and incubated overnight. The next day these petri dishes were placed on a Stuart SSL3 gyrorocker platform on top of an 11 cm high elevated platform, which was set to rotate at 60 RPM and placed in a humidified 5% CO₂ 37 °C incubator. Within all experiments, the endpoints of the different experimental conditions were synchronized, i.e. the 'static' samples were maintained for 48 hours in static conditions before lysis/fixation.

Quantitative MS analysis of flow-treated SILAC-labeled ECs

SILAC-labeled BOECs were seeded in the ring-shaped culture dishes as described above and subjected to flow when appropriate. Mass spectrometry sample preparation and acquisition, as well as cell surface labeling were performed as previously described [27]. Briefly, SILAC labeled BOECs were washed and incubated for 30 minutes at 4 °C with 3 mM EZ-Link sulfo-NHS-LC-biotin (ThermoFisher Scientific). Excess label was quenched with 100 mM Glycine, and cells were lysed in 4% SDS, 100 mM DTT, 100 mM Tris, pH 7.5, supplemented with HALT phosphatase and protease inhibitor cocktail (Thermo Scientific). Proteins were proteolytically digested with trypsin (Promega, cleaves after Lys and Arg) or chymotrypsin (Thermo Scientific, cleaves after Tyr, Trp and Phe) using filter-aided sample preparation (FASP) [28]. Biotin-labeled peptides were pulled-down using a SigmaScreen Streptavidin high capacity coated plates (Sigma Aldrich), and subjected to C18 StageTip [29] desalting and mass spectrometry analysis. For proteome samples 50 µg tryptic digests

were fractionated using a Strong Anion eXchange fractionation using Empore Anion and Cation Exchange-SR Extraction Disks (3M) [30], and peptides were desalted using C18 (3M) StageTips [30]. Peptides were separated by nanoscale C18 reverse chromatography coupled on line to an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific) via a nanoelectrospray ion source at 2.15 kV.

Label-free Quantitative MS analysis of flow/TNF α treated ECs

After 48 hours of flow-exposure, 10 ng/ml TNF α was added to the medium when appropriate and flow/static incubation was continued for 24 hours. Cells were prepared for MS analysis as described [31] with minor modifications. Briefly, ECs were lysed in 1% sodium deoxycholate 10 mM TCEP, 40 mM chloroacetamide, 100 mM Tris-HCl pH 8.0 supplemented with 1x HALT protease/phosphatase inhibitor (Thermo Scientific). Lysates were incubated for 5 min at 95 °C and sonicated for 10 min in a sonifier bath (Branson model 2510), after which trypsin (Promega) was added in a 1:50 (w/w) protein ratio. Peptides were loaded on in-house prepared SDB-RPS (Empore) StageTips, and eluted into 3 fractions subsequently using: buffer 1 (100 mM ammonium formate, 40% (v/v) acetonitrile, 0.5% (v/v) formic acid), buffer 2 (150 mM ammonium formate, 60% (v/v) acetonitrile, 0.5% (v/v) formic acid) and buffer 3 (5% (v/v) ammonium hydroxide, 80% (v/v) acetonitrile). Samples were vacuum dried and peptides were dissolved in 2% (v/v) acetonitrile, 0.1% (v/v) TFA, and subjected to MS-analysis. Peptides were separated by nanoscale C18 reverse chromatography coupled on line to an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Scientific) via a nanoelectrospray ion source at 2.15 kV. Buffer A was composed of 0.5% acetic acid and buffer B of 0.5% acetic acid, 80% acetonitrile. Peptides were loaded for 17 min at 300 nL/min at 5% buffer B, equilibrated for 5 minutes at 5% buffer B (17-22 min) and eluted by increasing buffer B from 5-27.5% (22-122 min) and 27.5-40% (122-132 min), followed by a 5 minute wash to 95% and a 6 min regeneration to 5%. Survey scans of peptide precursors from 375 to 1500 m/z were performed at 120K resolution (at 200 m/z) with a 4×10^5 ion count target. Tandem mass spectrometry was performed by isolation with the quadrupole with isolation window 0.7, HCD fragmentation with normalized collision energy of 30, and rapid scan mass spectrometry analysis in the ion trap. The MS2 ion count target was set to 3×10^4 and the max injection time was 20 ms. Only those precursors with charge state 2–7 were sampled for MS2. The dynamic exclusion duration was set to 30 s with a 10 ppm tolerance around the selected precursor and its isotopes. Monoisotopic precursor selection was turned on. The instrument was run in top speed mode with 3 s cycles. All data were acquired with Xcalibur software.

MS data analysis and visualization

Raw files were processed in the Maxquant 1.5.3.30 computational platform [32] to identify proteins and peptides by querying the uniprot database for human proteins (release 3-2017, 70947 entries) using the andromeda search engine. Default settings of Maxquant

included the following parameters: 1) precursor mass tolerance of 4.5 ppm, 2) fragment mass tolerance of 20 ppm, 3) fixed modification: cysteine carbamidomethylation, 4) variable modifications: methionine oxidation and N-terminal acetylation, 5) an FDR of 1% for proteins and peptides, 6) a minimum score of 0 for unmodified and 40 for modified peptides, and 7) a maximum of two missed cleavages.

For the TNF α -flow dataset, the label-free quantification (LFQ) was enabled with at least 2 ratios per protein. For SILAC samples multiplicity was set to 3, SILAC pairs were entered and the re-quantify option was enabled. The 'match between runs' option was enabled for all samples and protein quantification was based on unique peptides. For the cell surface proteome samples an optional modification for the biotin label (339.16166 Da) was added. Two subsequent streptavidin pull-downs were treated as fractions of 1 experiment. Peptides were generated using trypsin (whole cell proteome and cell surface proteome) or chymotrypsin (cell surface proteome). All assigned sequences, as well as all identified proteins of the SILAC proteome, cell surface proteomes and the label-free quantifications can be found in Tables S7-S14. Maxquant output tables were opened in Rstudio version 1.1.383 (R version 3.4.2) and reverse, potential contaminants and 'only identified by site' entries were filtered out. No other data points such as outliers were excluded from the subsequent analyses. SILAC ratios were log₂ transformed and filtered for at least 3 valid values in at least one of the conditions. Selection criteria for class I phosphopeptides, i.e. a localization probability of >0.75 and a score difference of >5 [33], were used for biotinylated peptides. Statistical analysis was performed using a linear model without intercept. The distribution of SILAC ratios was inspected and was normally distributed. A Benjamini-Hochberg multiple testing correction was applied to the p-values. Values were considered significant and relevant if $p < 0.05$ and log₂ fold change >1 for SILAC ratios and LFQ intensity values.

GO-term enrichment analysis for the SILAC data was performed by comparing all affected proteins to all quantified proteins using the hypergeometric method from goseq 1.24.0 [34], with an FDR adjusted p-value cutoff of 0.05. Enriched terms were grouped and visualized using gogadget [35] hierarchical clustering of overlap indices (with the ward.D agglomeration method and Euclidean distance). STRINGdb analysis was performed using the stringApp plug-in 1.2.2 [36] for Cytoscape 3.6.1 [37] with a confidence interval cutoff of 0.7. For the LFQ data and the cell surface proteome data GO-term enrichment analysis was performed in Cytoscape 3.4.0 [37] using the BiNGO plug-in [38] and this was visualized in Graphpad Prism 7.04. For the LFQ data this analysis was performed by comparing all affected proteins to all quantified proteins. For the cell surface data biotin-modified proteins were compared to all quantified proteins. Scatter plots of MS data were visualized using Graphpad Prism 7.04.

Immunofluorescence imaging

For immunofluorescence and immunoblot analyses, BOECs (pool of 3 donors) were

grown in Endothelial Cell Growth Medium (Promocell). Tissue culture treated polymer coverslips (Ibidi, #1.5) were mounted in the ring-shaped flow channel before cells were seeded. After flow/static incubation, cells were washed 3x with PBS, and subsequently fixed for 15 minutes in 4% paraformaldehyde (PFA, Electron Microscopy Sciences) in PBS. Samples were washed 3x time with PBS and PFA was quenched by incubating 15 minutes in 50 mM NH_4Cl /PBS. Next, samples were washed with PBS, blocked and permeabilized 15 min in blocking buffer (1% [w/v] BSA [Merck], 0.1% saponin in PBS), and subjected to antibody staining in blocking buffer. Between staining steps samples were washed 3x. After primary and secondary antibody incubation, samples were washed 2x with PBS, incubated 5 min with 1 $\mu\text{g}/\text{ml}$ HOECHST 33342 (Life tech) in PBS, washed with PBS and mounted in Aqua poly/mount (Polysciences Inc). Imaging was performed on a Leica TCS SP8 confocal scanning microscope system, equipped with a 63x Plan-Apochromat 1.40 oil immersion objective. Images were processed using the ImageJ [39] Fiji [40] package. The following antibodies were used: Rat IgG2b α ITGB4 555719 (BD Biosciences); Rat IgG2a α ITGA6 MAB13501 & Sheep polyclonal α LAMA4 AF7340 (R&D systems); Rabbit IgG polyclonal α FBLN2 PA5-51665 (Thermo Scientific); mouse IgG1 α EFEMP1 SC-365224 & Goat IgG polyclonal α VE-cadherin C-19 (Santa Cruz Biotechnology); mouse IgG2a α ITGB1 P4C10 (Millipore); mouse IgG2a α LAMA5 4C7 (Abcam). Secondary: Goat α mouse AF488 A11001, Goat α Rat-AF568 A11077, Donkey α Sheep-AF488 A11015, Donkey α Goat-AF568 A11057, Goat α Rabbit-AF568 A11011, Streptavidin-AF488 S11223 (ThermoFisher Scientific).

Immunoblot analysis

For immunoblot analysis BOECs were subjected to flow as described. Next, BOECs were washed 3x with PBS, lysed in 2x sample buffer (125 mM Tris, 2% SDS, 20% glycerol, 0.02% Bromophenol blue, 20 mM DTT), boiled for 10 min, passed 5x through a 29G needle, spun down 15 min at 16,000 xg, and filled up to 1x loading buffer. An estimated 20 μg sample was loaded per well on a NuPAGE 3-8% Tris-Acetate gel (ThermoFisher Scientific). Proteins were separated and transferred to an iBlot nitrocellulose blot (ThermoFisher Scientific). Blot was blocked for 1 hour in blocking buffer (4% BSA, 0.1% Tween-20 in TBS (Tris-buffered saline), incubated overnight in Sheep polyclonal α LAMA4 (AF7340, R&D systems) in blocking buffer, washed 3x with TBS 0.1% Tween-20, incubated 1 hour in Donkey α Sheep-HRP (713-035-147, Jackson ImmunoResearch) in blocking buffer, and developed using BM chemiluminescence blotting substrate (Roche). The loading control was stained using Rabbit IgG α α -tubulin (Ab52866, Abcam) and Swine α Rabbit-HRP (P0399, DAKO).

Experimental Design and Statistical Rationale

For the SILAC-based MS assessment, a pool of BOECs from 3 healthy donors was split in 3, maintained for 5 passages and subjected to the experimental conditions (n=3). For the label-free quantification-based MS analysis a pool of BOECs from 3 healthy donors was split in 12 and subjected to the experimental conditions (n=3). Statistical analysis was performed

using a linear model without intercept. A Benjamini-Hochberg multiple testing correction was applied to the p-values. All displayed immunofluorescence and immunoblot data are representative images of 3 independent biological replicates employing distinct pools of BOECs from 3 healthy donors.

RESULTS

Shear stress induces proteomic alterations that affect a range of cellular processes, including cell-matrix adhesion

To assess the effects of flow-induced shear stress, ECs were cultured to confluency in a ring-shaped flow chamber, and subjected to flow for 24 and 48 hours using a gyrorocker (Figure 1A). The endpoints of the samples were synchronized to equalize culturing times between samples. Flow exposure resulted in an altered morphology within 24 hours, and the ECs were elongated and completely aligned in the direction of the flow after 48 hours (Figure 1B). To assess flow-induced differences in protein expression profiles in a quantitative manner, ECs were metabolically labeled using SILAC, and after cell lysis, tryptic digests were subjected to mass spectrometry (MS) analysis (Figure 1A). Using this approach, we quantified 5200 proteins (Table S1), of which 104 were statistically significantly in- or decreased in flow conditions (Fig 2A and Table S1). Notably, the majority of these proteins ($n=83$) were more abundant in the flow-treated cells and only a fraction ($n=21$) decreased upon shear stress-stimulation (Figure 2A). All affected proteins were consistently in- or decreased in the 24 and 48 hour time-points. We complemented whole cell proteomics with the cell surface chemical footprinting approach that we have previously developed to detect intramolecular changes and altered protein-protein interactions [27] (Figure 1A). Therefore, ECs were labeled with a non-membrane permeable biotin label, which was predominantly

Figure 1. Mass spectrometry workflow and flow-induced morphological changes in endothelial cells. (A) Proteomics workflow. SILAC-labeled BOECs were treated with flow or incubated statically, and labeled with a sulfo-NHS-LC-biotin compound at 4 °C. SILAC-labeled cell lysates were mixed in a 1:1:1 protein ratio, proteins digested with trypsin or chymotrypsin and peptides subjected to streptavidin pull-down. 50 µg of the mixed lysates was used for strong anion exchange (SAX) fractionation. All samples were subjected to high resolution MS. (B) Immunofluorescence maximum intensity projections of BOECs subjected to flow for 0, 24 or 48 hours. Cells were stained for VE-cadherin (green) and with HOECHST (blue). Micrographs are representative for 3 independent experiments. The scale is 10 µm.

3

present at the apical surface of the cells and at the intercellular junctions, as indicated by junctional marker VE-cadherin (Figure S1). Using this approach we quantified a total of 1763 biotin-labeled sites, of which 340 were affected by shear stress (Table S2-3, Figure S2). In general, the SILAC ratios in the cell surface proteomics data corresponded closely with the SILAC ratios in the whole cell proteomes (Figure S2).

Flow-induced morphological changes in ECs were accompanied by altered protein levels of various flow-associated proteins, including the nitric oxide-producing enzyme NOS3 [41], HYAL2 (hyaluronidase 2) [42,43], TEK (TEK Receptor Tyrosine Kinase) [43], THBD (thrombomodulin) [17], ADAMTS1 (a disintegrin and metalloproteinase with thrombospondin motifs 1) [44], and THBS1 (thrombospondin) [45]. In addition to these hallmark proteins, we identified regulation of proteins that have not been previously associated with flow-exposure. To gain insight in the functional consequences of these changes, we subsequently conducted a gene ontology (GO)-term enrichment analysis. This analysis indicated that flow affected the abundance of proteins associated with a wide array of processes, ranging from tube development to nucleotide biosynthesis and cell adhesion (Figure 2B and Table S4). The top most statistically significant enriched processes encompassed ECM-, adhesion-, and cell surface-related terms (Figure 2C). The importance of these processes was further emphasized by a STRINGdb analysis which revealed a closely connected network of proteins with various ECM-related functions, including structural ECM proteins (laminins, VCAN [versican]), enzymes (MMP15 [matrix metalloproteinase 15], ADAMTS1&4), and cell adhesion molecules (integrins, BCAM [basal cell adhesion molecule]) (Figure 2D). In addition, this analysis exposed clusters of proteins related to regulation of the actin cytoskeleton (tropomyosin TPM1, TPM2, TPM4, as well as RHOB [ras homolog family member B], ARHGAP24 [Rho GTPase activating protein 24] and PLEKHG5 [pleckstrin homology and RhoGEF domain containing G5]), nitric oxide production (ASS1 [argininosuccinate synthase 1] and NOS3) and vasodilation (CALCRL [calcitonin receptor like receptor] and RAMP2 [receptor activity modifying protein 2]). Combined, these quantitative mass spectrometry data reveal an important role for cell-matrix adhesion in response to flow.

Flow induces remodeling of the laminin network and redistribution of laminin-associated proteins

One protein family that was considerably affected by flow exposure was the laminin protein family (Figure 2D). Laminins form $\alpha/\beta/\gamma$ heterotrimers and are key constituents of the endothelial basement membrane. The BM of ECs contains mainly laminins 411 ($\alpha 4$, $\beta 1$, $\gamma 1$) and 511, and in some tissues 421 and 521 [46]. Our whole cell proteomics data showed that laminin $\alpha 5$ (LAMA5), $\beta 2$ (LAMB2), $\beta 3$ (LAMB3) and $\gamma 2$ (LAMC2) levels increased, whereas the abundance of laminin $\alpha 4$ (LAMA4), $\beta 1$ (LAMB1) and $\gamma 1$ (LAMC1) remained unaffected (Figure 3A). In agreement with these data, cell surface proteomics revealed an increased abundance of LAMA5 and LAMB2 at the cell surface (Figure 3B). This effect was

Figure 3. Flow induces remodeling of the endothelial extracellular matrix. (A) Proteome and (B) cell surface proteome data of identified laminins. T or CT represents the enzyme used for protein digestion. The depicted numbers represent the biotin-modified lysine residue. (C) Schematic representation of laminin 411 with the linker region between domains LG3 and LG4. The blue lysine residues were found to be decreased under flow conditions (as depicted in panel B). (D) Immunoblot analysis of LAMA4 in ECs exposed to flow over time. α -tubulin was used as a loading control. (E) Protein distribution of LAMA5 and LAMA4 after flow-exposure. Green represents LAMA4 or LAMA5, blue is HOECHST. The scale bar is 10 μ m. Depicted are maximum intensity projections. (F) Proteome and (G) cell surface proteome data of identified fibulins. (H) Immunofluorescence analysis of EFEMP1 and FBLN2 abundance and distribution affected by flow. Red is VE-cadherin, blue is HOECHST. Depicted are maximum intensity projections. Micrographs are representative for 3 independent experiments. The scale bar is 10 μ m.

time dependent with more pronounced changes in protein levels after 48 h as compared to 24 h (Figure 3A-B). Interestingly, our cell surface proteomics approach indicated that the abundance of the C-terminus of LAMA4 decreased in flow conditions (Figure 3B), whereas the total level of LAMA4 was unaffected (Figure 3A). We hypothesized that this was due to a proteolytic cleavage resulting in the shedding of the LG4-5 region (Figure 3C), which reduces the molecular weight of the residual LAMA4 by about 43-45 kDa [47]. In line with this hypothesis, immunoblot analysis showed 2 forms of LAMA4 with a difference in molecular weight corresponding to the LAMA4 C-terminal fragment, of which the heavier band disappeared under flow exposure (Figure 3D).

To gain further insight into flow-induced alterations in the laminin network, we next assessed the cellular localization of LAMA4 and LAMA5 in static and flow conditions. Under static conditions, LAMA4 showed a diffuse staining pattern, whereas little LAMA5 staining was detected (Figure 3E). In agreement with our MS data, the staining intensity for LAMA5 was substantially increased in flow conditions, and appeared in a network-like pattern that was more aligned with the direction of the flow after 48 h as compared to 24 h (Figure 3E). In contrast to LAMA5, the protein levels of LAMA4 were not affected under flow conditions. Surprisingly, however, the localization of LAMA4 did change drastically upon exposure to flow (Figure 3E). Subsequent analysis of the relative localization of LAMA4 and LAMA5 indicated that these proteins displayed a high degree of co-localization, specifically after 48 h (Figure S2).

Laminins not only play a key role in the assembly of the BM [48,49], but are also involved in the determination of its molecular composition. In line with this concept, we observed that the laminin-associated proteins FBLN2 and EFEMP1 were amongst the most differentially abundant proteins in our mass spectrometry-based analysis (Figure 3F-G). The MS data indicated that the abundance of FBLN2 (fibulin-2) was increased upon exposure to flow, whereas the abundance of EFEMP1 (fibulin-3) was decreased. In agreement with these data, immunofluorescence analysis showed limited staining of FBLN2 in static conditions, and a bright staining upon exposure to flow, which appeared in a fibrillar pattern aligning in the direction of the flow (Figure 3H). In contrast to FBLN2, we observed cell-associated networks of EFEMP1 (fibulin-3) in static conditions, which mostly disappeared in flow conditions (Figure 3H). Combined, these data indicate that flow exposure results in a drastic reorganization of the structure and composition of the endothelial BM.

Shear stress induces a cell-aligned fibrillar distribution of integrin $\alpha 6\beta 4$ which co-localizes with the laminin network

The composition of the ECM can drive the molecular make-up of the associated cell-matrix adhesion complexes, including the adhesion receptors [50]. Most cell-matrix interactions are mediated by members of the integrin protein family [51], of which 11 subunits were identified by our MS approach. Three of these subunits, $\alpha 6$, $\alpha 9$ and $\beta 4$, were significantly increased in flow conditions (Figure 4A). These findings were confirmed in the

cell surface proteome analysis (Figure 4B), and indicate that the cell-matrix adhesion is affected by flow exposure. Next, we assessed the abundance and localization of the 2 integrin chains that were most prominently affected: $\alpha 6$ and $\beta 4$. As expected, a clear increase in the fluorescent signal was observed for both integrin chains (Figure 4C-D), thereby confirming our MS data. Surprisingly, both integrin $\alpha 6$ and $\beta 4$ showed an intense staining on the leading edge of the cells after 24 h of flow-exposure (Figure 4C-D). After 48 h of flow-exposure, a redistribution of these integrins had occurred into a mostly fibrillar pattern throughout the cells. Integrin $\alpha 6$ displayed a high degree of co-localization with integrin $\beta 4$ (Figure 4E), but not with $\beta 1$ (Figure S3).

Since integrin $\alpha 6\beta 4$ has been shown to interact with LAMA5 [52], we next assessed the localization of these molecules. In line with the described interaction of integrin $\alpha 6\beta 4$ and LAMA5, substantial co-localization of integrin $\beta 4$ and LAMA5 was observed, particularly after 48 h of flow exposure (Figure 5A). Surprisingly, a similar co-localization was observed between this integrin heterodimer and LAMA4 (Figure 5B). Taken together, these data indicate that the flow-induced remodeling of the endothelial BM is not limited to laminins, but includes altered protein levels and relocalization of laminin-associated proteins such as

Figure 4. Shear stress affects the distribution of matrix adhesion complex integrin $\alpha 6\beta 4$. (A) Proteome and (B) cell surface proteome data of identified integrins. T or CT represents the enzyme used for protein digestion. The depicted numbers represent the biotin-modified lysine residue. (C,D) Immunofluorescence maximum intensity projections of flow-treated (24 and 48 hours) and statically-incubated ECs stained for integrin $\alpha 6$ and $\beta 4$ (red). Blue is HOECHST. Scale bars are 10 μ m. (E) Co-localization of integrin $\alpha 6$ (red) and $\beta 4$ (green). Blue is HOECHST. Depicted are maximum intensity projections. Micrographs are representative for 3 independent experiments. Scale bar is 10 μ m.

Figure 5. Integrin $\alpha 6 \beta 4$ co-localizes with the laminin ECM. Co-stainings of ITGB4 (red) and LAMA4 or LAMA5 (green). Blue is HOECHST. Depicted are maximum intensity projections. Micrographs are representative for 3 independent experiments. Scale bars are 10 μ m.

fibulins and integrins, and suggests the formation of a cell-matrix interaction complex via integrin $\alpha 6 \beta 4$ and LAMA5.

Inflammation and flow-deprivation trigger separate pathways in ECs

We previously showed that TNF α stimulation of ECs affects ECM-related proteins and integrin subunits ITGA6 and ITGB4 as well [27]. These results are of particular interest as vascular inflammation and flow-exposure may be interlinked, since an inflammatory signature has been detected in regions of the vascular bed exposed to disturbed flow or low shear stress [14,53]. To dissect the interplay between flow-deprivation and inflammation at a system-wide level, we compared the proteomes of ECs exposed to flow, TNF α or a combination of both by employing label-free proteomics. Using this approach, we quantified the abundance of 5613 proteins, of which 533 changed among the different conditions (Table S5). A highly dissimilar effect was observed for flow-deprivation and TNF α stimulation compared to flow-exposed cells in a principal component analysis (Figure 6A) and k-means clustering of differentially abundant proteins (Figure 6B). Hallmark inflammatory proteins, such as ICAM1 (intercellular adhesion molecule 1), VCAM1 (vascular cell adhesion molecule 1), and SELE (E-selectin), were specifically increased upon exposure to TNF α (Figure 6C, top panel), whereas established flow-associated proteins, including NOS3, THBD and TEK, as well as the flow-responsive proteins identified in this study, were specifically in- or decreased in flow conditions (Figure 6C, bottom panel). As expected, affected proteins were enriched for cell-matrix adhesion in flow-exposed ECs and for immune-related processes in TNF α -stimulated ECs, respectively (Figure 6D, Table S6). Statistical analysis revealed that, although most of their effects were distinct, for a limited number of proteins TNF α stimulation

Figure 6. Flow induces a specific protein signature that is unrelated to endothelial inflammation. (A) Principal component analysis of four groups of samples: static, TNFα-, flow-, and flow & TNFα-treated ECs. (B) Heatmap representation of proteins changed significantly in either of the groups. Z-scored label-free quantification (LFQ) intensities are depicted in which red and blue represent increased and decreased values, respectively. (C) Heatmap representation of z-scored LFQ values of hallmark proteins of TNFα and flow-exposure. (D) Top 5 unique and shared GO-terms enriched in affected proteins of TNFα- or flow-treated samples. A pool of BOECs originating from 3 healthy volunteers was used.

to flow-deprivation did exert similar effects (Figure S4A). Furthermore, for 15 proteins, a synergistic or neutralizing effect was observed: flow deprivation modulated the endothelial response to TNFα (Figure S4B). Combined, these results indicate that flow-deprivation and TNFα-exposure exert fundamentally different effects on ECs with limited interplay.

DISCUSSION

Hemodynamic forces are crucial for the function of the endothelium in vascular homeostasis and thereby for vascular health. Despite this importance, limited information is available on the flow-responsive molecular wiring in vascular endothelial cells. Here, we present quantitative proteomic data of ECs exposed to flow for 24 and 48 hours. We employed Blood Outgrowth Endothelial Cells for their superior propagation capacity [26], which was necessary to obtain >95% SILAC amino acid incorporation required for protein quantification. Our MS data confirmed the flow-induced regulation of nitric oxide production and cytoskeletal rearrangements, and revealed an extensive remodeling of the endothelial basement membrane induced by flow. Specifically, we observed that flow exposure resulted in the formation of a laminin network and the expression of ECM adhesion complex integrin $\alpha 6 \beta 4$. Furthermore, we showed that a lack of flow does not directly induce the expression of inflammatory markers.

In line with previous studies that have demonstrated a crucial role for LAMA5 in EC alignment and production of vasodilatory agents in response to flow [54], our data indicate that the expression of LAMA5 is increased in flow conditions. This laminin has been shown to decrease the transmigration capacity of leukocytes both directly, by inhibiting the migration across the BM [55], and indirectly, by improving the barrier function of the associated ECs [56]. This suggests that leukocytes will primarily transmigrate in regions devoid of high shear stress. In line with this hypothesis, leukocyte extravasation mainly takes place in post-capillary venules, which are not exposed to high shear forces [57], and contain low levels of LAMA5 [58]. It is therefore tempting to speculate that the low LAMA5 content of these vessels is the direct result of low shear force exposure.

In this study, our combined proteomic and immunofluorescence data reveal that, in addition to the increased LAMA5 levels, the protein expression levels and localization of multiple other BM-related proteins are affected in flow conditions. Notably, both LAMA4 and LAMA5 adopted a network-like phenotype in flow conditions. A possible explanation for this observation lies in the N-terminal region of LAMA5, which has the capacity to bind the N-terminal regions of laminin β - and γ -chains, thereby supporting the polymerization of LAMA5-containing heterotrimers [59] into a laminin network. Remarkably, although LAMA4 lacks such a polymerization region [46,60,61], it also adopted a network configuration, however, exclusively when ECs were exposed to flow. We speculate that the expression of LAMA5 induces the formation of a laminin network, which also induces the relocalization LAMA4, possibly via the N-terminal polymerization regions of the β - and γ -subunits of the LAMA4-heterodimers.

In addition to the endothelial BM, our MS data indicated that various proteins involved in matrix adhesion were affected in flow conditions. Within the integrin family we detected a flow-induced increase in the expression levels of $\alpha 3$, $\alpha 6$, $\alpha 9$, and $\beta 4$ subunits, of which $\alpha 3$, $\alpha 6$, and $\beta 4$ form specific receptors for laminins [62]. This may suggest that in flow conditions, besides binding to collagen and the RGD-motif, ECs rely strongly on laminin-

binding integrins for cell adhesion. This is in agreement with the recent finding that the simultaneous blockage of integrin $\alpha 3$ and $\alpha 6$ results in an abrogation of the EC adhesion capacity to laminin 511 [54]. As the $\alpha 6$ and $\beta 4$ subunits were most affected in our MS data, we subsequently focused on these, and showed that integrin $\alpha 6\beta 4$ and LAMA5 co-localized in flow conditions. Combined with the finding that purified integrin $\alpha 6\beta 4$ and LAMA5 interact [52], this suggests that these proteins may be part of the same complex, and that integrin $\alpha 6\beta 4$ plays a role in cell adhesion to LAMA5 [63]. Integrin dimers are involved in various adhesion structures, including focal complexes, focal adhesions [64], fibrillar adhesions [65,66] and the recently described reticular adhesions [67]. These structures can be dynamic in their integrin content, however, the specific flow-induced integrin $\alpha 6\beta 4$ pattern did not match any of these structures. A possible explanation for this unknown integrin localization may be found in the laminin network formation, as integrins and their laminin substrates have previously been shown to affect each other's localization in a reciprocal fashion [68]. The question remains what the function of this complex is. Integrin $\alpha 6\beta 4$ is well-known in epithelial cells for its role in hemidesmosomes: multiprotein complexes that link intracellular keratin intermediate filaments to the ECM laminin 332 [69]. These complexes are essential in maintaining epithelial adhesion to the BM, and their disruption results in the blistering disease junctional epidermolysis bullosa and other severe problems involving epithelia [70,71]. Would integrin $\alpha 6\beta 4$ serve a similar purpose in ECs? It has been described that integrin $\alpha 6\beta 4$ is expressed by ECs *in vivo* [72], and that overexpression of this integrin results in the formation of adhesion complexes that link to the laminin BM and the vimentin cytoskeleton via plectin [73,74]. Furthermore, it has been shown that laminin 511 supported cell adhesion in flow conditions, whereas laminin 411 did not [54]. As integrin $\alpha 6\beta 4$ can interact with laminin 511, but not 411 [52], these data may suggest that, in endothelial cells, the LAMA5-integrin $\alpha 6\beta 4$ complex anchors ECs to the BM. This is supported by the observation that plectin (PLEC), which forms a link between integrin $\beta 4$ and the vimentin cytoskeleton [74], displays a higher abundance in flow-exposed cells. We speculate that the LAMA5-integrin $\alpha 6\beta 4$ link enables the cells to withstand higher shear forces.

In addition to ECM- and adhesion-related proteins, various other proteins were affected by flow-treatment, for which the function and molecular interactions are yet to be determined. For instance, parallel to the proteolytic processing of LAMA4, protein levels of metalloprotease MMP15 were increased. Other membrane-type MMPs have been shown to cleave various ECM components [75] including laminin 111 [76]. It is therefore tempting to speculate that MMP15 may have a role in LAMA4 processing. Furthermore, two proteins that are associated with the barrier function were affected: CLDN11 (claudin 11) and NDRG1 (N-Myc downstream regulated 1). Knock-down of NDRG1 has previously been associated with a decrease in epithelial barrier function [77], whereas CLDN11 is part of the tight junctions and thereby maintains the endothelial barrier function [78]. Our observations that CLDN11 levels were decreased and the abundance of NDRG1 was increased in flow

conditions may suggest that flow-exposure induces a switch in the junctional composition of ECs.

Combined, our data show that, in addition to the morphological change, improved barrier function and regulation of the vasomotor tone, flow exposure of ECs results in a drastic remodeling of the endothelial BM and the cell-BM interaction. This change is characterized by LAMA5-driven laminin network formation and cell-matrix adhesion via integrin $\alpha 6\beta 4$. These findings illustrate the plasticity of the endothelial BM and emphasize the role of blood flow in the vascular function. Additional studies are required to further dissect the implications of the observed integrin-laminin co-localization, including interventional studies using depletions of ITGA6, ITGB4, and LAMA5 to unravel their role in EC functions such as NO production, cell alignment and leukocyte transmigration. In addition, it is essential to assess the EC proteome in other flow conditions, including disturbed flow and different flow rates. Moreover, it will be important to evaluate whether these data can be extrapolated to ECs from distinct vascular beds, including aortic ECs and ECs originating from post-capillary venules. Finally, studies using EC-specific knock-out mice are required to answer the question how our findings translate to *in vivo* settings.

An important aspect of mechanosensing in a (patho)physiological context is the interplay between flow and inflammation. *In vivo*, regions of the vasculature not exposed to laminar flow have been shown to display a pro-inflammatory signature, including increased expression of adhesion proteins ICAM1 and VCAM1 [79–81]. Indeed, multiple *ex vivo* and *in vitro* studies suggest that flow can affect aspects of the endothelial inflammatory response [5–10]. Our data now show that, at a system-wide level *in vitro*, flow and inflammation trigger a fundamentally distinct endothelial response showing limited synergy and interplay. Although we cannot exclude that the apparent discrepancies between our results and the aforementioned studies may result from differences in experimental design, our data suggest that the absence of flow does not result in an inflammatory signature within the experimental timeframe. Possibly, such an inflammatory signature may develop after a prolonged period of culturing. Alternatively, other factors in the complex pathophysiological environment may contribute to the development of diseases related to flow deprivation [82]. Further research into the endothelium in its (patho)physiological environment, possibly using ECs harvested from patients with vascular diseases, is therefore needed to fully understand the basis of vascular disorders such as atherosclerosis and thrombosis.

ACKNOWLEDGMENTS

We thank Floris van Alphen for mass spectrometry assistance. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

SOURCES OF FUNDING

This work was supported by the Landsteiner Foundation for Blood Transfusion Research [LSBR fellowship 1517 to M. van den Biggelaar].

DISCLOSURES

None

DATA AVAILABILITY

The raw MS files and search/identification files obtained with MaxQuant have been deposited in the ProteomeXchange [83] consortium via the PRIDE partner repository [84] (<https://www.ebi.ac.uk/pride/archive/>) with identifier PXD014526.

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SUPPLEMENTAL FIGURES

Figure S1. The cell surface biotin label is mainly present at the apical membrane of ECs. (A) Immunofluorescence maximum intensity projections of BOECs subjected to flow for 0, 24 or 48 hours and labeled with sulfo-NHS-LS-biotin. The scale bar is 20 μm . Cells were stained for biotin (green), VE-cadherin (red) and with HOECHST (blue). (B) Staining as in (A), now depicted with an orthogonal projection on the location of the yellow lines. The scale is 20 μm .

Figure S2. The cell surface proteome corresponds to the proteome and enriches for cell surface proteins. (A) Heatmap representation of biotin sites that were significantly affected in flow conditions. Each row represents a biotinylated site. Biotin sites have been matches to the corresponding median proteome SILAC ratios. (B) Top 20 enriched GO-terms in the cell surface proteome compared to the whole cell proteome and surface proteome combined.

Figure S3. LAMA4 and LAMA5 co-localize in flow conditions. Co-stainings of LAMA4 (green) and LAMA5 (red). Blue is HOECHST. Depicted are maximum intensity projections, scale bars are 10 μm .

Figure S4. Integrin $\beta 1$ distribution does not correspond with integrin $\alpha 6$ localization. Co-staining of ITGA6 (red) with ITGB1 (green). Blue is HOECHST. Depicted are maximum intensity projections. The scale bar is 10 μm .

Figure S5. Proteins displaying similar or synergistic effects upon flow-deprivation and TNF α -stimulation.(A) Proteins displaying similar effects upon flow-deprivation or TNF α exposure. (B) Proteins displaying synergistic or neutralizing effects.

SUPPLEMENTAL TABLES

The following supplemental tables are available upon request.

Table S1. Flow-induced proteomic alterations in ECs.

Table S2. Flow-induced changes in the cell surface proteome, unambiguous localizations.

Table S3. Flow-induced changes in the cell surface proteome, ambiguous localizations.

Table S4. GO-term enrichment analysis of proteins affected by flow.

Table S5. Flow- and TNF α -induced proteomic alterations in ECs.

Table S6. GO-term enrichment analyses of flow- and TNF α -induced changes in the EC proteome.

Table S7. All assigned peptides from the SILAC proteome data (Table S1).

Table S8. All assigned proteins from the SILAC proteome data (Table S1).

Table S9. All assigned peptides from the trypsin-digested biotin pull-down (Table S2-3).

Table S10. All assigned proteins from the trypsin-digested biotin pull-down (Table S2-3).

Table S11. All assigned peptides from the chymotrypsin-digested biotin pull-down (Table S2-3).

Table S12. All assigned proteins from the chymotrypsin-digested biotin pull-down (Table S2-3).

Table S13. All assigned peptides from the label-free protein quantification of cells stimulated with flow, TNF α or both (Table S5).

Table S14. All assigned proteins from the label-free protein quantification of cells stimulated with flow, TNF α or both (Table S5).

CHAPTER 4

PROTEOMIC ANALYSIS OF CYTOKINE- INDUCED MONOCYTE DIFFERENTIATION INTO MACROPHAGES, DENDRITIC CELLS AND OSTEOCLASTS REVEALS UNIQUE AND SHARED PROTEIN SIGNATURES

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Manuscript in preparation

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ABSTRACT

Monocytes can give rise to multiple highly specialized cell types to perform a wide array of functions, ranging from pathogen phagocytosis to bone resorption. This differentiation is induced by the binding of cytokines to dedicated receptors on the surface of monocytes, which results in the initiation of genetic programs that enable cells to perform their specialized functions. Given their common background, it is not surprising that monocyte-derived cells share abilities and cellular markers, yet their specialized functions require a dedicated set of proteins. In order to dissect the monocyte differentiation process and to define cell type-specific marker proteins, we differentiated circulating monocytes into dendritic cells, M1 and M2 macrophages, and osteoclasts, and assessed their proteomes by quantitative mass spectrometry throughout the differentiation process. Statistical analysis indicated that monocyte differentiation is a linear process characterized by a common core of proteins that is similarly affected among the distinct differentiation paths. Throughout the specialization process a cluster of RNA-binding and RNA-processing proteins was downregulated, whereas the abundance of proteins associated with metabolic processes was increased. Analysis of the specialized cells after 10 days of differentiation uncovered existing and putative novel dendritic cell markers. Combined, we here present a comprehensive proteomic analysis of monocyte differentiation uncovering shared and distinct proteomic features of differentiating monocytes and monocyte-derived cells.

INTRODUCTION

Cells from the monocyte hematopoietic lineage fulfill a wide array of functions within the host, ranging from bone remodeling to innate immune responses and to the initiation and orchestration of adaptive immune responses [1,2]. To perform these specialized functions, monocyte-derived cells (i.e. macrophages, dendritic cells (DCs) and osteoclasts) share abilities such as phagocytic and endocytic mechanisms, as well as the ability to present antigens from internalized proteins. These common characteristics also led to the initial classification of these cells within the mononuclear-phagocyte system [3]. To induce the differentiation of monocytes into the various specialized cell types, monocytes express multiple cytokine receptors on their cell surface [4]. Binding of these dedicated receptors to their respective ligands initiates signaling cascades which eventually induce the genetic programs necessary for differentiation. This process takes place during inflammation, when monocytes are locally exposed to cytokines and consequently give rise to macrophages and DCs [5].

Although monocyte-derived cells share their origin, they are highly specialized and occupy unique niches. Macrophages and DCs are widely distributed throughout the body and are both considered professional antigen-presenting cells [6]. However, whereas macrophages are phagocytic pathogen killers, DCs play major role in bridging innate and adaptive responses [1,7,8]. In contrast to these inflammatory effector functions, osteoclasts are known for their unique ability to resorb bone and have been described to modulate hematopoiesis [9].

These functionally distinct cell types can be cultured and differentiated *in vitro* from primary monocytes. As cellular capabilities arise during differentiation and maturation, this allows for the characterization of the development of these cell types. Many of the physiological functions, such as phagocytosis for macrophages [10], or bone resorption for osteoclasts [11], are retained in culture conditions. While there have been several proteomic assessments of monocyte-derived cells [12–15], it has remained unknown how the overall proteome changes during the differentiation process and how this compares between the different cell types.

In the present study, we used label-free quantitative proteomics to gain insight into the transformation that monocyte-derived cells undergo during differentiation. We assessed the proteomes of primary human monocytes and *in vitro* differentiating DCs, M1 and M2 macrophages, and osteoclasts, at five time points. This revealed that the differentiation kinetics for all assessed cell types were similar and uncovered a common core proteome of monocyte-derived cells. In addition, we compared protein profiles with idealized values in order to identify cellular markers. This revealed both existing, as well as previously unidentified proteins that might be used as DC markers. Overall, we here present a comprehensive proteomic map of monocyte differentiation revealing common and unique features of monocyte-derived cells.

MATERIALS AND METHODS

Monocyte isolation and differentiation

All blood samples were obtained and used in accordance with Dutch regulations and with approval from the Sanquin Ethical Advisory Board in accordance with the Declaration of Helsinki. Written informed consent was given by all participants. In order to map similarities and differences between differentiating monocyte-derived cells, we isolated CD14⁺ peripheral blood mononuclear cells (PBMCs) from separate buffy coats of 3 healthy volunteers as described previously [16]. Briefly, blood diluted 1:1 with PBS (Fresenius Kabi) was loaded onto a Ficoll-paque layer (Merck) and centrifuged for 20 min at 400 x g. Ring fractions were collected and CD14⁺ cells were isolated using MACS anti-CD14 microbeads (Miltenyi Biotec) according to manufacturer's instructions. In addition, we isolated PBMCs from pooled buffy coats of 40 healthy volunteers to account for individual variance. Therefore, monocytes from 40 pooled buffy coats were pre-isolated using counterflow centrifugal elutriation as described [17], after which CD14⁺ cells were isolated using MACS anti-CD14 microbeads. Per condition 2.5×10^6 cells were seeded into a 6 wells plate (Thermo Fisher Scientific). Cells were differentiated into dendritic cells (DCs) using 100 U/ml GM-CSF (CellGenix) and 80 U/ml IL-4 (CellGenix) in Cellgro medium (Fisher Scientific), M1 macrophages using 100 U/ml GM-CSF (CellGenix) in RPMI 1640 supplemented with HEPES (Lonza) supplemented with 10% FBS (Bodinco), M2 macrophages using 50 ng/ml M-CSF (Peprotech) in RPMI 1640 supplemented with HEPES (Lonza) supplemented with 10% FBS (Bodinco), or osteoclasts (OS) using 25 ng/ml M-CSF in alpha MEM (Gibco) supplemented with 10% FBS (Bodinco). Osteoclast medium was replaced at day 3 and 7 with fresh alpha MEM containing 25 ng/ml M-CSF and 100 ng/ml RANKL (Peprotech). All media were supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin. Macrophages and osteoclasts were cultures on a glass coverslip, DCs were grown in non-tissue culture treated wells. Samples of undifferentiated monocytes and differentiating cells at days 2, 4, 6, 8 and 10 were collected and processed for proteomic analysis. Cells were visually inspected for viability and osteoclast multinucleated cells.

Mass spectrometry sample preparation and analysis

Cells were washed twice with PBS, non-adherent cells were collected by centrifugation at 300x g for 5 min. Adherent and non-adherent cells were lysed together in 50 µl 4% SDS, 100 mM DTT, 100 mM Tris, pH 7.5, supplemented with 1x HALT protease and phosphatase inhibitor (Thermo Scientific). Lysates were subjected to filter-aided sample preparation (FASP) [18] followed by desalting using C18 StageTips [19]. Mass spectrometry analysis was performed as described previously [20]. In brief, tryptic digests were separated by nanoscale C18 reverse-phase chromatography coupled on-line to an orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific) via a nanoelectrospray ion source (Nanospray Flex Ion Source, Thermo Scientific). All data were acquired using Xcalibur software.

Proteome data processing and analysis

Raw files were analyzed with the Maxquant (1.5.3.30) computational platform [21]. Proteins and peptides were identified using the Andromeda search engine by querying the human Uniprot database (release 3-2017, 70947 entries) using standard settings with the following adjustments. Protein quantification was based on unique peptides, and the 'match between runs' option was enabled. Output tables were loaded into RStudio 1.1.456/R 3.5.3. Contaminants and reverse values were filtered out. Proteins were considered quantified based on the presence of 3 of 4 valid values in at least one condition. Missing values were imputed by normal distribution (width = 0.3 sd and down shift = 1.8 sd of the non-missing data), assuming these proteins were close to the detection limit [22]. Statistical analysis was performed with moderated t-tests using the limma package [23], a $P < 0.05$ and an absolute $\log_2$ fold change > 1 were considered significant and relevant. Clustering was performed using K-means with an optimal number of clusters (K) determined by gap statistics [24]. GO analyses were performed with clusterProfiler [25]. Heatmaps were generated with ComplexHeatmap [26]. Manual database annotations were based on the Human protein atlas version 18.1 [27], which includes Cluster of differentiation, GPCR, FDA approved targets, Transcription factors, and Ribosomal proteins, Granule proteins [28], Meta Adhesome [29], Primary immunodeficiency genes [30], RNA binding proteins [31], RECON1 [32], and MitoCarta 2.0 [33]. Enrichment was tested with fisher exact tests followed by Benjamini-Hochberg multiple testing correction. Correlation between sample data and cell type specific idealized theoretical values at the final sample stage was performed using Pearson correlation. Proteins with correlation coefficients > 0.8 for a cell type were considered specific and z-scored values of these were visualized in a PCA plot. The MS raw files and MaxQuant search/identification files are available via ProteomeXchange [34] with identifier PXD016245.

RESULTS

High resolution quantitative proteomics of monocyte-derived cell differentiation

To determine proteome dynamics during the differentiation from monocytes towards macrophages (M1 and M2), dendritic cells and osteoclasts, monocytes were obtained from blood of healthy donors, or from a mixed buffy coat of 40 healthy donors. In literature, many different protocols have been used to induce the differentiation of monocytes, however, most of these rely on the same specific cytokines: GM-CSF and IL-4 for DCs, GM-CSF for M1 macrophages, M-CSF for M2 macrophages and osteoclasts using M-CSF and RANKL [16,35–37]. For our differentiation study, these cytokines were used and samples were collected on day 2, 4, 6, 8, and 10 and subjected to mass spectrometry analysis. For differentiation towards osteoclasts, monocytes were initially only stimulated with M-CSF. From day 3, the medium of these cells was replaced by medium containing M-CSF and RANKL [38,39]. Undifferentiated monocytes were used as day 0 samples (Figure 1A). In total, we identified 6029 proteins, of which 4409 were quantified in at least 3 samples in at least one of the subsets (Figure 1B, Table S1). To validate the differentiation of monocytes into the distinct specialized subtypes, we assessed the protein levels of typical markers for monocytes (CD14), DCs (CD209 and HLA-1A), and osteoclasts (ITGB3 and CTSK [40,41]) (Figure 1C). The M1 and M2 macrophage subtypes are more difficult to distinguish using traditional markers, as many protein markers, including MRC1 for M1 macrophages (Figure 1C), are shared with other monocyte-derived cells, or were not detected (CD80, CD163). Other typical features, such as secreted cytokines, including CCL2/8/15/19/20, IL1 β /6/8/12/15/23, and CXCL9/10/11/13 [42,43], or ARG1 and NOS2 [44] could not be detected. A clear morphological distinction was observed as M1 macrophages appeared round, whereas M2 macrophages frequently displayed a “fried egg”-shape (Figure S1). Pearson correlation coefficients of the label-free quantification (LFQ) intensity values were calculated for the different subsets and time points (Figure 1D). This indicated a high overall correlation of the proteomes between the different subsets with lower correlation coefficients between monocytes and subsequent stages, as well as between DCs and other subsets. Principal component analysis separated the differentiation stages of all subsets from the monocyte samples (Figure 1E). Time-dependent differentiation effects were mainly captured by PC1. DCs clustered furthest from all other cell types, followed by M1 macrophages indicating that the proteomes of these cell types were more distinct from other subsets. The overlap of M2 macrophages and osteoclasts indicated that these differentiation subtypes were highly similar.

Cell differentiation kinetics are similar between monocyte-derived cells

To further assess the proteomic changes during monocyte differentiation, we performed moderated t-tests on the 4409 quantified proteins. This analysis showed that, for each subset, a remarkably large portion of proteins (35-44%) was altered during

Figure 1. Temporal profiling of monocyte-differentiation towards Dendritic cells, M1 and M2 macrophages and Osteoclasts. (A) Schematic representation of the experimental setup. Primary human monocytes were isolated and differentiated towards M1 and M2 macrophages, dendritic cells and osteoclasts. Samples for proteomics analysis were taken on days 2, 4, 6, 8 and 10. Undifferentiated monocytes were used as day 0. (B) Number of identified and quantified proteins per sample. The dashed line indicates the total amount of quantified proteins. (C) Protein levels of selected hallmark proteins during monocyte differentiation. (D) Heatmap of Pearson correlation coefficients of monocytes and the developmental stages of monocyte-derived cells. The color scale represents Pearson correlation coefficients. (E) Principal component analysis of differentiation of dendritic cells (DC, squares), M1 macrophages (M1, diamonds), M2 macrophages (M2, triangles), monocytes (Mono, circles) and osteoclasts (Osteo, inverse triangles). Color scale represents time in days.

cellular differentiation (Figure 2A). To assess the development of monocyte differentiation throughout the differentiation process, we next determined the cumulative global change, i.e. the ratio of significant proteins at a sampled time point over the total amount of significant proteins for each of the subsets. This indicated that, specifically during the first 6 days, monocyte differentiation is a gradual process, which develops in a similar manner for the distinct cell types (Figure 2B). To assess whether proteins display a gradual or step-wise changes throughout the differentiation process, we determined at which differentiation stage reached their maximum or minimum values (Figure 2C). Although there were proteins that peaked at early time points, the largest portion of affected proteins had a maximal/minimal value at the final day of sampling, indicating that most proteins displayed a gradual profile. To further visualize these proteomic dynamics throughout the differentiation process, we clustered the protein profiles based on gap statistics (Figure 2D-E). Most proteins showed

Figure 2. Distinct monocyte-derived cell types display similar differentiation kinetics. (A) Bar graph showing the amount of proteins displaying significantly changed abundances during the 10 days of differentiation. (B) Line plot of the cumulative global change of differentiation: the ratio of significant proteins at a given time point over the total number of significant proteins for a specific cell type. (C) Pie graphs showing the proportions at which sampling day a maximum or minimum protein level was reached. (D) Heatmap representation of the proteins that changed throughout monocyte differentiation clustered by gap statistics. The color scale represents z-scored LFQ values. DC: Dendritic cells, M1: M1 macrophages, M2: M2 macrophages, Osteo: Osteoclasts. (E) Protein profiles of the clusters visualized in panel D.

consistently increasing or decreasing levels, or an increase or decrease to a plateau level (Figure 2E).

Core proteome of monocyte-derived cells

Next, we set out to determine the core proteome of differentiating monocytes using two approaches. First, we selected proteins that displayed similarly increased or decreased abundances at all sampled time points for all subsets. This yielded a selection of 133 proteins separated in 3 clusters (Figure 3A). Two of these described enhanced proteins levels (cluster 1 and 3), and included tetraspanins CD9 and CD81, which modulate FcεR-mediated signaling on antigen presenting cells [45], as well as ICAM1 and ITGB2, that mediate interactions between immune cells. These clusters were enriched for proteins involved in inflammatory and apoptotic regulation GO terms (Figure 3B and Table S2). Cluster 2 contained proteins with decreased abundances during differentiation, including DNA replication-associated proteins and cohesin subunits STAG2 and SMC1A [46], that are crucial for mitosis. In parallel, we focused on proteins that were similarly increased (342) or decreased (446) in abundance at the final sampled time point (day 10) (Figure 3C). The strongest decrease was observed for

Figure 3. Monocyte-differentiation is characterized by a core of proteins that changes regardless of specialization type. (A) Line plots of 133 proteins that displayed similar profiles throughout monocyte-differentiation. K-means clustering defined 3 dominant kinetics. Black lines and colored dots represent median protein values within a cluster for each cell type, grey lines represent protein LFQ intensity values. (B) Bar graph showing the top most significantly enriched gene ontology (GO)-terms for the clusters in panel A. (C) Dot plot of proteins that were similarly up (342) or down (446) in abundance at the final sampled time point (day 10). Colored dots represent median protein values within a cluster for each cell type, grey dots represent protein LFQ intensity values. (D) Bar graph of top 3 most significantly enriched GO terms for the clusters in panel C. (E) Enrichment analysis of the in- and decreased proteins from panel C in manually curated databases. From top to bottom: RECON1 (metabolic reconstruction), RNA binding proteins, Granules, Primary immunodeficiency genes, Clusters of differentiation, Transcription factors, MitoCarta 2.0 (mitochondria), Meta Adhesome, FDA approved targets, G-protein-coupled receptors, and ribosomal proteins.

MPO, which is primarily known for its role in neutrophils, but is also present in monocytes [47]. The highest increase was seen for MRC1 (CD206), which has been described as a marker for macrophages and monocyte-derived DCs [48,49]. GO term enrichment on these endpoint proteins showed strong enrichment of general terms, i.e. ‘chromosome’ for decreased proteins and ‘lipid metabolic process’ for proteins with increased abundances (Figure 3D and Table S3). Due to generic nature of these GO terms, we also performed enrichment analysis based on manually curated databases of proteins function (Human protein atlas version 18.1 [27], which includes Cluster of differentiation, GPCR, FDA approved targets, Transcription factors, and Ribosomal proteins; Granule proteins [28], Meta Adhesome [29], Primary immunodeficiency genes [30], RNA binding proteins [31], RECON1 [32], MitoCarta 2.0 [33]) (Figure 3E). We observed a highly significant enrichment of proteins with enhanced abundances for the RECON1 database, which contains metabolic proteins. The strongest enrichment for decreased proteins was observed for RNA binding protein and granule protein databases. Together, these data showed that proteins that are shared at the end stage of differentiation reflect general differentiation processes (e.g. metabolism) whereas a core set of proteins that follow the same dynamics during entire differentiation process contained proteins associated with immune functions.

Proteomic characterization of cellular identity

In order to classify cell identity-specific protein markers using our proteomics dataset, we assessed the proteomic profiles of end stage differentiated DCs, M1- and M2 macrophages and osteoclasts. Principal component analysis of all proteins present at the final stage of differentiation revealed that between subsets, M2 macrophages and osteoclasts differed the least and that DC proteomes were most distinct (Figure 4A). Next, we selected proteins that were statistically significant between monocytes and the differentiated end stages (Figure 4B), and compared these with theoretical marker profiles (Figure S3A) using Pearson correlations. We used a correlation coefficient threshold cutoff of >0.8 to define cell type specific proteins (Figure S3B and C). For M2 macrophages, there were no proteins that fulfilled our specificity criteria, whereas for DCs, M1 and osteoclasts, 36, 3 and 7 proteins were cell type specific, respectively. These 46 proteins were visualized in a PCA plot based on z-scored values (Figure 4C). A clear partition was present between DCs, M1 macrophages and osteoclasts. To assess the validity of this approach we selected previously established markers for DCs, macrophages and osteoclasts and plotted their kinetics during cell subset differentiation (Figure 4D). This showed that DC marker CD209 was present in an early stage of monocyte differentiation, as well as CCL24 in M1 macrophage-developing cells. For osteoclasts, CTSK levels increased and CCL24 decreased after addition of RANKL in the culture medium at day 3. In addition to well-established DC markers (e.g. CD1B, CD1C, CD209, CD86, ALOX15), we identified putative novel markers for DCs. After annotating Uniprot keywords to the 46 specific proteins, we selected correlating DC proteins with ‘Cell membrane’ annotations, which are not known to be established DC markers, and contain

Figure 4: Proteomics assessment of cellular identity. (A) Principal component analysis of final sampled time-point (10 days) of dendritic cells (DC, red squares), M1 macrophages (M1, green diamonds), M2 macrophages (M2, blue triangle) and osteoclasts (Osteo, purple inverse triangles). (B) Heatmap representation of proteins with statistically significantly higher or lower abundances at day 10 compared to monocytes. The color scale represents z-scored LFQ intensity values. (C) Principal component analysis of z-scores of proteins that matched an ideal marker profile (figure S2A) with a pearson correlation of >0.8. Dotted lines represent manually defined partitions. (D) Profile plots showing LFQ intensity values of established markers for dendritic cells (CD209, green ribbons), M1 macrophages (CCL24, red ribbons) and osteoclasts (CTSK, blue ribbons). (E) Profile plots showing LFQ intensity values of putative dendritic cells markers (CCL18, red ribbons; CCL22, green ribbons; FCER2, beige ribbons).

extracellular domains (Figure 4E and Table S4). These included FCER2, ANPEP, and PTPRE, of which PTPRE and ANPEP display a minor difference between DCs and the other cell types. In contrast, the expression of FCER2 is mostly limited to DCs, with considerably lower intensities in M1 cells (Figure 4E). In addition, the levels of secreted proteins CCL22, and to a minor extent CCL18 (Figure 4E), may present biomarkers for DC identity. These data demonstrate that idealized protein values can be used to select for cellular markers.

DISCUSSION

Circulating monocytes can give rise to dendritic cells, macrophages and osteoclasts. The development of monocytes into either of these specialized cell types is well-studied, yet numerous differentiation protocols have been used to obtain monocyte-derived cells [12,15,50–52]. In the present study we performed a temporal analysis of monocyte differentiation using common differentiation protocols employing IL-4 and GM-CSF (DCs), GM-CSF (M1 macrophages), M-CSF (M2 macrophages), and M-CSF and RANKL (Osteoclasts). We assessed the proteomic dynamics of the monocyte differentiation using label-free quantitative mass spectrometry. Our data provide insight into common and unique features of these monocyte-derived cells. Although proteomic analyses have been performed on differentiating monocytes [12–15], a comprehensive approach comparing the differentiation of DCs, M1 macrophages, M2 macrophages and osteoclasts has not been reported so far.

Proteins that were similarly affected during monocyte differentiation into the four specializations showed a strong inflammatory signature. While this would be expected from DCs and macrophages, there does not seem to be a direct role for osteoclasts in host immune responses. It has, however, been described that osteogenesis and bone resorption are induced in inflammatory conditions [53]. Our observations may therefore reflect proteins that do not mediate the inflammatory process, but are responsive to inflammatory cues nonetheless.

The monocyte-derived DC proteome was most distinct from the other assessed cell types. This was unexpected given the great differences in appearance between osteoclasts and the other cell subsets. In contrast to their morphological differences, the proteomes of osteoclasts and M2 macrophage proteomes were remarkably similar. Likewise, Osteoclasts resembled M1 macrophages to a high degree. This argues that the proteomes reflect the distinct function of these cell types rather than their contrasting morphology.

In order to define a specific marker set to distinguish these cell types, we evaluated proteins whose levels were specifically increased in only one of the cell types. Due to the stringency of our cutoff no specific proteins could be found for the M2 macrophages. M1 macrophages retained high levels of ALOX5AP, and produced CCL24 and GLRX [5,54]. The dynamics of CCL24 are suggestive of an initial common differentiation route of Osteoclasts and M1 and M2 macrophages: after a few days of culturing their differentiation paths diverge. Surprisingly, CCL24 is identified as a marker for M1 macrophages in our data, whereas this chemokine is generally considered to be associated with M2 macrophages [55,56]. Although in our hands CCL24 levels were initially high in M2 macrophages, from day 8 onwards there was as strong decline. Although *in vivo* macrophages have been suggested to form a spectrum rather than the M1 versus M2 polarization model [57] and there is controversy in the literature on the M1/M2 model of macrophages dichotomy [58,59], it remains unclear what lies as the basis of this observation. Osteoclasts had a more pronounced signature, which included typical markers such as CKB [60] and CTSK [40]. In addition, SLC9B2, also known as NHA2, was previously shown to be selectively expressed

in osteoclasts [61], and high protein levels of AK5 and PLS3 have also been observed in this cell type [38]. We further identified osteoclast-specific proteins which indirectly link to the osteoclast function: OXR1 interacts with V-ATPases, which regulate bone remodeling [62,63] and FLNB mutations have been found in human skeletal disorders and its knock-out in mice resulted in impaired skeletal development [64]. As expected, based on the proteomic distinctness discussed above, the highest amount of specific proteins were found for DCs. These include well-known DC proteins such as ALOX15 [65,66], CD1B, CD1C, and DC-SIGN (CD209). In addition, in line with literature [67], the co-stimulatory molecule CD86, as well as the metalloprotease MMP12 were most abundant in DCs.

In addition to studying the dynamics of monocyte development, we used our dataset to define additional cell-specific markers. Based on its DC specificity, cell surface localization and differentiation dynamics, we selected FCER2 as a putative DC marker. This low affinity receptor for IgE molecules is also known as CD23 and exists as a membrane-bound, as well as soluble form after proteolytic processing [68]. Multiple cell types, including B and T lymphocytes, intestinal epithelial cells and bone marrow stromal cells have been shown to express this receptor [68]. Its use as a biomarker is therefore limited to the combined usage with other DC-specific proteins. In addition to FCER2, our data indicated that the expression of 2 cytokines, CCL18 and CCL22, was also highly selective for DCs. CCL18 is expressed by immature DCs at high levels, whereas DC maturation induced by LPS, TNF α or CD40L strongly suppresses this production [69]. In agreement with our data, CCL22 expression has been observed in macrophages [70], yet DCs express this cytokine much more potently. FCER2, CCL18 and CCL22 may serve as future DC markers, although further investigation using primary human material may provide consensus on the practical applicability of these putative markers.

Although DCs, macrophages and osteoclasts are terminally differentiated, a considerable plasticity is still present in these cells. For instance, upon transplantation of tissue-resident macrophages from one organ to another, these cells adopt the organ-specific transcriptional profile of their new location [71]. Furthermore, it has been demonstrated that murine splenic DCs can transdifferentiate into osteoclasts if presented with a bone marrow microenvironment [72,73]. This shows that the cellular identity of terminally differentiated cell remains dependent on environmental cues and it implies that vastly different phenotypes can be achieved with a limited set of determining genes/proteins. This is in line with our observation that a substantial part of the proteomic changes detected during monocyte-differentiation is shared between DCs, M1- and M2-macrophages and osteoclasts. In order to identify the proteins that enable this phenotype switching, the core proteome of differentiating monocytes should be compared to other distant cell types which can give rise to multiple specialized cells.

Taken together, our study provides a comprehensive proteomic comparative analysis that provides insight into the development of monocytes into macrophages, dendritic cells and osteoclasts. This study highlights a common core of proteins that changes during

monocyte development and emphasizes the similarity of M1 and M2 macrophages and osteoclasts, and the distinct proteome of DCs. Furthermore, our data revealed a distinct set of proteins that are specifically induced in the development of dendritic cells, and may serve as DC markers. The applied approach to identify cell-specific markers is not limited to our current dataset, but can be employed in any omics dataset describing distinct cell types to identify unique markers and possibly therapeutic targets. Combined, our data shed light on the similarities and differences in the development of monocyte-derived DCs, macrophages and osteoclasts, and identify FCER2, CCL18 and CCL22 as putative DC markers.

ACKNOWLEDGMENTS

The work presented here was supported by the Sanquin Blood Supply Foundation [PPOP 13-002] and Landsteiner Foundation for Blood Transfusion Research [LSBR-1517 to M.v.d.B.]. The authors thank Floris van Alphen, Mark Hoogenboezem, Erik Mul and Simon Tol for their technical assistance during elutriation cell separation.

AUTHOR CONTRIBUTIONS

K.M., M.v.d.B., A.B.M., and A.J.H. designed the study and supervised research. E.P.B., M.J.M. and L.C.N. performed experiments. A.J.H. and E.P.B. analyzed data, E.P.B., A.B.M. and A.J.H. wrote the manuscript. All authors read and approved the final version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests

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SUPPLEMENTAL FIGURES

Figure S1. Cell morphology after 10 days of differentiation. Micrographs of monocyte-derived DCs, M1 and M2 macrophages and osteoclasts.

Figure S2. Theoretical marker correlation. (A) Bar graphs showing profiles of theoretical marker dynamics for dendritic cells (DC), M1 macrophages (M1), M2 macrophages (M2) and osteoclasts (Osteo). (B) Dot plots showing ranked Pearson correlation coefficients. Dotted line represents a cutoff threshold of 0.8. (C) zoom of panel B.

SUPPLEMENTAL TABLES

The following supplemental tables are available upon request.

Table S1. Identified proteins in differentiating monocytes.

All proteins identified in either of the differentiating monocyte samples. Listed are $\log_2$ transformed label-free quantification values of each of the separate proteome analyses. Proteins that are significant in either of the analyses were assigned to a cluster. NaN = not a number (not quantified).

Table S2. Gene ontology enrichment analysis of core common monocytic proteome.

Gene ontology enrichment analysis of statistically differentially abundant proteins during differentiation. Cluster annotation, ontology, GO term ID and description are shown. Gene IDs are based on ENTREZ identifiers.

Table S3: Gene ontology enrichment analysis of end-stage common monocytic proteome.

Gene ontology enrichment analysis of statistically differentially abundant proteins at the final samples stage of differentiation. Cluster annotation, ontology, GO term ID and description are shown. Gene IDs are based on ENTREZ identifiers.

Table S4: List of DC specific proteins.

Statistically differentially abundant proteins ranked according to Uniprot membrane annotation and Pearson correlation with theoretical DC marker dynamics. Gene names, Uniprot identifiers, membrane annotation and Pearson correlation coefficients are listed.

CHAPTER 5

ENDOCYTOSIS BY MACROPHAGES: INTERPLAY OF MACROPHAGE SCAVENGER RECEPTOR-1 AND LDL RECEPTOR-RELATED PROTEIN-1

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Adapted from: *Haematologica*. 105 (2020) e133-e137

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ABSTRACT

Multiple receptors may be involved in the uptake of a singular ligand from the circulation. For Von Willebrand Factor (VWF), these include LDL receptor-related protein-1 (LRP-1), Macrophage scavenger receptor-1 (MSR-1, SR-AI or CD204), Macrophage Galactose-type lectin (MGL), Siglec-5 and the Asialoglycoprotein receptor (ASGPR). We have previously shown that human monocyte-derived macrophages (MDMs) internalize VWF in a flow-dependent manner. In the present study, we aimed to gain insight into the interplay of multiple receptors to the cellular internalization of a single ligand like VWF. Cell surface proteomics identified LRP-1, MSR-1 and one peptide originating from SIGLEC5 or -14 on MDMs. Subsequent label-free quantification indicated that, of these receptors, LRP-1 and MSR-1 were most abundant. To assess the relative contribution of these receptors on VWF uptake, cellular uptake experiments were performed using VWF-fragments D'D3A1 and D'D3, which contain binding sites for LRP-1 and MSR-1. Immunofluorescence microscopy analysis and flow cytometry studies revealed that both fragments were internalized by MDMs in a flow-independent manner. In contrast, U87MG cells, that express LRP-1 but not MSR-1, only internalized D'D3A1. This is in agreement with the observation that LRP-1 contains a binding site for the A1 domain. In line with these data, blocking MSR-1 on MDMs inhibited the uptake of D'D3, but not D'D3A1. Surprisingly, blocking LRP-1 in MDMs inhibited the uptake of both fragments. In addition, we showed that MSR-1 associated directly with LRP-1, and that D'D3 internalization was induced in U87MG cells upon supplying soluble MSR-1. Combined, these data suggest that both MSR-1 and LRP-1 contribute to VWF uptake by MDMs, and that MSR-1-mediated uptake is facilitated by LRP-1.

INTRODUCTION

The concentration of plasma proteins spans more than 10 orders of magnitude. Yet the concentration of each of the individual proteins is tightly controlled within a functional range. This concentration is dependent on protein expression and release, as well as clearance from plasma. Hemostatic protein Von Willebrand Factor (VWF) is produced and stored in endothelial cells and megakaryocytes [1,2]. This glycoprotein is critical during primary hemostasis in the recruitment of platelets at sites of vascular damage, and shields coagulation factor VIII from rapid clearance [3]. The cellular mechanisms that contribute to the clearance of VWF from the circulation are poorly understood. Insight into these mechanisms is critical to increase our understanding of not only the role of VWF in primary hemostasis but also in bleeding disorders that are associated with reduced plasma levels of VWF [4,5].

Multiple cell types and receptors have been postulated to contribute to VWF clearance [6–8]. Studies in mice revealed that the liver and spleen are the most active sites for VWF clearance [9,10]. Specifically the macrophages in these organs have been reported to contribute considerably, as they were found to internalize high levels of VWF [9,11,12]. The finding that chemical inactivation of macrophages in the liver of mice drastically increased VWF levels also indicates a role for macrophages in VWF clearance [9,12,13]. This hypothesis is further supported by *in vitro* experiments which confirmed that macrophages can bind and internalize VWF [14–16]. The numerous receptors that have been implicated to contribute to the clearance of VWF include LDL receptor-related protein-1 (LRP-1) [14,17,18], Macrophage scavenger receptor-1 (MSR-1, SR-AI or CD204) [15], Macrophage Galactose-type lectin (MGL) [16], Siglec-5 [19], Stabilin-2 (STAB2) [20], C-Type Lectin Domain Family 4 Member M (CLEC4M) [21], and the Asialoglycoprotein receptor (ASGPR) [12,22]. However, the relative contribution of these receptors to the uptake of VWF by macrophages is completely unclear.

Previously, we have shown that human monocyte-derived macrophages (MDMs) can effectively internalize VWF via LRP-1 [14]. However, this internalization only takes place in conditions of flow-induced shear stress. This finding is compatible with the observation by others that full-length VWF only binds LRP-1 under shear stress conditions [17]. This shear force may induce a configurational change in VWF from a globular shape to an elongated shape, thereby exposing binding sites for clearance receptors.

In the present study, we employed cell surface proteomics on MDMs to evaluate the expression of putative VWF clearance receptors on these cells, and identified LRP-1 and MSR-1 on these cells. To study their relative contribution to VWF internalization in a flow-independent manner, we used N-terminal VWF fragments comprising the D'D3 and D'D3A1 domains. These fragments contain binding sites for LRP-1 in the A1 domain [18], and MSR-1 in the D'D3 and A1 regions [23], respectively. We applied functional assays on U87MG cells, that express LRP-1 but not MSR-1, and MDMs to unravel the role of MSR-1 and LRP-1 in the internalization of VWF fragments. Combined, our data imply that both MSR-1

and LRP-1 are involved in the uptake of VWF by MDMs, and that MSR-1-mediated VWF internalization is facilitated by LRP-1.

MATERIALS AND METHODS

Expression and purification of recombinant proteins

HPC4 mouse hybridoma (HB9892) was obtained from ATCC and cultured in IMDM supplemented with 10% heat-inactivated fetal calf serum (FCS). HPC4 antibody was purified with protein G sepharose according to the manufacturer's protocol. VWF fragments D'D3 and D'D3A1 were designed in pcDNA3.1(+) with two point mutations at position C1099S and C1142S in order to prevent dimerization as described [24]. An HPC4 tag was fused to the C-terminus of both fragments for purification and detection purposes. Sequences were codon optimized for expression in human cells. Coding regions were verified by sequence analysis using the BigDye Terminator Sequencing kit (Applied Biosystem). Recombinant proteins were transiently expressed in HEK293 Freestyle cells using Polyethylenimine (PEI) transfection. Cells were grown in Freestyle Expression medium (Thermo Fisher Scientific). Five days after transfection, proteins were purified from medium by immunoaffinity chromatography using CNBr-Sepharose 4B coupled with an anti-HPC4 antibody purified from mouse hybridoma HB9892 cells. Fragments were loaded on the anti-HPC4 column in 20 mM Tris-HCl (pH 7.4), 100 mM NaCl, 10 mM CaCl₂. The column was washed with 20 mM Tris-HCl (pH 7.4), 1 M NaCl, 10 mM CaCl₂. Proteins were eluted with 20 mM Tris-HCl (pH 7.4), 100 mM NaCl, 5 mM EDTA. Protein-containing fractions were dialyzed against 50 mM HEPES (pH 7.4), 150 mM NaCl, 10 mM CaCl₂, 50% (v/v) glycerol and stored at -20 °C. Protein concentrations were assessed using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Glutathione-S-transferase – Receptor Associated Protein (gst-RAP) and LRP1 Cluster-II expression and purification was performed as described previously [25,26].

Culture of MDMs and U87MG cells

Blood samples of healthy volunteers were used in accordance with Dutch regulations and after approval from the Sanquin Ethical Advisory Board in accordance with the Declaration of Helsinki. Written informed consent was given by all participants. Human monocytes were isolated from blood using MACS CD14-microbeads (Miltenyi Biotec) and were differentiated to macrophages using 50 ng/ml hrM-CSF (PeproTech) in RPMI-1640 (Lonza), supplemented with 10% FCS (Bodinco), 100 U/ml penicillin, and 100 µg/ml streptomycin as described previously [14]. U87MG cells were obtained from ATCC (HTB-14) and cultured in phenol red-free DMEM-F12 with HEPES and L-glutamine (Thermo Fisher Scientific) supplemented with 10% FCS. U87MG cells were maintained in culture flasks coated with collagen type I (50 µg/ml, BD Biosciences).

Uptake of VWF fragments

Cells were washed with HEPES buffer (10 mM HEPES (pH 7.4), 135 mM NaCl, 10 mM KCl, 5 mM CaCl₂, 2 mM MgSO₄) and incubated with VWF fragments for 30 minutes at 37 °C in the absence or presence of a 5x molar excess of RAP, LRP-cluster II or mouse monoclonal

anti-human SR-A1/MSR (clone #351620, R&D systems). After incubation, cells were washed and fixed in 4% paraformaldehyde (Electron microscopy sciences) in PBS for 15 minutes.

Antibodies for immunofluorescent labeling

Primary antibodies used for labeling were: mouse monoclonal EEA-1 IgG1 (BD biosciences), mouse monoclonal anti-HPC4 IgG1 (see above, purified from mouse hybridoma HB9892), rabbit polyclonal HPC4 IgG (Cell Signaling technology) and mouse monoclonal IgG2b anti-MSR-1 (Clone #351615, R&D Systems). Secondary antibodies were: goat anti-mouse Alexa Fluor 488, goat anti-mouse Alexa Fluor 568 and F(ab')₂-Goat anti-Rabbit IgG Alexa Fluor 488 (all from Thermo Fisher Scientific).

Immunofluorescence labeling and confocal imaging

After fixation, cells were washed with TBS followed by blocking and permeabilization with staining buffer (1% BSA, 0.1% saponin, 5 mM CaCl₂ in TBS). Cells were sequentially incubated with primary and secondary antibodies diluted in staining buffer and counter stained with Hoechst 33342 and HCS CellMask deep red (Thermo Fisher Scientific) for quantification purposes. Samples were imaged on a Leica SP8 confocal laser scanning microscope (Leica microsystems) equipped with a HC PL APO CS2 63x/1.40 oil immersion objective. Tile scans were collected for quantification and analyzed using ImageJ (version 1.52d, Wayne Rasband; National Institutes of Health) and Graphpad prism 7 (GraphPad Prism Software Inc.) software.

Flow cytometry

Adherent cells were detached using a citric saline solution of 1% (w/v) KCl and 0.25% (w/v) Sodium citrate. After restoring the osmotic balance with the HEPES buffer described above, cells were placed in a V-bottom 96 wells plate using 2×10^5 cells per condition. Cells were incubated with VWF fragments for 30 minutes at 37 °C, washed and fixed as described above. To stain for the VWF fragments directly, we conjugated anti-HPC4 antibodies to Pacific blue using a Pacific Blue protein labeling kit (Thermo Fisher Scientific). We used the same staining protocol as described for immunofluorescence labeling. After incubating cells with antibodies, cells were washed twice with staining buffer and placed in TBS supplemented with 2.5 mM CaCl₂ for analysis on a BD FACSCanto or BD FACSCanto II (BD biosciences). Data was analyzed using FlowJo 10.4 (FlowJo, LLC) and Graphpad prism 7 (GraphPad Prism Software Inc.).

Cell surface labeling and mass spectrometry

10 cm Ø petri dishes of macrophages or U87MG cells were washed 3x with HEPES buffer and incubated for 30 min with 2 ml/dish 3 mM EZ-link Sulfo-NHS-LC-Biotin (Thermo Scientific) at 4 °C. Excess label was quenched by washing 4x with ice-cold HEPES buffer with

100 mM glycine, and cells were lysed at RT with 100 μ l 4% SDS, 100 mM Tris-HCl, 0.1 M DTT, 1x HALT protease inhibitor (Thermo Scientific). Lysates were processed using the FASP method [27]. 50 μ g of the tryptic digests was subjected to strong-anion exchange using Empore Anion and Cation Exchange-SR Extraction disks (3M) as described [27], with elution buffer pHs of 11, 8, 6, 5, 4 and 3. The flow-through of fractions 8 and 11 were collected, acidified to pH <2.5 using trifluoroacetic acid and subjected to C18 desalting (named flow-through). Fractions were desalted using C18 StageTips [28]. The remainder of the tryptic digests was subjected to biotin pull-down by incubating peptides for 2 hours in 50 mM NH_4HCO_3 , 150 mM NaCl pH 8.3 in 3 wells/sample of a SigmaScreen Streptavidin High Capacity Coated plates. Another round of pull-down was performed with the flow-through. Captured peptides were diluted using 70% acetonitrile, 5% formic acid. Samples were vacuum-dried to remove the acetonitrile and desalted in C18 StageTips.

Mass Spectrometry analysis and data processing

Peptides were separated on nanoscale C18 reverse chromatography coupled on line to an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific) via a nanospray ion source (Nanospray Flex Ion Source, Thermo Scientific) as described previously [29]. For the fractionated proteome the elution gradient was adjusted to 5-25% buffer B (pH 11), 5-30% buffer B (pH 5, 6, 8, flow-through) and 5-35% buffer B (pH 3, 4). For the biotin pull-down samples, the MS acquisition settings were adjusted to perform the MS² analysis in the orbitrap analyzer, as has been described before for phosphopeptides [29]. All data were acquired with Xcalibur software. To identify proteins and peptides, raw files were analyzed with the MaxQuant (1.5.3.30) computational platform [30], using the Andromeda search engine by querying the human Uniprot database (release 3-2017, 70947 entries) using standard settings with the following adjustments. Protein quantification was based on unique peptides, the 'match between runs' option was enabled. In the cell surface samples, a variable modification comprising the biotin added mass (339.16166 Da), was added. Perseus 1.5.6.0 was used to estimate protein copy numbers with the proteomic ruler plugin [31]. These data, as well as the cell surface data, were loaded in Rstudio 1.1.383 (R version 3.4.2) [32]. Reverse values, potential contaminants and 'only identified by site' values were filtered out, as well as peptides without at least 2 valid values in 1 of the groups. Peptide numbers per protein were counted for the surface data, and these were coupled to the copy number estimates obtained from the proteome data. ComplexHeatmap 1.14.0 [33] was used to generate heatmaps. The raw MS files and search/identification files obtained with MaxQuant are available via ProteomeXchange [34] with identifier PXD011490.

VWF fragment binding to MSR-1

Microtiter plates were coated over night at 4 °C with human recombinant soluble MSR-1 (R&D Systems) or BSA using 50 μ l/well of a 2.5 μ g/ml solution. Wells were rinsed with wash buffer (50 mM Tris pH 7.4, 150 mM NaCl, 2 mM CaCl_2 , 0.1% Tween-20) and

blocked with working buffer (50 mM Tris pH 7.4, 150mM NaCl, 2 mM CaCl_2 , 0.1% Tween-20, 0.1% PolyVinylPyrrolidone) for 1 h at 37 °C to prevent non-specific binding. A concentration series of the VWF fragments diluted in working buffer were incubated for 2 h at 37 °C. On wells coated with BSA only the lowest and highest concentration of each fragment was examined. After incubation, unbound proteins were washed away and bound protein was detected by incubating sequentially with anti-HPC4 (10 $\mu\text{g}/\text{ml}$) and goat-anti-mouse HRP (1:5000, Southern biotech), both for 1 h. Peroxidase activity was detected using a tetramethylbenzidine substrate solution (TMB). Absorbance was measured at 450 nm.

Surface plasmon resonance analysis

SPR analysis was performed using a BIAcoreT200 biosensor system (Biacore AB). For assessment of VWF fragment binding to LRP-1, LRP-1 purified from placenta (Biomac) was immobilized at $\sim 2 \text{ fmol}/\text{mm}^2$ to a CM5-sensor chip via primary amino groups, using the amine-coupling kit as prescribed by the supplier. Varying concentrations of D'D3 and D'D3A1 ranging from 0 to 250 nM were passed over the immobilized LRP-1 for 240 s. For assessment of sMSR-1 and Cluster-II binding to RAP, gst-RAP was coupled directly to a CM5 sensor chip at $8 \text{ fmol}/\text{mm}^2$. Concentrations ranging from 0 to 40 nM of Cluster-II and sMSR-1 were passed over immobilized gat-RAP for 240 s in SPR buffer. For assessment of sMSR-1 binding to LRP-1 Cluster-II, recombinant LRP-1 Cluster-II was directly coupled to a CM5 sensor chip at a density of $\sim 18 \text{ fmol}/\text{mm}^2$. A concentration range of 0-80 nM sMSR-1 was passed over immobilized Cluster-II for 240 s. For the competition experiment we used 40 nM sMSR-1 and titrated in a concentration series of 0, 20, 40, and 80 nM Cluster-II. In all experiments, the proteins were diluted in SPR buffer containing 20 mM Hepes (pH 7.4), 150 mM NaCl, 5 mM CaCl_2 and 0.05% (v/v) Tween-20 and binding was measured at 25 °C at a flow rate of 30 $\mu\text{l}/\text{min}$. After each binding experiment the sensor chip was regenerated by washing repeatedly with 1M NaCl, 50 mM EDTA. Binding curves were corrected for the binding response that was measured in the absence of an immobilized protein. Binding responses were plotted using Graphpad prism 7 software.

RESULTS

Cell surface proteomics identifies VWF clearance receptors LRP-1 and MSR-1 on MDMs

Previously, we have shown that human monocyte-derived macrophages (MDMs) internalize VWF via a mechanism that depends on LRP-1 [14]. To identify putative other VWF clearance receptors on MDMs, we now analyzed the cell surface proteome of MDMs using mass spectrometry analysis. To this end, extracellular proteins were labeled with the membrane impermeable sulfo-NHS-biotin after which samples were subjected to mass spectrometry processing and enrichment of biotin-labeled peptides using a biotin pull-down approach (Figure 1A). Mass spectrometry analysis of the collected proteins resulted in the identification of 1340 potential cell surface proteins (Supplementary Table S1). Among the most labeled proteins were LRP-1, CD109 and TFRC (Figure 1B). Several peptides of the VWF receptors LRP-1 and MSR-1 were identified as well as one peptide that is shared by Siglec-5 and Siglec-14 (Figure 1C). Siglec-5 was previously described to be involved in VWF clearance but is very similar to Siglec-14 [19]. The estimated copy numbers of these receptors revealed a markedly higher expression of LRP-1 and MSR-1 compared to Siglec-5/14. We therefore focused our study on the possible dual mechanism by which LRP-1 and MSR-1 may cooperate

Figure 1. Cell surface proteomics uncovers cell surface receptors on MDMs. Whole-cell proteome and cell surface proteome of MDMs were analyzed by mass spectrometry to map putative VWF clearance receptors (n=3). (A) Workflow of sample preparation and data processing. Briefly, cells were labeled with a non-membrane permeable biotin label and lysed. Extracted proteins were digested with trypsin and the resulting peptides were either subjected to biotin pull-down or protein fractionation. All fractions were analyzed using high resolution LC-MS/MS. From the label-free quantification (LFQ) intensities of the fractionated proteomes protein copy numbers were estimated. In parallel, the number of biotin-modified residues was established per protein. (B) The cell surface top 10 of proteins for which the most biotin-modifications were measured. Numbers indicate the number of modified sites identified per protein in each of the samples. The color coding represents log₁₀ values of the estimated copy numbers per cell. (C) Identified VWF clearance receptors that were previously described, represented as in panel B.

in the cellular uptake of VWF by MDMs.

The D'D3A1 region of VWF contains multiple binding sites that facilitate uptake

To study the internalization of VWF in a flow-independent manner, we employed N-terminal VWF fragments comprising the D'D3 and D'D3A1 regions (Figure 2A). Both fragments were equipped with a C-terminal HPC4-tag for purification and imaging purposes. Upon incubation with MDMs for 30 minutes, a clear punctuate pattern was observed for both fragments, which showed substantial co-staining with early endosomal marker EEA1 (Figure 2B). In agreement, flow cytometry assessment of MDMs incubated with the respective fragments displayed a dose-dependent increase in fluorescence, which was more pronounced for D'D3A1 (Figure 2C). These observations indicate that receptors on MDMs can interact with the VWF fragments in static conditions which results in their internalization.

LRP-1 facilitates the uptake of D'D3A1 via the A1 domain

To further dissect the role of LRP-1 in these observations, we employed the U87MG cell line, which has been shown to express relatively high levels of LRP-1 [35]. Our mass spectrometry approach confirmed the expression of LRP-1 in these cells and showed that MDMs and U87MG cells contained similar levels of LRP-1 (Supplementary Figure S1 and Supplementary Table S1). Although we cannot fully exclude their presence using this approach, MSR-1 and other VWF clearance receptors were not identified on U87MG cells (Supplementary Table S1). Microscopy and flow cytometry studies revealed that U87MG cells effectively internalized D'D3A1, but, in contrast to MDMs, not D'D3 (Figure 2D-E). The presence of LRP-antagonist Receptor Associated Protein (RAP), which can efficiently block binding to LRP-1 and its related receptors [25,36,37], abrogated the internalization of D'D3A1 by these cells (Figure 2F-G). Likewise, the cellular uptake of D'D3A1 was blocked in the presence of an excess of purified LRP-1 domain Cluster II (hereafter called Cluster II). These findings indicate that LRP-1 mediates the uptake of D'D3A1 by U87MG cells, and are in line with the finding that the A1 domain of VWF comprises a binding site for LRP-1 [18]. Indeed, surface plasmon resonance (SPR) analysis showed that, in contrast to D'D3, D'D3A1 did bind to Cluster II, thereby confirming that the A1 domain of D'D3A1 is crucial for its interaction with LRP-1 (Supplementary Figure S2).

MSR-1 binds D'D3 and D'D3A1, yet is only required for D'D3 internalization

In parallel, we investigated the role of MSR-1 in the internalization of VWF fragments D'D3 and D'D3A1. Binding studies showed a dose-dependent interaction of the extracellular domain of MSR-1 (sMSR-1) with both VWF fragments (Supplementary Figure S3). This finding is in line with the observation of Wohner *et al.* that VWF contains binding sites for MSR-1 in the D'D3 and A1 domain [15]. However, internalization experiments with MDMs revealed that blocking MSR-1 only affected the cellular uptake of D'D3 (Figure 3). As the used antibody inhibits both the binding of D'D3 and D'D3A1 to MSR-1 (Supplementary

Figure 2. Uptake of VWF fragments D'D3A1 and D'D3 in MDMs and U87MG cells. (A) Schematic representation of the domains of VWF and the generated fragments. Both fragments were equipped with a C-terminal HPC4 tag for purification and detection purposes. (B,D) Uptake of 100 nM D'D3A1 or D'D3 in MDMs and U87MG cells. Cells were stained with antibodies against HPC4 (green) and EEA1 (red), and Hoechst (blue). Scale bar represents 10 μ m. Co-localization results in a yellow staining. (C,E) Flow cytometry measurements of fragment uptake in MDMs (panel C) and U87MG cells (panel E). Cells were incubated with concentrations of fragments of 0, 63, 125, 250 and 500 nM. Data show the fold-increase (mean $\pm$ SD) of the geometric mean fluorescent intensity (GeoMFI) after correction for background staining in cells incubated with buffer only. Data were obtained from 4 independent experiments. (F) U87MG cells were incubated with 75 nM D'D3A1 or D'D3 with or without a 5x molar excess of RAP or LRP-1 Cluster-II. Cells were stained with anti-HPC4 (green), anti-EEA1 (red) and Hoechst (blue). Scale bar represents 10 μ m. Sites of co-localization result in a yellow staining (G) Quantification of confocal images. Number of particles per cell were counted in tile scans from 4 independent experiments. For each experiment 300-500 cells were analyzed. Data represent mean $\pm$ SD.

Figure S4), this indicates that the observed uptake of D'D3A1 in the presence of the α MSR-1 antibody proceeds via an alternative mechanism, possibly via LRP-1.

LRP-1 enables internalization of both D'D3 and D'D3A1

So far, we have established that U87MG cells, that do not express MSR-1, internalize D'D3A1 in an LRP-1 mediated manner. In addition, we showed that D'D3 is internalized by MDMs via MSR-1, whereas D'D3A1 uptake is independent of MSR-1. To address whether LRP-1 mediates the D'D3A1 endocytosis by MDMs, we incubated MDMs with the fragments in the presence of the LRP-1 antagonist RAP or an excess of Cluster II. As expected, RAP and Cluster II minimized the uptake of the D'D3A1 fragment in MDMs (Figure 3B). Surprisingly, however, the uptake of D'D3 was affected in a similar fashion (Figure 3A,C). This suggests that internalization of both D'D3 and D'D3A1 is dependent on LRP-1, in spite of the notion that the LRP-1 binding site resides in the A1 domain of VWF.

MSR-1 associates with LRP-1 for internalization

To explain the remarkable dependence of D'D3 uptake by MDMs on both LRP-1 and MSR-1, we hypothesized that MSR-1-induced D'D3 internalization may be mediated by LRP-1. We therefore assessed whether sMSR-1 can directly interact with LRP-1 using SPR analysis. To this end, sMSR-1 was passed over immobilized Cluster II on an SPR sensor chip. A dose dependent-binding response was observed that could be effectively blocked upon prior incubation of sMSR-1 with Cluster II (Figure 4A). This finding implies that sMSR-1 may indeed interact with LRP-1. Subsequent confocal microscopy analysis showed that, upon incubation with U87MG cells, sMSR-1 was transported to the early endosomes via a process that was inhibited in presence of an excess of RAP or Cluster II (Figure 4B). These observations imply that sMSR-1 is endocytosed by U87MG cells via an LRP-1 dependent mechanism. In line with this hypothesis, we found that sMSR-1 could induce the internalization of D'D3 by U87MG cells (Figure 4C). Taken together, these data suggest that sMSR-1 acts as a bridging receptor between LRP-1 and D'D3, thereby mediating the uptake of this fragment via LRP-1. To assess whether this mechanism of endocytosis also holds true for another MSR-1 ligand, we incubated fluorescent acLDL with the U87MG cells in presence and absence of sMSR-1. No uptake of acLDL was observed in the absence of sMSR-1, however, as for D'D3, the presence of sMSR-1 induced the localization of acLDL to the early endosomes (Figure 4D).

Figure 3. Uptake of D'D3 by MDMs is mediated by MSR-1 and LRP-1. (A, B) Blocking MSR-1 only affects D'D3 interaction, whereas blocking LRP-1 affects both fragments. MDMs were incubated with 75 nM D'D3A1 or D'D3 with or without a 5x molar excess of anti-MSR1 blocking antibodies, RAP or LRP-1 Cluster-II. Cells were stained with anti-HPC4 (green), anti-EEA1 (red) and Hoechst (blue). Co-localization results in a yellow staining. In MSR-1 blocking conditions, red staining represents EEA-1 as well as MSR-1 as these antibodies were from the same species and isotype. Scale bar represents 10 μ m. (C) Quantification of confocal images. Number of particles per cell were counted in tile scans from 4 independent experiments. For each experiment 300-500 cells were analyzed. Data in panel C represents mean $\pm$ SD.

Figure 4. MSR-1 associates with LRP-1 to mediate D'D3 uptake. SPR and cell-based experiments to explore the interaction between MSR-1 and LRP-1. (A) Binding of soluble MSR-1 (sMSR-1) to 18 fmol/mm² immobilized LRP-1 Cluster-II in the absence and presence of increasing concentrations of soluble Cluster-II. The concentration of sMSR-1 was kept at 40 nM and the concentration of soluble Cluster-II was 20, 40 or 80 nM. (B) Uptake of 100 nM sMSR-1 by U87MG cells in the absence or presence of a 5x molar excess of RAP or Cluster-II. Cells were stained with anti-MSR-1 (green), anti-EEA1 (red) and Hoechst (blue). Co-localization results in a yellow staining. (C) Uptake of 100 nM D'D3 in the absence and presence of 200 nM sMSR-1. Cells were stained for anti-HPC4 (green), anti-EEA1 (red) and Hoechst (blue). (D) Uptake of 2.5 µg/ml acLDL in the absence and presence of 200 nM sMSR-1. The scale bar represents 10 µm.

DISCUSSION

In this study we dissected the clearance mechanisms of VWF by MDMs, by using N-terminal VWF fragments D'D3 and D'D3A1. These data provide evidence for a role for LRP-1 as a co-receptor for MSR-1 in the uptake of ligands by MDMs. Using a cell surface proteomics approach, we showed that, of the receptors that have been implicated in VWF clearance, MDMs contain LRP-1 and MSR-1 (Figure 1). Uptake studies indicated that MDMs can internalize D'D3 and D'D3A1, and confirmed that the A1 region is involved in LRP-1 dependent uptake (Figure 2). In addition, we showed that D'D3 internalization is dependent on MSR-1 as well as LRP-1 (Figure 3). Finally, we showed that the uptake of D'D3 and acLDL via MSR-1 is mediated by LRP-1 (Figure 4). Together, these results imply that D'D3A1 can be internalized via two mechanisms, either directly via the association of the A1 domain to LRP-1, or via interaction of the D'D3 assembly to MSR-1 followed by internalization via LRP-1.

The clearance of VWF can be affected in patients with Von Willebrand Disease (VWD). This disorder is characterized by the decreased function, or even the functional absence of VWF in the circulation. Many mutations in VWD patients that are associated with increased VWF clearance are located in the D'D3A1 region [6,38]. This same region contains binding sites for clearance receptors MSR-1 and LRP-1 [15,18], yet cells containing these uptake receptors, including the MDMs used in this study, are not able to internalize VWF in common *in vitro* conditions. Only when subjected to flow-induced shear stress, MDMs can internalize VWF in an LRP-1 mediated fashion [14,17]. We now show that N-terminal VWF fragments comprising the D'D3 or D'D3A1 domains can be internalized by MDMs in static conditions as well. This suggests that VWF contains cryptic regions for MSR-1 and LRP-1 binding that are exposed in flow conditions as well as in our N-terminal fragments. Such a mechanism is in line with other VWF interactions that rely on flow-exposure, including platelet binding and ADAMTS13 interaction [39–41].

LRP-1 recognizes over 30 ligands, and is therefore involved in a number of diverse physiological processes [42,43]. It is surprising that one receptor, albeit as large as LRP-1, provides interaction sites for all of these structurally diverse ligands. Our data now provide evidence for a role for LRP-1 as a co-receptor for MSR-1-mediated VWF uptake. The use of co-receptors could drastically reduce the number of interaction partners of LRP-1, and thereby facilitate the indirect interaction of LRP-1 with many structurally diverse ligands. Furthermore, the use of co-receptors allows for fine-tuning of LRP-1 ligand endocytosis by regulating the expression of its co-receptors. Such a mechanism could prevent that abundant LRP-1 ligands outcompete lower level interaction partners. The hypothesis that LRP-1 functions as a co-receptors for MSR-1 is supported by the finding that the internalization of acLDL, another MSR-1 ligand, can be induced in U87MG cells by supplementing the soluble MSR-1 receptor. Would such a mechanism also be in place for other MSR-1 ligands [44], such as coagulation factor X [45]?

A similar mechanism in which LRP-1 functions as a co-receptor has been proposed

for the uptake of the uPA-PAI-1 complex via the urokinase plasminogen activator receptor (uPAR), although the details are different [43]. The uPAR can bind uPA with and without its inhibitor PAI-1. The uPAR-uPA complex is stable, whereas the uPAR-uPA-PAI-1 complex is internalized via LRP-1 [43]. Also in this case, LRP-1 serves as a co-receptor which, in concert with a primary receptor (uPAR), mediates the internalization of a ligand (uPA-PAI-1). We and others have previously shown that the LRP-1 dependent uptake of FVIII also proceeds via a dual receptor mechanism. In addition to the interaction with LRP-1, FVIII requires prior binding to other structural elements in the cell surface to mediate the internalization by the cells [46,47]. These findings indicate that LRP-1 may serve as a co-receptor for multiple primary receptors, and thereby mediate the endocytosis of many structurally distinct ligands.

Studies in mice have shown that LRP-1 and MSR-1 play a physiological role in VWF clearance as well [15,17]. However, many issues involving this mechanism remain to be resolved. For instance, others have shown that PMA-stimulated THP-1 cells can bind VWF and internalize it via a shear-independent mechanism [13,15]. In our hands, MDMs only internalize VWF in a shear-dependent manner. This indicates that the model system can have a drastic effect on the experimental outcomes. In contrast to the monocytic leukemia-derived THP-1 cell-line, that requires PMA-stimulation to obtain their macrophage-like characteristics, MDMs can be differentiated from circulating monocytes using the cytokine M-CSF (macrophage colony stimulating factor). This suggests that MDMs provide a better representation of *in vivo* macrophages, although it remains to be established to which extent MDMs resemble Kupffer cells, the primary suspects of macrophage-mediated VWF-clearance. Understanding the differences between the various cellular models remains a topic for further investigation. The same holds true for the expanding list of putative endocytic receptors for VWF. The relative importance of these receptors to mediate the uptake of VWF remains an open question.

Based on the data presented in this study, we propose the following model to explain the relationship between the two abundant VWF receptors on MDMs. Both LRP-1 and MSR-1 associate with regions within D'D3A1, thereby initiating two endocytic pathways that are both regulated by LRP-1. The first pathway follows a direct association of the VWF A1 domain to LRP-1. In the second pathway, VWF interacts to MSR-1 via regions in the D'D3 assembly, which subsequently associates to LRP-1 for endocytosis.

ACKNOWLEDGEMENTS

This work was supported by research funding from Sanquin Blood Supply foundation (PPOP 13-002).

AUTHOR CONTRIBUTIONS

E.P.B., M.P., E.F.J.J., H.M., M.S. and M.J.M. performed experiments, E.P.B., K.M., M.v.d.B., A.B.M. and M.J.M. designed the research, E.P.B. and M.J.M. analyzed the data and E.P.B., A.B.M. and M.J.M wrote the manuscript.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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SUPPLEMENTAL FIGURES

Figure S1. Most identified biotin-labeled peptides on MDMs and U87MG cells. The cell surface top 25 of proteins for which the most biotin-modifications were measured. Numbers indicate the number of modified sites identified per protein in each of the samples. The color coding represents log₁₀ values of the estimated copy numbers per cell. (A) Most labeled peptides on MDMs and U87MG cells. (B) The largest differences between MDMs and U87MG cells. (C) The largest differences between U87MG cells and MDMs.

Figure S2. Association of D'D3A1 to LRP-1 is dependent on the A1 domain. SPR analysis of D'D3A1 and D'D3 binding to immobilized LRP-1 (2 fmol/mm²). For each fragment a concentration series of 0, 31.25, 62.5, 125 and 250 nM was passed over the chip.

Figure S3. The ability of VWF to bind sMSR-1 resides in the D'D3 region. Immunosorbent assay on D'D3A1 and D'D3 binding (0-200 nM) to wells coated with sMSR-1 or bovine serum albumin (BSA, control). Bound fragments were detected via TMB-hydrolysis following labeling with an anti-HPC4 antibody and polyclonal peroxidase-labeled goat anti-mouse antibody. Control data points represents the binding of 0 and 200 nM D'D3A1 to BSA.

Figure S4. Anti-MSR-1 competes with VWF-fragment binding to sMSR-1. Immunosorbent assay on D'D3A1 and D'D3 binding (40 nM) to wells coated with sMSR-1. Bound fragments were detected via TMB-hydrolysis following labeling with a polyclonal rabbit anti-HPC4 antibody and polyclonal peroxidase-labeled swine anti-rabbit antibody.

SUPPLEMENTAL TABLES

The following supplemental table is available upon request.

Table S1. Protein copy number estimates and cell surface peptides of MDMs and U87MG cells. Cell surface peptides and estimated cell surface protein copy numbers per cell of MDMs (M1-3) and U87MG cells (U1-3). NA = not available.

CHAPTER 6

SUMMARIZING DISCUSSION

Cells selectively express surface receptors that respond to extracellular signals. Activation of these receptors induces signaling pathways that guide a variety of biological processes, including intercellular communication, cell differentiation and ligand endocytosis. In this thesis, we studied the molecular mechanisms that result from receptor activation in the vasculature at a system-wide level by virtue of mass spectrometry-based proteomics. Specifically, we dissected the receptor-induced adaptive response of endothelial cells to pro-inflammatory cytokines and flow-exposure. Furthermore, we investigated the cytokine-induced receptor-mediated terminal differentiation of monocytes towards macrophages, dendritic cells and osteoclasts. Finally, we unraveled the interplay of two endocytic receptors in the internalization of a single ligand. In this chapter, we summarize and discuss the main findings of these studies.

The receptor-mediated adaptive endothelial response in inflammation

In Chapter 2, we set out to unravel the EC response to receptor-activation by pro-inflammatory cytokines TNF α and IL-1 β . Surprisingly, we observed that TNF α and IL-1 β , through the activation of separate dedicated receptors, provoked virtually the same endothelial response. This apparent redundancy raises the question why ECs would express distinct receptors for these two cytokines. These findings may be explained by a putative synergistic effect of IL-1 β and TNF α with each other, or with other stimuli. The stimulation of an EC with multiple cytokines may amplify the endothelial response or fine-tune it for specific situations. An amplification has for instance been observed for VCAM1 after combined TNF α and interferon- γ stimulation [1]. Furthermore, it is important to consider the wider biological context of these endothelial responses. For specific conditions immune cells may require an identical response from ECs, but a distinct response from other cell types. The production of two separate cytokines with partially overlapping target cell populations may realize such a cellular response. Alternatively, a possible explanation may lie in the proteins that were specifically regulated, including CLDN11 and MX1. These proteins may serve essential functions to fine-tune the inflammatory response of ECs. Future studies are required to determine how the signaling cascades that underlie the proteomic response to IL-1 β and TNF α are interlaced. Furthermore, to fully appreciate the responsive potential of ECs to environmental stimuli, a systematic evaluation of EC responses to a wide range of stimuli, and combinations thereof, is necessary.

In addition to the comparison between the endothelial responses to IL-1 β and TNF α , we focused on the temporal profiling of these responses. A selection of the proteins affected by cytokine-stimulation, including leukocyte adhesion receptors ICAM1 and SELE, showed increased proteins levels after 4 hours of cytokine-stimulation (Chapter 2, Figure 2C-D). In contrast, many other proteins, for instance proteins involved in self-antigen presentation (HLAs, B2M, ERAP1, TAP1&2 and TAPBP), displayed increased levels after ≥ 16 hours. This suggests the presence of a two-step mechanism of EC activation. In an early stage during inflammation, the adhesion and extravasation of leukocytes is supported, whereas other

processes, including antigen presentation and the production of antiviral factors, are induced at a later stage. This two-step mechanism may be in place to only exploit the full potential of the inflammatory response, including the activation of the adaptive immune system, if the innate immune system is not sufficient to clear a potential infection. In our experiments, cells were continuously exposed to TNF α or IL-1 β . Future experiments are required to determine what the minimum cytokine-exposure time is to induce the secondary burst of protein expression.

To specifically focus on cell surface proteins, we have developed and applied a mass spectrometry-based footprinting method. This method relies on the covalent labeling of surface exposed lysine residues with a biotin-label, followed by cell lysis, protein digestion and biotin pull-down. The use of this method resulted in the quantification of additional proteins, provided information on the surface localization of proteins, and lead to the identification of site-specific changes in ITGA5 and ICAM1 (Chapter 2, Figure 3). We speculate that the observed changes in ITGA5 and ICAM1 result from ITGA5 activation and ICAM1 dimerization, respectively [2–4]. Activation of ITGA5 has been shown to be induced by TNF α , and promotes neutrophil adhesion [5]. Dimerized ICAM1 displays a higher affinity for its ligand integrin α M β 2 on leukocytes [4] and thereby aids in leukocyte extravasation as well. These results suggest that cytokine-stimulation of ECs not only induces the expression of adhesion molecules, but also induces changes within adhesion receptors that result in an increased leukocyte-binding affinity.

These proteomic data provide a rich resource on the molecular details of endothelial inflammation. We identified numerous cytokine-responsive proteins that have not been associated with endothelial inflammation before, including multiple proteins on the cell surface. As blocking antibodies against ICAM1, VCAM1, PECAM1 and CD99 do not completely prevent leukocyte transmigration [6–11], these may include additional therapeutic targets to prevent leukocyte extravasation. In addition, these findings indicate that 1) separate receptors and associated signaling pathways can induce highly similar responses, 2) endothelial inflammation consists of early and late-responsive proteins, and 3) leukocyte adhesion is further enhanced in inflammatory conditions by ICAM1 dimerization and ITGA5 activation.

Flow-exposure of ECs induces basement membrane remodeling

In addition to the adaptive response that ECs display in inflammatory conditions, ECs respond to hemodynamic forces generated by blood flow through specialized mechanosensors. These mechanosensors can be activated by shear stress or cyclic strain and thereby affect the cellular phenotype and behavior. The effect of hemodynamics on EC morphology is clearly visible *in vivo* as different regions within the aortic branch exhibit distinct phenotypes [12]. When exposed to laminar flow, ECs exhibit a spindle-like phenotype and cells are aligned in the direction of the flow. In contrast, weak or turbulent flow patterns result in a polygonal phenotype. Morphological changes associated with flow-exposure are

accompanied by a change in endothelial function that comprises increased nitric oxide production, inhibition of cell proliferation and increased barrier function [13,14]. However, little is known about the mechanoreceptor-induced changes at the molecular level that underlie the flow-induced shift in endothelial phenotype and function. To unravel the molecular details that underlie the endothelial response, we dissected the endothelial proteome after 24 and 48 hours of flow-exposure in Chapter 3. In accordance with literature, our proteomic data demonstrated altered levels of proteins involved in nitric oxide production and cytoskeletal remodeling, the two hallmarks of EC flow-exposure [15–17]. In addition to these processes, our data indicated that many other proteins and biological processes that were affected in flow conditions, were most prominently related to the ECM and cell-ECM adhesion (Chapter 3, Figure 2). We therefore subsequently focused on two protein families involved in cell-ECM adhesion: laminins and integrins.

The laminin content of endothelial basement membranes (BM) can have considerable implications for BM-associated cells [18]. Highly relevant for the endothelial BM, the transmigration of leukocytes can be inhibited by the presence of LAMA5 [19,20]. We observed that flow-exposure results in increased levels of LAMA5, which suggests that leukocyte extravasation mainly takes place in regions deprived of high shear exposure. In line with this hypothesis, the primary extravasation sites of leukocytes are found in the post-capillary venules, which are not exposed to high shear forces [21] and are largely devoid of LAMA5 [22]. It is therefore tempting to speculate that the lack of LAMA5 in these regions is a direct result of low flow exposure of ECs. In addition to the protein levels of LAMA5, immunofluorescence studies revealed that, in flow conditions, LAMA5 formed a subcellular network (Chapter 3, Figure 3E), and, after 48 hours of flow-exposure, LAMA4 adopted a similar configuration. Furthermore, we demonstrated that both the protein levels as well as the localization of laminin-associated proteins FBLN2 and EFEMP1 were affected by flow-exposure. These findings suggest that flow-exposure elicits major changes in the endothelial basement membrane. However, it remains to be established how these findings translate to an *in vivo* setting. Does the laminin network formation also take place in regions of the vasculature exposed to laminar flow? And how does the turbulent flow found in bifurcations affect the laminin network?

In addition to the changes in laminin levels, our MS data indicated that particularly laminin-binding integrins were elevated in flow conditions, as integrins $\alpha 3$, $\alpha 6$, and $\beta 4$, that were increased in flow conditions, specifically bind to laminins [23]. Furthermore, our immunofluorescence data suggest that integrin $\alpha 6$ and $\beta 4$ form an adhesion complex, that has not been described so far, and co-localizes with the laminin network (Chapter 3, figure 3-5). In epithelial cells the integrin $\alpha 6\beta 4$ complex has been shown to connect the cytoskeleton to the ECM, thereby absorbing mechanical forces [24]. The disruption of these integrin complexes results in rapid friction- or trauma-induced detachment of epithelial layers. This is clearly illustrated in patients with ITGB4 mutations, that suffer from severe blistering diseases, as a result of rapid detachment of the epidermis from the dermis

Figure 1. Flow induces the relocation of vimentin (VIM), integrin α V β 3 (ITGAV, ITGB3) and podocalyxin (PODXL). (A-D) Maximum intensity projections of BOECs exposed to flow. Green represents VIM, ITGAV, ITGB3 and PODXL, respectively, red is ITGB4, Blue is HOECHST. The scale bar is 10 μ m. (E) Log₂ SILAC ratios of the proteins depicted in panels A-D. Grey open circles and black closed circles represent 24 and 48 hours of flow exposure compared to the static condition, respectively.

[25,26]. Based on these findings and our immunofluorescence data, we speculate that integrin $\alpha 6 \beta 4$ may provide similar mechanical support in the endothelium, by linking the cytoskeleton to the laminin basement membrane. In contrast to other integrins, that link to actin filaments, the $\alpha 6 \beta 4$ heterodimer associates with vimentin intermediate filaments [27,28]. In line with the integrin $\alpha 6 \beta 4$ -vimentin link and the affected localization of integrin $\alpha 6 \beta 4$, immunofluorescence micrographs showed that the structure of the vimentin network is affected in flow conditions (Figure 1A). Furthermore, in agreement with a role for integrin $\alpha 6 \beta 4$ in mechanical support of ECs, the knock-down of vimentin leads to detachment of ECs exposed to flow [29]. Moreover, replacement of the laminin 511 network with laminin 411 or 111, which can both not interact with integrin $\alpha 6 \beta 4$ [27], leads a decreased adhesion strength [30]. Combined, these findings support the hypothesis that flow induces basement membrane remodeling and BM-adhesion to withstand the higher mechanical forces induced by flow.

Besides integrin $\alpha 6 \beta 4$, we assessed the localization of a well-known EC-ECM adhesion complex: integrin $\alpha V \beta 3$. As for LAMA4, the protein levels of integrin $\alpha V \beta 3$ in static and flow conditions were similar (Figure 1E), however, the localization of this integrin heterodimer was affected by flow-exposure as well (Figure 1B-C). Both the αV and the $\beta 3$ subunit displayed a high degree of co-localization with ITGB4, which indicates that these integrin complexes may be in close proximity. A possible explanation for this observation lies in the ability of integrin $\alpha V \beta 3$ to bind to an RGD motif in LAMA5 [31]. However, it remains to be elucidated what the function of this integrin pair is in flow-exposure of ECs.

In addition to the proteins we studied in detail, our data indicate that the levels of numerous other proteins were affected by hemodynamics. The role of many of these proteins in relation to flow-exposure is currently unknown and remains a topic for further studies. One noteworthy example is PODXL (podocalyxin). Our MS data indicated that PODXL protein levels were about six-fold higher in flow conditions (Figure 1E). However, immunofluorescence assessment of this protein revealed a more complex picture (Figure 1D). We observed basolateral clusters of PODXL in static conditions, whereas a diffuse staining pattern was observed at the apical side after flow exposure. This observation may be explained by the trafficking of PODXL from vesicles to the apical surface. A similar observation has been made during the polarization of epithelial cells: PODXL is initially present on the entire cell surface, however, contact with a glass or matrigel surface resulted in rapid internalization in vesicular compartments and subsequent redistribution to the apical surface [32]. Remarkably, knock-down of PODXL in endothelial cells resulted in a reduced endothelial laminin adhesion capacity [33] and barrier function [34]. This suggests that the increased abundance of PODXL in flow-exposed ECs contributes to the improved laminin adhesion and barrier function demonstrated in flow-adapted ECs. However, the mechanisms underlying these observations remain to be elucidated, and may provide the basis for future studies.

In the vasculature, a wide array of distinct flow patterns is present, ranging from pulsatile

in arteries and arterioles, to laminar in the capillaries and veins, and turbulent/oscillatory at bifurcations. These flow types can vary in amplitude, frequency and flow strength. In order to dissect the cellular responses to these distinct flow types, and thereby determine the associated (patho)physiological consequences, a systematic evaluation of the various flow parameters is essential. Therefore, a new flow system is required that 1) provides direct control of the flow type and strength, and 2) allows for simultaneous testing of multiple flow types. A possible way to meet these criteria is by using multiple cone-and-plate flow devices that are compatible with disposable petri dishes, and allow for the precise control of the cone rotation. By regulating the cone's direction and rotational velocity, this system may allow for the simulation laminar, pulsatile, oscillatory and turbulent flow patterns. Such a systematic analysis will teach us about the endothelial response to various flow conditions, and may provide clues about pathological mechanisms of flow-related diseases.

Furthermore, it would be of considerable interest to assess the short-term effects of physical stress, such as flow-induced shear stress, on EC proteins. The identification of many shear stress sensors [17,35], and several shear stress-dependent interactions in the vasculature (VWF-ADAMTS13 [36], GP1b α -VWF [37], SELP-PGSL1 [P-selectin glycoprotein ligand 1] [38]) suggests that shear stress can directly alter the conformation of multiple proteins. The quantitative footprinting approach we developed now allows for the unbiased screening of stress-sensitive proteins, and provides site-specific information on the induced changes.

The endothelial response to its microenvironment

As described in Chapter 1, the function of the endothelium is, among others, dependent on the inflammatory, hemodynamic and hemostatic status of its environment. Based on these parameters, ECs can mediate biological processes, such as leukocyte transmigration and leakage of plasma proteins. It has been suggested that the endothelial response to inflammatory and hemodynamic triggers is interconnected, as several studies have shown that typical inflammatory markers, including ICAM1, VCAM1 and SELE, have been found to be upregulated in flow-deprived regions [39–41]. Therefore, in Chapter 3, we explored the interplay of flow and inflammation. Surprisingly, we found that flow and TNF α induced a fundamentally different endothelial response (Chapter 3, Figure 6). In general, TNF α and flow exposure affected a distinct set of proteins: TNF α affected proteins were associated with inflammation, whereas many of the proteins affected by flow-exposure were linked to ECM-adhesion. However, for a limited number of proteins, including adhesion molecules ITGB4 and BCAM, we did detect interplay of TNF α - and flow stimulation. Compared to flow conditions, the levels of ITGB4 and BCAM were decreased in static conditions, as well as upon stimulation with TNF α . This suggests that, for these molecules, the deprivation of flow affects ECs in a similar manner as TNF α does. The downregulation of these molecules in static conditions may be the first step towards the inflammatory profile observed in flow-deprived regions of the vascular bed.

The data in this thesis describing the endothelial response to inflammation and flow may provide additional targets for therapeutic use. For instance, it is remarkable that both in inflammatory and in flow conditions the ECM and the cell-ECM interaction were affected. As the subendothelial ECM has been shown to affect the transmigration of leukocytes [19,20], it may present an interesting target for the treatment of undesired leukocyte transmigration. Our data indicate that flow-exposure of ECs results in increased LAMA5 levels in the subendothelial basement membrane, and suggest that the EC-ECM interaction is stronger compared to static conditions. The presence of LAMA5 in the BM has been associated with decreased neutrophil transmigration [20]. Therefore, therapeutic agents that induce the upregulation of LAMA5 and integrin $\alpha 6 \beta 4$ may provide the potential to counteract leukocyte extravasation. This may be achieved by the activation of transcription factors that are upregulated in flow conditions. For example KLF2 is activated in flow conditions [42], and this activation can be enhanced *in vitro* using tannic acid [43]. Alternatively, statins may be used, which have been shown to potently induce ITGB4 expression in ECs [44], thereby increasing adhesion potential. Statins are already clinically used to treat and prevent cardiovascular diseases, including atherosclerosis, for their LDL and cholesterol-lowering, as well as their anti-inflammatory properties [45]. Possibly some of the pleiotropic effects observed in statin treatments may be due to the increased ITGB4 levels in ECs. Future studies should dissect the molecular mechanisms underlying the endothelial response to statins and evaluate the effect of tannic acid on the inflammatory status of endothelial cells for potential use in a clinical setting.

The interplay between flow- and TNF α exposure raises the question of how flow-adaptation of ECs affects other vascular processes. Is there for instance an effect of flow-adaptation on the EC response to hemostatic stimuli? The observation that the levels of thrombin receptor PAR-1 (F2R) are decreased in flow conditions [46] suggests that there is. And do other processes that affect EC behavior affect each other as well? In Chapter 2, we showed that TNF α and IL-1 β produce essentially the same response. Perhaps the combination of these cytokines results in an even stronger behavioral effect. In order to appreciate the full spectrum of EC behavior, it is necessary to systematically assess the endothelial response to individual signals and combinations thereof in a system-wide fashion. These studies will contribute to our understanding of the endothelial response to its environment, and may provide clues about the onset of endothelial dysfunction.

Cytokine-induced monocyte differentiation

In addition to the transient effects on the inflammatory status of ECs, cytokines can induce the terminal differentiation of immune cells. In Chapter 4 we employed proteomics to unravel the terminal differentiation of monocytes into dendritic cells, M1 and M2 macrophages, and osteoclasts. These cells are highly specialized and serve distinct functions. Osteoclasts are specialized in bone resorption, dendritic cells are characterized by their immune modulatory function and macrophages by their ability to engulf and digest

large particles. This differentiation is induced by cytokines and can be mimicked *in vitro* while preserving most of the physiological characteristics of these cells [47–51]. In Chapter 4, we analyzed undifferentiated monocytes, and each of the distinct cell types throughout their differentiation. Our proteomic analysis indicated that there was a high correlation between the different cell subsets in general. Lower correlations were observed between monocytes and the other differentiation stages, as well as between DCs and the other cell types. Analysis of cells that were differentiated for 10 days indicated that established protein markers, including CD209 and CD1B/C for DCs, as well as CKB and CTSK for osteoclasts, were expressed by the respective subsets. In addition, we identified PTPRE and FCER2 as markers for DCs.

The differentiation of the monocyte-derived cells was a continuous process characterized by a common core of affected proteins among the distinct cell types, comprising decreasing levels of DNA-replication and RNA-binding proteins, and increasing levels of inflammatory, apoptotic and metabolic proteins. The strong inflammatory signature is surprising for osteoclasts given their bone resorbing function. However, it has been established that osteoclasts are responsive to inflammatory stimuli, as these cells are involved in inflammation-induced bone erosion [52]. Furthermore, like DCs, osteoclasts are antigen presenting cells able to activate T-cells [53]. This indicates that osteoclasts have a role in immune-regulation as well, which may explain the detected inflammatory signature.

In agreement with other reports, we found that lipid metabolism is induced in monocyte-derived cells [54,55]. This function is necessary for macrophages to handle the lipids that are acquired through the phagocytosis of other cells [56]. However, it remains to be elucidated why DCs and osteoclasts upregulate their lipid metabolizing machinery as well.

We speculate that the common core of proteins that was similarly affected amongst the distinct cell types is required by monocytes for their terminal differentiation, regardless of specialization. The other observed changes may provide the unique capabilities of the fully differentiated cells. Remarkably, these specialized monocyte-derived cells still display a certain degree of plasticity. This has been illustrated by the transplantation of tissue-resident macrophages to other tissue. After their relocation, these cells adopted the tissue-specific transcription profiles from their new location [57]. Another clear example from this plasticity is the ability of monocyte-derived cells to adopt a different specialization. For instance murine DCs have been demonstrated to be capable of adopting an osteoclast signature when presented with a bone-marrow microenvironment [57,58]. This highlights the importance of surface receptors in the identity of cells. Together, our data highlight the common core of proteins that is affected during monocyte differentiation and underline the similarity between M1 and M2 macrophages and osteoclasts.

The interplay of surface receptors in ligand endocytosis

In addition to the receptors that induce cellular responses, endocytic receptors are

in place to facilitate the internalization of their ligands. For example the plasma levels of hemostatic protein VWF are maintained by the clearance of this protein from the circulation. Remarkably, multiple receptors and even various cell types have been reported to be involved in the clearance of this glycoprotein [59–71]. In Chapter 5 we aimed to get insight into the interplay of multiple receptors to the cellular internalization of a single ligand like VWF. A previous study from our lab showed that monocyte-derived macrophages (MDMs) can effectively internalize VWF via an LRP-1 dependent mechanism, provided that flow is present [67]. This finding is in line with previous reports showing that macrophages interact with VWF [72] via LRP-1 [65–67], and that chemical inactivation of macrophages increases plasma VWF concentrations [73]. The proteomic assessment described in Chapter 5 indicated that MDMs contained two receptors that were previously associated with VWF interaction: LRP-1 and MSR-1 (Chapter 5, Figure 1). To assess the relative contribution of these receptors to VWF uptake, we used N-terminal VWF fragments D'D3 and D'D3A1, that contain binding sites for LRP-1 and MSR-1 [66,68]. We showed that these fragments can be internalized by MDMs in a flow-independent manner (Chapter 5, Figure 2). Further functional studies indicated that both of these receptors were involved in VWF interaction and this led us to propose the following model. VWF can interact with LRP-1 via the A1 domain, which directly leads to its internalization. In parallel, VWF can associate with MSR-1 via the D'D3 region, and this complex subsequently binds to LRP-1 binding for endocytosis. This dual receptor model is in agreement with the previous observation that blocking LRP-1 with antibodies inhibits the internalization of VWF, but not its docking to the cell surface [67].

These data raise the question why two distinct receptors would be in place for the endocytosis of a single ligand. A possible explanation may be provided by the affinity of these receptors for VWF. The affinity of LRP-1 for another ligand, coagulation factor VIII (FVIII), is relatively low [74]. Therefore, it has been proposed that pre-concentration of FVIII via its membrane-interacting domains is required to induce its cellular uptake [75]. A similar mechanism may be in place for VWF. After pre-concentration via MSR-1, the VWF-MSR-1 complex can interact with LRP-1 to induce its uptake. We speculate that the use of co-receptors is a general mechanism for endocytosis via LRP-1. This may also explain the extensive list of LRP-1 interactors, which includes over 30 ligands [76,77]. The observed co-receptor mechanism of LRP-1 and MSR-1 implies that other MSR-1 ligands can be taken up by cell types expressing LRP-1 but not MSR-1, once the soluble MSR-1 is provided. In line with this hypothesis, we showed that, in addition to D'D3, the MSR-1 ligand acLDL could be internalized by U87MG cells in the presence of soluble MSR-1. In addition, the uPA-PAI-1 complex provides another example of an LRP-1 mediated uptake mechanism that requires a co-receptor [77]. Combined, these data suggest that the use of co-receptors may be a common mechanism to allow for ligand internalization via LRP-1.

These *in vitro* studies also bring up the question how our findings translate to a physiological context. In mice, it has been shown that the monocyte/macrophage-

Figure 2. The abundance of MSR-1 and LRP-1 throughout monocyte differentiation. LFQ intensity values of MSR-1 and LRP-1 in samples from monocytes differentiating towards dendritic cells (DC), M1 macrophages (M1), M2 macrophages (M2) and osteoclasts (OS). Depicted are mean values and standard deviations of 4 independent experiments.

specific knock-out of LRP-1 results in increased VWF levels in plasma [65]. Furthermore, the knock-out of MSR-1 resulted in decreased VWF propeptide/antigen ratios, which may indicate that the clearance of VWF is inhibited [68]. In line with our *in vitro* data, these results suggest a role for MSR-1 and LRP-1 in VWF clearance. In comparison to LRP-1, which is expressed in a variety of cell types, including macrophages, neurons, and hepatocytes [78], MSR-1 expression is more exclusive. However, contrary to what the name suggests, MSR-1 can also be expressed by other cells than macrophages [79]. The proteomic assessment of monocyte-derived cells described in Chapter 4 indicated that all monocyte-derived effector cells expressed LRP-1 and MSR-1 (Figure 2). Hence, this suggests that also DCs and osteoclasts contain the receptors necessary to internalize VWF fragments D'D3 and D'D3A1. In line with this concept, DCs have been shown to interact with VWF *in vitro*, although no internalization was observed in these experiments [80]. However, as previous studies indicated that specifically macrophages are involved in the clearance of VWF [63,73,81], it appears unlikely that DCs and osteoclasts contribute significantly to VWF clearance.

A previous study in our lab indicated that full-length VWF requires flow-induced shear stress for clearance by MDMs [67]. These data are further supported by the finding that the LRP-1-VWF interaction only takes place in flow conditions [65]. A role for shear stress in VWF clearance would not be surprising as other functional aspects of this glycoprotein, including platelet binding and ADAMTS13 interaction, are dependent on shear stress as well [65,82–86]. However, it does raise the question where macrophages would be exposed to shear stress in a physiological situation. A possible clue comes from the finding that radiolabeled VWF infused in mice mainly ends up in the liver [73]. The liver contains specialized macrophages, named Kupffer cells, that are present inside liver sinusoids. Within these capillaries, a shear

stress has been measured of around 13 dyn/cm² [87], which is well above the 4 dyn/cm² that is required for macrophage-VWF interactions *in vitro* [67]. Together, these data indicate that Kupffer cells play a prominent role in the clearance of VWF.

The finding that both MSR-1 and LRP-1 have a role in the uptake of VWF fragments poses various questions. For instance whether simultaneous blocking of MSR-1 and LRP-1 prevents the interaction with macrophages altogether, both *in vitro* and *in vivo*. And does direct LRP-1 binding play a role in VWF clearance at all, or is the LRP-1-blocking effect only due to its co-receptor function? Answers to these questions may improve our understanding of the clearance of VWF and FVIII from the circulation.

Concluding remarks

The data presented in this thesis provide insight into receptor-induced cellular responses in the vasculature. These studies emphasize the role of the subendothelial basement membrane in inflammation and flow-adaptation, and describe the development of the inflammatory response of ECs. Furthermore, our data highlight shared and unique features of monocyte differentiation, and unveil the combined effort of two receptors in the endocytosis of a single ligand. These studies emphasize that mass spectrometry-based proteomics can be applied to address a wide array of biological questions within the vasculature. In addition to probing protein levels, we assessed protein localization and evaluated the presence of post-translational modifications. A major challenge for the future is to integrate these data on protein levels, localization, and PTMs with transcriptomic data, to describe molecular processes that underlie cell function.

A particularly understudied aspect of cell function is the dynamics of cell surface protein interactions. We therefore developed a peptide-based cell surface proteomics approach based on the chemical footprinting of extracellular proteins. This method allowed us to identify intramolecular changes induced by external stimuli. We envision that this method can be applied to characterize dynamic interactions of cells with their environment, including cell-cell interactions involved in vessel integrity and transendothelial migration, as well as cell-matrix interactions between the basement membrane and ECs or platelets.

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APPENDICES

NEDERLANDSE SAMENVATTING ABOUT THE AUTHOR ACKNOWLEDGEMENTS/DANKWOORD

SAMENVATTING

Elke cel moet kunnen reageren op bepaalde prikkels om zijn functie te vervullen. Daarom brengen cellen een specifieke set celmembraanreceptoren tot expressie die een interactie aan kunnen gaan met extracellulaire moleculen. Deze extracellulaire interacties resulteren in een intracellulaire respons die kan leiden tot een verandering in het gedrag van een cel. Dergelijke veranderingen kunnen tijdelijk zijn of voor langere tijd effect hebben. Het meeste onderzoek binnen celbiologie richt zich op de rol van individuele eiwitten binnen een proces. Maar om cellulaire processen onbevooroordeeld te kunnen bestuderen zouden alle eiwitten, oftewel het proteoom, gelijktijdig in kaart moeten worden gebracht. Door de technologische ontwikkeling van de massaspectrometrie (MS) is dit de afgelopen decennia tot de mogelijkheden gaan behoren. In dit proefschrift bestuderen we de rol van receptoren in het bloedvatstelsel met behulp van MS. Hierbij kijken we voornamelijk naar twee typen cellen: endotheelcellen en macrofagen. Endotheelcellen vormen de binnenste bekleding van het vaatstelsel. Doordat deze cellen hecht met elkaar verbonden zijn ontstaat er een functionele scheiding tussen het bloed en weefsel die ervoor zorgt dat eiwitten en cellen niet uit bloedvaten kunnen lekken en *vice versa*. Naast de fysieke barrière zijn endotheelcellen ook actief betrokken bij een aantal processen, waaronder ontsteking, mechanische stress en hemostase. Om deze functies te kunnen vervullen brengen endotheelcellen een scala aan receptoren tot expressie. Op basis van de informatie van deze receptoren reguleren endotheelcellen onder andere de vasculaire tonus, de barrierefunctie van het endotheel zelf, en het uitscheiden van de inhoud van zogenaamde Weibel Paladelichaampjes (WBPs). Ontregeling van de functie van het endotheel, endotheel dysfunctie genaamd, kan leiden tot een verscheidenheid aan vaataandoeningen, waaronder een verhoogde bloeddruk, beroerte en trombose. Om te begrijpen hoe deze ziektebeelden kunnen ontstaan door de dysfunctie van endotheel is het van belang om in kaart te brengen hoe endotheelcellen reageren op een veranderende omgeving. Daarnaast richt dit proefschrift zich op de interactie van macrofagen met hun omgeving. Macrofagen zijn erop gericht om een verscheidenheid aan ongewenste deeltjes, zoals fragmenten van apoptotische cellen, pathogenen en kankercellen, te internaliseren en op te ruimen. Daarvoor bevindt zich op de celmembraan van macrofagen een verscheidenheid aan receptoren. Hoe de samenstelling van deze verzameling verandert gedurende de ontwikkeling van macrofagen en in hoeverre deze overeenkomt met de receptorverzameling van gerelateerde cellen, zoals dendritische cellen en osteoclasten, is onbekend. Bovendien is het van belang om aan het licht te brengen hoe deze receptoren met elkaar kunnen samenwerken.

In het inleidende **Hoofdstuk 1** wordt de context van dit onderzoek beschreven. Hier wordt uitvoerig stilgestaan bij de functie van endotheel, en hoe deze

verandert gedurende inflammatie en blootstelling aan stroming. Daarnaast wordt de achtergrond beschreven van de ontwikkeling van monocysten in macrofagen, dendritische cellen en osteoclasten, en wordt er stilgestaan bij de klaring van stollingseiwitten Von Willebrand Factor (VWF) en factor VIII (FVIII). Dit hoofdstuk bevat bovendien een overzicht van de verschillende MS strategieën die in dit proefschrift gebruikt zijn. Tot slot wordt benoemd welke vragen worden onderzocht in de volgende hoofdstukken.

In **Hoofdstuk 2** onderzoeken we hoe endotheelcellen reageren op de activatie van receptoren door ontstekingsopwekkende cytokines $\text{TNF}\alpha$ en $\text{IL-1}\beta$. Daartoe brengen we het proteoom van gestimuleerde en onbehandelde cellen in kaart middels MS. Hierbij zijn we in het bijzonder geïnteresseerd in celmembraaneiwwitten, aangezien deze vrijwel alle interacties met de omgeving faciliteren. We laten zien dat $\text{TNF}\alpha$ en $\text{IL-1}\beta$ vrijwel dezelfde respons induceren in endotheelcellen, hoewel deze cytokines verschillende receptoren activeren. Daarnaast laten we in dit hoofdstuk zien dat de ontwikkeling van de ontstekingsreactie grofweg twee fasen kent. In eerste instantie, binnen 4 uur, observeren we verhoogde hoeveelheden van eiwitten die een rol spelen bij leukocyt transmigration, waaronder ICAM1 en SELE. Pas in een later stadium, na 16 uur, zien we ook verhoogde hoeveelheden van eiwitten die betrokken zijn bij bijvoorbeeld antigeenpresentatie en eiwitten met een antivirale werking. Dit duidt op een tweetrapsmechanisme waarbij alleen gedurende een langdurige ontsteking endotheelcellen zelf worden bewapend om een eventuele infectie te bestrijden. Tevens bestuderen we in dit hoofdstuk de forforylering van eiwitten en integreren we de verscheidene analyses in één model. Hieruit blijkt dat inflammatie veranderingen teweeg brengt in een aantal functionele processen, waaronder leukocytmigration, cytokineproductie en antigeenpresentatie. Bovendien benadrukt deze analyse het belang van de extracellulaire matrix tijdens de ontstekingsreactie, en laat zien dat er vele tientallen veranderde eiwitten zijn waarvan het niet bekend is hoe deze een rol spelen tijdens een ontsteking. Tot slot introduceren we in dit hoofdstuk een methode om specifiek celmembraaneiwwitten chemisch te labelen en te verrijken, om deze vervolgens te analyseren door middel van MS. Deze methode biedt de unieke mogelijkheid om veranderingen binnen eiwitten waar te nemen, omdat de labelefficiëntie van een bepaalde locatie in een eiwit afhankelijk is van de toegankelijkheid voor het label. We laten zien dat een residu in het eiwit ITGA5 beter beschikbaar is voor het label, wat waarschijnlijk veroorzaakt wordt door de activatie van dit eiwit en de bijbehorende conformatieverandering.

Naast ontsteking heeft ook de bloedstroming een grote invloed op het fenotype en de functie van het endotheel. Laminaire stroming resulteert in een langwerpige fenotype waarin de cellen zijn uitgelijnd in de richting van de stroming, terwijl gebrek aan stroming of een turbulente stroming leidt tot een polygonale vorm. Dit polygonale fenotype is geassocieerd met dysfunctie van het endotheel en

kan leiden tot vasculaire aandoeningen, waaronder atherosclerose. In **Hoofdstuk 3** onderzoeken we het effect van activatie van mechanoreceptoren door stroming op de functie van endotheelcellen. Door middel van MS observeren we dat, in overeenstemming met de literatuur, het blootstellen van endotheelcellen aan stroming leidt tot veranderingen in het cytoskelet en een verhoogde hoeveelheid stikstofmonoxide-synthase. Daarnaast openbaren onze data dat er in het bijzonder veranderingen zijn in de extracellulaire matrix en celadhesie-eiwitten. We laten zien dat LAMA4 onder invloed van stroming wordt geknipt, en dat er een drastische verandering plaatsvindt in de morfologie van de extracellulaire matrix. Deze verandering wordt gekenmerkt door de vorming van een laminine netwerk. Microscopiedata suggereren dat integrine $\alpha 6\beta 4$, dat verhoogd tot expressie komt onder stromingsomstandigheden, een verbinding verzorgt tussen dit lamininenetwerk en de cel. Omdat de afwezigheid van laminaire stroming wordt geassocieerd met een ontstekingssignatuur, onderzoeken we daarnaast of de aanwezigheid van stroming de ontstekingsreactie kan beïnvloeden. Onze data tonen aan dat stroming en ontsteking een vrijwel compleet verschillende verzameling van eiwitten beïnvloeden. Bovendien heeft de aanpassing naar stromingscondities nauwelijks effect op de ontstekingsreactie. Dit impliceert dat andere factoren in de micro-omgeving van endotheelcellen uiteindelijk resulteren in het waargenomen ontstekingsprofiel in regio's zonder laminaire stroming.

Naast een adaptieve respons kan de activatie van receptoren door cytokines leiden tot de terminale differentiatie van cellen. In **Hoofdstuk 4** hebben we onderzocht hoe monocyten, na receptoractivatie door cytokines, differentiëren tot dendritische cellen (DCs), M1 en M2 macrofagen of osteoclasten. We bestuderen daarvoor het proteoom van deze cellen gedurende het differentiatieproces met behulp van MS. Om er zeker van te zijn dat de monocyten gedifferentieerd zijn in de gespecialiseerde celtypen, valideren we de expressie van celtype-specifieke eiwitten op deze cellen. We laten zien dat een grote kern van eiwitten vergelijkbare veranderingen ondergaat tijdens de differentiatie naar de verschillende celtypen. Veel van de eiwitten in deze groep die verhoogd tot expressie komen zijn geassocieerd met ontstekingsreacties of met lipidenmetabolisme. Dit laatste is eenvoudig te verklaren voor macrofagen, omdat deze cellen moeten kunnen omgaan met de opgenomen lipiden uit bijvoorbeeld apoptotische cellen, maar het blijft de vraag waarom osteoclasten en dendritische cellen deze eiwitten nodig hebben. Naast deze veranderingen, die vergelijkbaar zijn voor alle celtypen, komen er uit deze analyses eiwitten naar voren die typerend zijn voor één van de gespecialiseerde celtypen. Voornamelijk DCs wijken af van de andere gespecialiseerde celtypen en zijn goed te onderscheiden van de andere celtypen door de expressie van FCER2, CCL18 en CCL22.

Hoofdstuk 5 richt zich op opname van een enkel ligand door meerdere

receptoren. Hierbij onderzoeken we specifiek de opname van stollingseiwit Von Willebrand Factor (VWF) door macrofagen. VWF beschermt stollingsfactor VIII (FVIII) tegen klaring uit de bloedsomloop. De klaring van VWF en FVIII is van groot belang voor de behandeling van hemofilie A, een aandoening die wordt gekenmerkt door de functionele afwezigheid van FVIII. Deze afwezigheid leidt tot bloedingsneigingen en kan worden gecorrigeerd door periodieke intraveneuze toediening van FVIII. De korte halfwaardetijd van FVIII en VWF in het bloed vereist een infusiefrequentie van 2-3 keer per week. Door het remmen van de opname van VWF/FVIII, en daarmee verlengen van de halfwaardetijd van deze eiwitten, kan deze frequentie worden gereduceerd. Het is bekend dat macrofagen een belangrijke rol spelen in de opname van VWF, en verscheidene receptoren worden geassocieerd met deze klaring. Eerder onderzoek binnen onze afdeling heeft aangetoond dat macrofagen afgeleid van monocytin inderdaad in staat zijn om VWF op te nemen, zij het uitsluitend onder invloed van stroming. Door middel van MS laten we in hoofdstuk 5 zien dat macrofagen, van de receptoren geassocieerd met VWF-opname, alleen LRP-1 en MSR-1 bevatten. Om de relatieve bijdrage van deze receptoren aan VWF-opname verder te bestuderen maken we gebruik van N-terminale VWF fragmenten D'D3 en D'D3A1. We laten zien dat deze fragmenten kunnen worden opgenomen door macrofagen, onafhankelijk van stroming. Door middel van functionele proeven laten we zien dat VWF kan worden opgenomen door macrofagen door interactie van het A1 domein met LRP-1. Daarnaast tonen we aan dat het D'D3 deel van VWF een interactie aan kan gaan met MSR-1, en dat dit complex vervolgens via LRP-1 kan worden opgenomen. Bovendien tonen we dat deze laatste route ook geldt voor MSR-1 ligand acLDL. Gecombineerd suggereren deze data dat zowel MSR-1 als LRP-1 bijdragen aan de opname van VWF door macrofagen en dat de opname door MSR-1 via LRP-1 verloopt.

Ten slotte worden in **Hoofdstuk 6** de resultaten van de beschreven studies samengevat en bediscussieerd. Daarnaast bevat dit hoofdstuk microscopiedata van endotheelcellen blootgesteld aan stroming die niet zijn opgenomen in hoofdstuk 3. Tot slot bevat dit hoofdstuk enkele suggesties voor vervolgstudies op basis van de beschreven resultaten.

ABOUT THE AUTHOR

Eelke Béguin was born on January 24th 1988 in Zevenaar, the Netherlands. In 2006 he graduated from pre-university education at Liemers College and started his bachelor's studies Biomedical Sciences at the University of Utrecht. After graduating in 2009, he worked as a technician focused on protein expression at the department of Cellular Architecture and Dynamics at the University of Utrecht for 5 months. Subsequently, Eelke travelled through South-America for 6 months. In 2010, he started his master's studies Molecular and Cellular Life Sciences at the University of Utrecht. He performed his first 9 months internship at the virology division of the department of Veterinary Sciences at the University of Utrecht, where he studied the role of enzyme CELA2B in Mouse Hepatitis Virus entry. Next, he did a 3 months internship at the Department of Pharmaceutics where he worked on the cell-free expression of virus-like particles. Afterwards, he performed a 6 months internship at the Australian Disease Research Centre at the University of Queensland, Brisbane, Australia, on the localization of dengue virus non-structural protein 1 (NS1). He concluded his master studies with a thesis discussing the diagnostic opportunities for Hepatitis E. In 2014, he started his PhD training at the department of Plasma Proteins, presently named Molecular and Cellular Hemostasis, at Sanquin Research in Amsterdam, of which the results are described in this dissertation. Currently, Eelke is working as a scientist at Idris Oncology in Leiden where he specializes in hematology and cell biology.