Membrane microdomains and their role in human disease: a novel strategy for characterization disease specific alteration

Doctoral Thesis

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2010

This work was performed at the Institute of Clinical Chemistry and Laboratory Medicine at the University of Regensburg between October 2003 and March 2007 under the supervision of Prof. Gerd Schmitz and Dr. Evelyn Orsó.

~ To my parents ~

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List of Abbrevations

ABCA1	ATP-binding cassette transporter 1
AP3	Adaptor protein complex 3
APC	Allophycocyanin
ApoA-I	Apolipoprotein A-I
BSA	Bovine serum llbumin
CAD	Coronary artery disease
CD	Cluster of differentiation
CXCR4	Chemokine receptor 4
DAF	Decay accelerating factor / CD55
DIG	Detergent insoluble glycolipid-enriched domains
DMPE	1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine
DRM	Detergent resistant membrane
E-LDL	Enzymatically modified low density lipoprotein
GPI	Glycosylphosphatidylinositol
GPL	Glycerophospholipid
FCDR	Flow cytometric detergent resistance
FITC	Fluorescein-isothiocyanate
FSC	Forward scatter
GDF5	Groth differentiation factor 5
GM1	Monosialotetrahexosylganglioside
GTPase	Guanosine-5'-triphosphate hydrolase
HA	Haemagglutinin
HDL	High density lipoprotein
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
hsCRP	High-sensitivity C-reactive protein
Hsp	Heat shock protein
kDa	Kilo Dalton
LBP	Lipopolysaccharide binding protein
L_β	Solid gel phase
LDL	Low density lipoprotein

L_c	Fuid, crystalline state
L_o	Liquid-ordered state
LPS	Lipopolysaccharide
MBCD	Methyl β cyclodextrin
M-CSF	Macrophage colony-stimulating factor
MDCK	Madin-Darby canine kidney cells
MHC	Major histocompatibility complex
MFI	Mean fluorescence intensity
NF-κB	Nuclear factor 'kappa-light-chain-enhancer' of activated B cells
NOS	Nitric oxide
NOS	Nitric oxide synthase
NPC	Niemann-Pick type C disease
Ox-LDL	Oxidized low density lipoprotein
PAMP	Pathogen associated molecular pattern
PBS	Phosphate-buffered saline
PC	Phosphatidylcholine
PE	Phycoerythrin
PE	Phosphatidylethanolamine
PGN	Peptidoglycan
PerCP	Peridin chlorophyll protein
PS	Phosphatidylserine
SDS	Sodium dodecylsulfate polyacrylamide
SSC	Sideward scatter
SIRS	Systemic inflammatory response syndrome
TD	Tangier disease
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor α
T_m	Melting temperature
TMR	Tetramethylrhodamine
LTA	Lipoteicoic acid
TGN	Trans-golgi network
TX-100	Triton X-100

1. Introduction

1.1 Monocytes: the cutting edge between inflammation and atherosclerosis

Chronic inflammation is well known to play a key role both in the development and progression of the atherosclerotic plaque and its thrombotic complications, and it has been proposed as a mechanistic link between hyperlipidaemia and atherogenesis (1). The contribution of inflammatory processes in atherosclerosis has been well established, and inflammatory parameters such as high-sensitivity C-reactive protein (hsCRP) are being used for cardiac risk (2). Monocyte infiltration is crucially involved in chronic inflammatory reactions and in the pathogenesis of atherosclerosis by secreting and/or being activated by cytokines, chemotactic and adhesion molecules or growth factors (3-6). The discovery and implementation of novel biomarkers and therapeutics is of importance for stratification and management of patients with atherogenetic risk factors.
Research related to cardiovascular diseases often depends on the availability of vascular tissue, the lack of appropriate *in vitro* human disease models, and the functional complexity of the cellular dysregulations. Circulating blood cells may at least reflect part of the vascular response, and therefore provide a reasonable cell-source for atherosclerosis research: (i) monocytes are easy to access and they are critical components in atherogenesis (ii) circulating monocytes are in direct contact with the affected endovascular lumen and therefore may represent 'vascular disease reporters', (iii) monocytes have a defined pattern of surface markers that is helpful for their purification to homogeneity for gene expression analysis, (iv) immortalized human monocytic cell lines are available, which retain differentiated phenotypes, and can thus support *in vitro* studies or verification experiments.
In order to identify disease markers involved in inflammatory diseases, circulating monocytes from patients with defined disease groups can be isolated and subjected to detailed transcriptomic, proteomic, lipidomic, etc. analysis. Based on these results, novel biomarkers for diagnostic assays and therapeutic monitoring can be established for the daily practice in laboratory medicine.

1.2 Peripheral blood monocyte subpopulations and their correlation with inflammation and atherosclerosis

Circulating human blood monocytes represent a heterogeneous group of cells, and in healthy individuals at least five different monocyte subpopulations can be distinguished by cell expression densities of various antigens involved in extravasation, uptake of atherogenic lipoproteins, differentiation and inflammation. Monocyte subpopulations can be discriminated by multiparametric flow cytometry based on cellular expression densities of the 55 kDa lipopolysaccharide (LPS) receptor CD14, the FcγIIIa receptor CD16, the neural cell adhesion molecule CD56, a sialic-acid-binding immunoglobulin-like lectin (Siglec) CD33, a costimulatory molecule CD40, and the splice variants of the receptor tyrosine phosphatase CD45 (7).

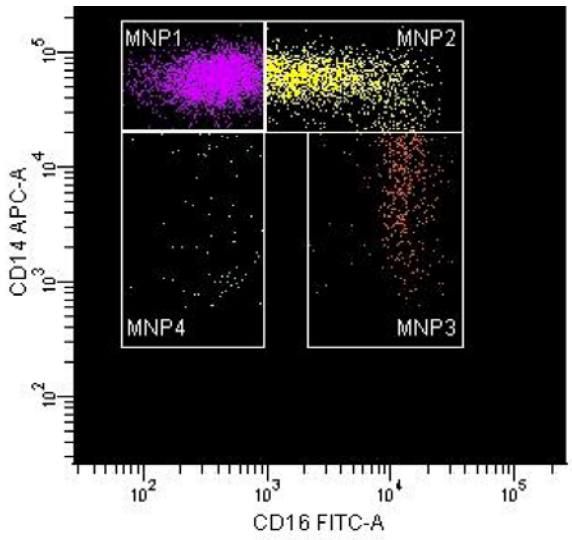

Figure 1 Classification of major monocyte subpopulations in circulating peripheral blood.

Flow cytometric characterization of monocyte subsets based on their relative expression for CD14 and CD16. The major subsets (1-4), and their relative expansions (density of dots) are indicated. Proatherogenic factors promotethe expansion of subset-3, while antiatherogenic factors diminish its occurence.

The immunophenotype of circulating monocytes clearly reflects various disease states. The Fcγ receptors, primarily CD16a/FcγRIII, together with the LPS receptor CD14, appear to be key players in defining monocyte subpopulations. Expansion of $CD14^{dim}$, $CD16^{+}$ monocytes (i.e. subset-3, see Figure 1) was observed in inflammation (8). Furthermore, this subpopulation of monocytes showed a positive correlation with elevated concentrations of plasma cholesterol, or triglycerides, or homozygous occurence of the apolipoprotein *E4* allele, or genetic deficiency of lysosomal acid lipase (7,9,10). The $CD14^{dim}$, $CD16^{+}$ monocytes are negatively correlated to plasma HDL-cholesterol and other anti-atherogenic compounds (10,11). Changes in the distribution

of monocyte subsets have also been reported with other diseases, including cancer, acute and chronic inflammation (12-18). Moreover, a functional genetic polymorphism of CD14 is associated with myocardial infarction (19,20). The pool size of $CD14^{dim}$ $CD16^{+}$ monocytes correlates with plasma lipids and lipoprotein metabolism as well as inflammation and the acute phase reaction stressing a link between peripheral blood monocyte heterogeneity and cardiovascular risk factors (4).

2. Membrane microdomains/ lipid rafts: state of the art

In the 1930´s, the very early concept of cell membranes proposed that living cells are surrounded by a thin film of lipoidal material. This was coated on both sides with water-soluble proteins, in a kind of lipoprotein sandwich. This model allowed for selective permeability of the membrane, meaning that certain molecules can pass through while others are barred (21). This model was superseded by the fluid mosaic model introduced in the 1970´s by Singer and Nicholson, proposing a uniform lipid bilayer with randomly distributed proteins (22). The model anticipated free rotational and lateral diffusion, but restricted transverse mobility of proteins and lipids within the plane of the membrane.
This concept of the plasma membrane has undergone a revolutionary change in the last few decades. Large number of evidence have indicated that membrane lipids are not homogenously distributed, but spontaneously form separate liquid-ordered phase(s) in the more loosely packed liquid-disordered phase (23,24). In liquid-ordered phase phospholipids with saturated hydrocarbon chains are tightly packed with cholesterol, nevertheless, they retain lateral mobility within the plane of the plasma membrane. These lateral microdomains, also called `lipid rafts`, are enriched in sphingomyelin, glycosphingolipids and cholesterol, which are thought to be located mainly in the outer membrane leaflet, connected with phospholipids and cholesterol in the cytoplasmic membrane leaflet (Figure 2) (23,24).

2.1 Lipid rafts: the origin of the concept

Lipid rafts were historically proposed to be lateral assemblies of specific lipids, in particular, cholesterol and glycosphingolipids, involved in the sorting of proteins to the apical surface of polarized epithelial cells (23). This theory was based on biochemical data of lipid phase separation and on the polarized distribution of certain lipids and proteins in epithelial cells, especially the apical localized glycosylphosphatidylinositol (GPI)-anchored proteins (25). The first operational definition of lipid rafts came with the demonstration that sphingolipids and GPI-anchored proteins are resistant to cold nonionic detergent extraction, classically to Triton X-100 (TX-100), and “float” like a “raft” to the top of a density gradient as a separable, cholesterol-dependent fraction (24).

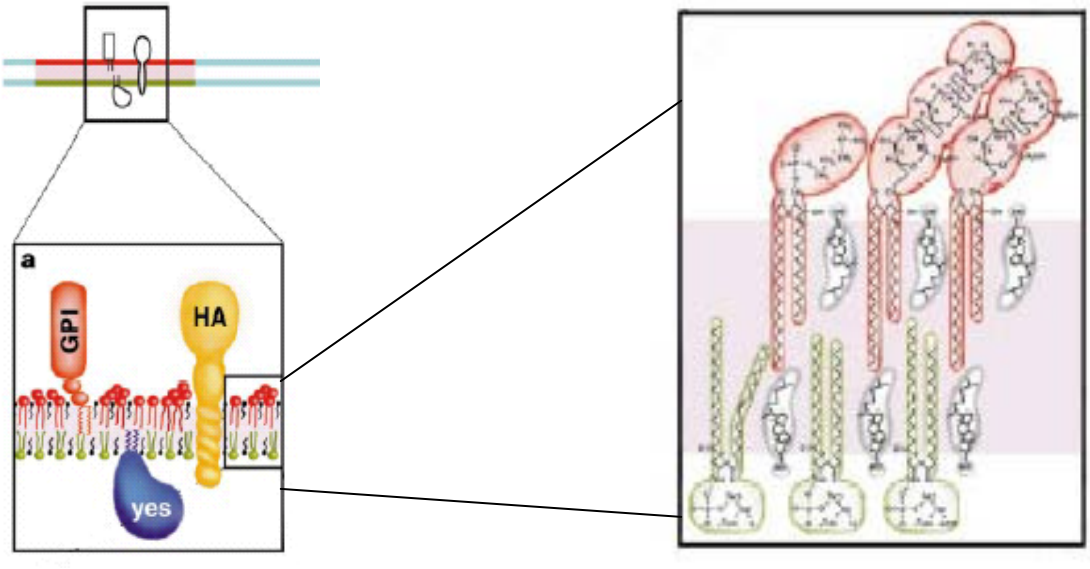

Figure 2. Model for the organization of lipid rafts in the plasma membrane.

Lipid rafts (red) segregate from the other regions (blue) of the lipid bilayer. (a) Rafts contain proteins mainly attached to the exoplasmic leaflet of the bilayer by glycosylphosphatidylinositol (GPI)-anchor, to the cytoplasmic leaflet by acyl chains (Src-family kinase Yes is shown) or proteins associating through their transmembrane domain, like haemagglutinin (HA). (b) The lipid bilayer in lipid rafts is asymmetric, the exoplasmic leaflet is enriched in sphyngomyelin (red) and glycosphingolipids (red), while the inner leaflet contains special glycerolipids (green), such as phosphatidylserine (PS) and phosphatidylethanolamine (PE). Cholesterol (gray) is present in both leaflets and fills the space between the sphingolipids. *Modified from Simons & Ikonen, 1997, Nature (23)*

Compositional analyses of detergent-resistent membranes (DRMs) showed a high proportion of cholesterol, sphingolipids and a variety of phosphatidylcholines (PCs) (26). Lipid microdomains also present a unique protein composition of constitutive raft-associated proteins connected to the lipid layer by saturated lipid moieties, which traps these proteins in an ordered environment (26,27). Proteins with high raft-affinity are anchored with GPI to the outer plasma membrane leaflet or to cholesterol itself, while proteins linked to the inner leaflet are mainly with double-acylated chains. The latter group includes several signalling molecules, such as Src-family kinases, or α-subunits of heterotrimeric G-proteins and the sonic Hedgehog proteins (23,28,29).

2.2 Caveolae

The link between rafts and caveolae has, in the past, been controversial discussed. Cells that lack caveolins, such as cells from the haematopoeitic lineage, lack the flask-shaped invaginations but do contain DRMs (23). The consensus view is now that caveolae are a subset of lipid rafts. Caveolae are only the morphologically identifiable type of membrane microdomains and have been recognized as stable flask-shaped invaginations of the plasma membrane (30,31). They are also enriched in cholesterol and sphingolipids and only distinguishable from bulk lipid rafts by the presence of caveolin (Figure 3).

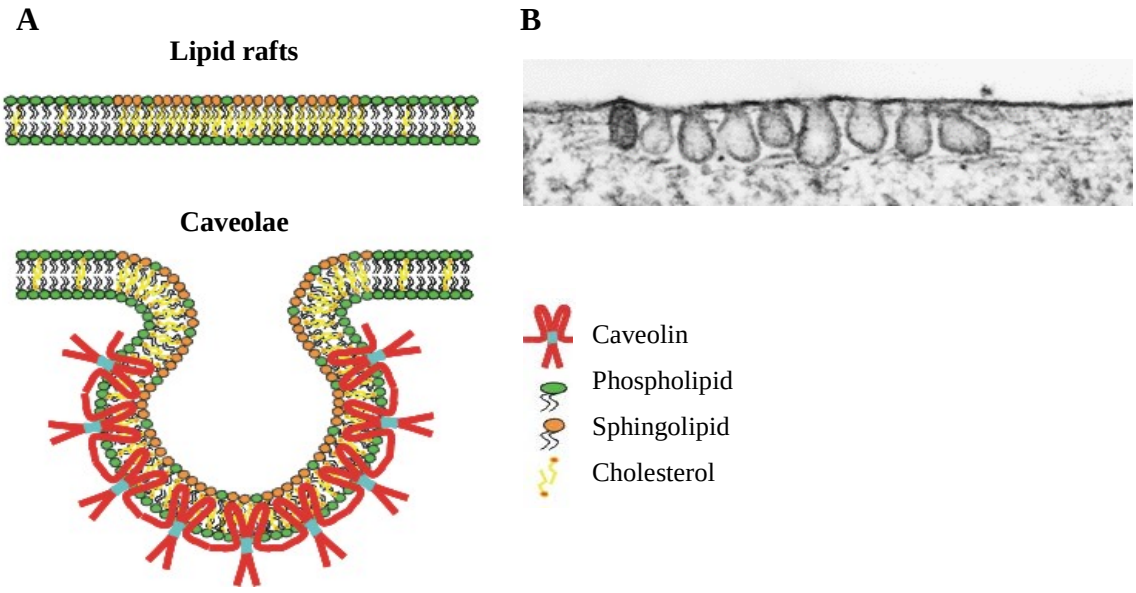

Figure 3. Model for the organization of lipid rafts and caveolae in the plasma membrane.
(A) Lipid rafts: the liquid-ordered phase is dramatically enriched in cholesterol (shown in yellow) and exoplasmic oriented sphingolipids (sphingomyelin and glycosphingolipids) (shown in orange). In contrast, the liquid-disordered phase is composed essentially of phospholipids, such as phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine (shown in green). Caveolae: the liquid-ordered and liquid-disordered phases are illustrated as in lipid rafts. Upon integration of the caveolin-1 protein, liquid-ordered domains form small flask-shaped invaginations called caveolae. Caveolin-1 oligomers are red and the caveolin-1 oligomerization domain is shown in blue. (B) Electron micrograph of a fibroblast in cross-section showing caveolae as deep indentations in the plasma membrane. *Modified from Razani et al., 2002, Pharmacological Reviews (32)*

Caveolae are formed from lipid rafts by polymerization of caveolins — hairpin-like palmitoylated integral membrane proteins that tightly bind cholesterol (33,34). The caveolin proteins (caveolin-1, -2, and -3) serve as the structural components of caveolae, while also functioning as scaffolding proteins, capable of recruiting numerous signaling molecules to caveolae, as well as regulating their activity. Several functions of caveolae have been proposed, such as endocytosis, cell signalling, cholesterol homeostasis and lipid trafficking (23,35). Moreover, caveolae have been implicated in various human diseases, including diabetes, cancer, cardiovascular disease, atherosclerosis, pulmonary fibrosis, and a variety of degenerative muscular dystrophies (32,34,36).

2.3 Biophysical properties of domains formation

2.3.1 Lipid phase behaviour of lipid microdomains

Lipid bilayers can exist in many physical states, such as solid gel (L_β) and fluid, crystalline states (L_c) (37,38). Pure phospholipids exist in a solid, ordered gel phase at low temperatures. Above a melting temperature (T_m), which is characteristic of each lipid, pure lipids form the fluid, disordered L_c phase. Most of the phospholipids in cell membranes have low T_m values, therefore, at 37°C (above T_m) are believed to be in the L_c phase. In the solid-like gel state lipids are ordered and tightly packed with the acyl chains extended and have a little lateral mobility. In contrast, in the L_c state the acyl chains are packed loosely, and have relatively rapid lateral diffusion (Figure 4A).

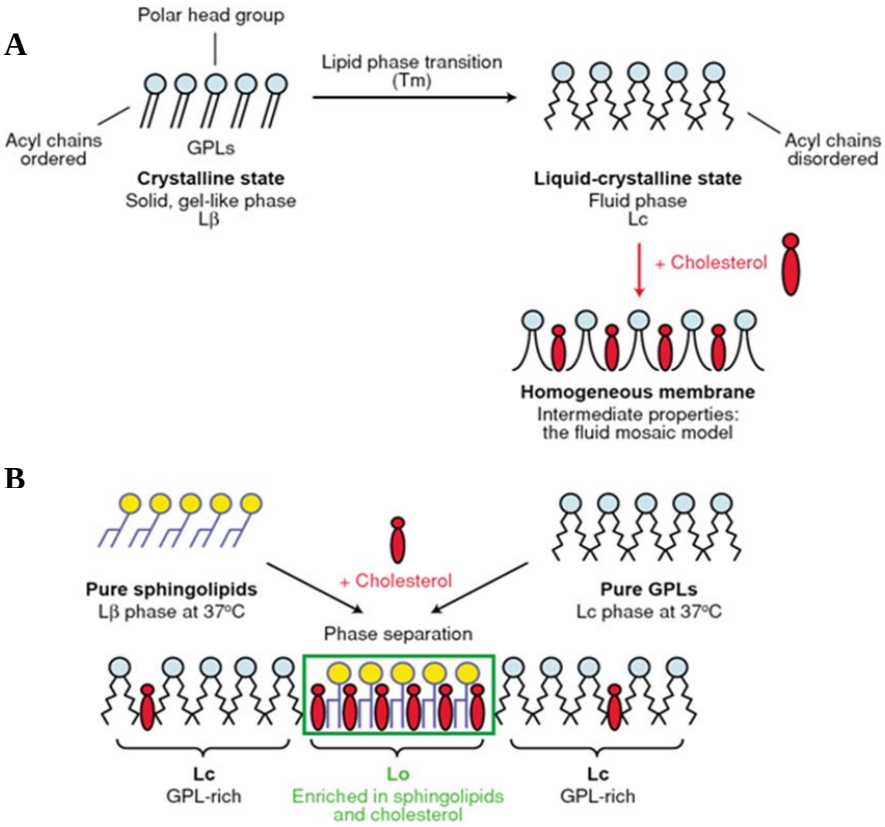

Figure 4. Cholesterol favours phase separation of membrane lipids

(A) The addition of cholesterol to pure glycerophospholipids (GPLs) abolishes the normal thermal transition between L_β and L_c phases, giving membrane properties intermediate between the two phases. (B) In contrast, cholesterol interacts preferentially (although not exclusively) with sphingolipids and favours the phase separation between sphingolipids and GPLs. In the plasma membrane, GPLs form a

relatively cholesterol-poor L_c phase, whereas sphingolipids form a liquid-ordered (L_o) phase highly enriched in cholesterol. *Modified from Fantini et al., 2002 (39)*

Phase separation between lipid in different physical states has been well characterised in model membranes (40-44). L_o phase domains can co-exist in bilayers with domains in the more familiar gel or liquid crystalline (L_c) phases (45).

The L_o phase, which requires sterol to form, has properties intermediate between gel and L_c phases. It has been observed in mixtures of cholesterol with lipids whose highly saturated acyl chains gives them a high T_m in the pure state (46,47). Cholesterol is thought to contribute to the tight packing of lipids in L_o domains by filling interstitial spaces between lipid molecules (Figure 4B). Both saturated chain phosphatidylcholines and sphingolipids can form the L_o phase when they are mixed with cholesterol. However, the formation of L_o domains is seen only within certain ranges of cholesterol concentration (48).

2.3.2 Detergent resistence in model membranes

Model membrane studies demonstrated that lipid bilayers in tightly packed configurations (L_o state) are less susceptible to solubilisation by low amounts of cold non-ionic detergents, presumably tight packing reduces access to the hydrophobic core by the detergent molecules (49,50). These studies showed that detergent insolubility is a useful method to study phase behaviour in model membranes. Ahmed et al. studied the phase state of a series of sterol-rich mixtures containing varying amounts of order- and disorder-preferring lipids by a fluorescence-quenching assay. This method determined the amount of order-preferring lipid required for phase separation. DRMs could only be detected in mixtures that contained L_o-phase domains before the addition of TX-100 (51). Dietrich et al. presented using two-phase supported lipid bilayers that separate L_d and L_o domains can be visualized by microscopy (52). TX-100 selectively solubilized L_d-phase domains, leaving the L_o-phase domains relatively unchanged as DRMs. Both of these approaches showed that TX-100 did not substantially change the relative amounts of membrane in the two phases.

2.3.3 Detergent resistance in biological membranes (DRM´s)

The characteristic partitioning of raft-associated lipids into ordered lipid phases renders them relatively insoluble in certain detergents, such as TX-100 at 4°C. Accordingly, rafts can be readily purified as detergent-insoluble, glycolipid-enriched complexes (DIGs) (35) or detergent-resistant membranes (DRMs) by ultracentrifugation on sucrose density gradients. The migration of DRMs to these low-density layers is consistent with the relatively high lipid content of these fractions.

Sphingolipids and (GPI)-anchored proteins (whose acyl chains are long and saturated), as well as cholesterol, are enriched in the cold detergent–insoluble fractions (48,50). Depletion of cholesterol or sphingomyelin from biological membranes causes a reduction in detergent insolubility, which is consistent with the idea that L_o-like lipid organizations are responsible for the DRMs in cell membranes (48,50). Many proteins including exoplasmic GPI-anchored proteins, cytosolic lipid-linked non-receptor tyrosine kinases, transmembrane proteins, caveolin, and a specific subset of lipids are insoluble in non-ionic detergent. A rigorous physical explanation for detergent-insolubility of membrane components is not available; detergent-insolubility of lipids as a phenomenon strongly correlates with the co-existence of liquid-ordered phases in a fluid (liquid-disordered) membrane.

Taken together, these data strongly suggested that DRMs correspond to lipid membrane microdomains/lipid rafts, and the TX-100 extraction procedure has become the most popular method of raft isolation from natural and artificial membranes. However, this method requires isolation at 4°C, a temperature that could artefactually increase or even induce raft formation in the plasma membrane, leading to the questioning of the existence of rafts by several investigators (53-55). Nevertheless, it is possible to work within the confines of this artefactual system. Detergent solubility/ insolubility are not strict criteria in themselves; however, many of the major breakthroughs in the raft field originate from observations of changes in DRM association upon induction of physiologically relevant stimuli. The analysis of DRM partitioning explored many raft-related phenomena, including roles in disease pathogenesis, membrane trafficking and identifying ‘raftophilic’ peptide moieties (56).

2.4 Current view of lipid rafts

The Keystone Symposium 2006 on Lipid Rafts and Cell Function agreed in a consensus definition of membrane rafts (57): “Membrane rafts are small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein-protein and protein-lipid interactions (Figure 5).”

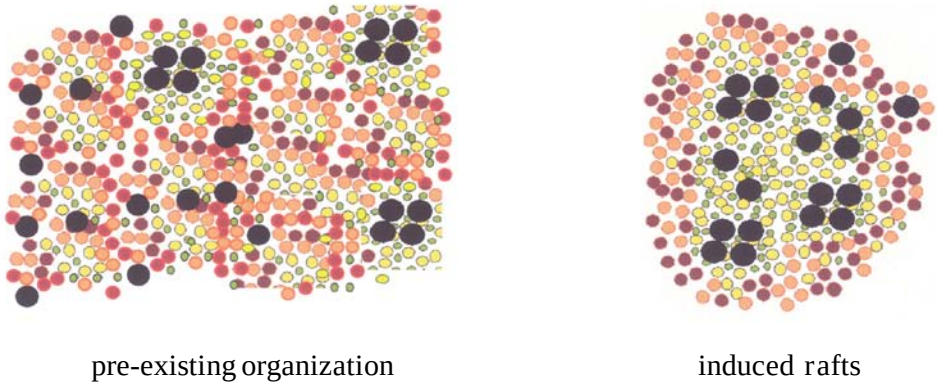

Figure 5. Current view of lipid rafts: actively generated spatial and temporal organization of raft components.

Data from GPI-anchored protein studies in living cells, suggest that preexisting lipid assemblies are small and dynamic, and coexist with monomers. They are actively induced to form large-scale stable ‘rafts’. Black circles, GPI-anchored proteins; red and pink circles, nonraft associated lipids; yellow circles, raft-associated lipids; green, cholesterol. *Modified from Mayor et al., 2004, Traffic (58)*

2.5 Functions of lipid rafts

Raft microdomains are thought to be involved in several cellular functions, including endocytosis, pinocytosis, membrane sorting and trafficking, compartmentalization of receptors, cell signalling and serve as an entry site for various pathogens or toxins (23,59-63). Table 1 presents a classification of membrane microdomains according to their trafficking and sorting response, as well as, their relation to diseases (64).

2.5.1 Membrane sorting and trafficking

Lipid rafts have been implicated in protein and lipid sorting at various places in the cells (65). Membrane microdomains are involved in sorting mechanism in apical membrane in endothelial cells, in Rab GTPase localization, the AP3 pathway, in exocytic/biosynthetic as well as endocytic transport routes and in the formation of lipid droplets (Figure 6).

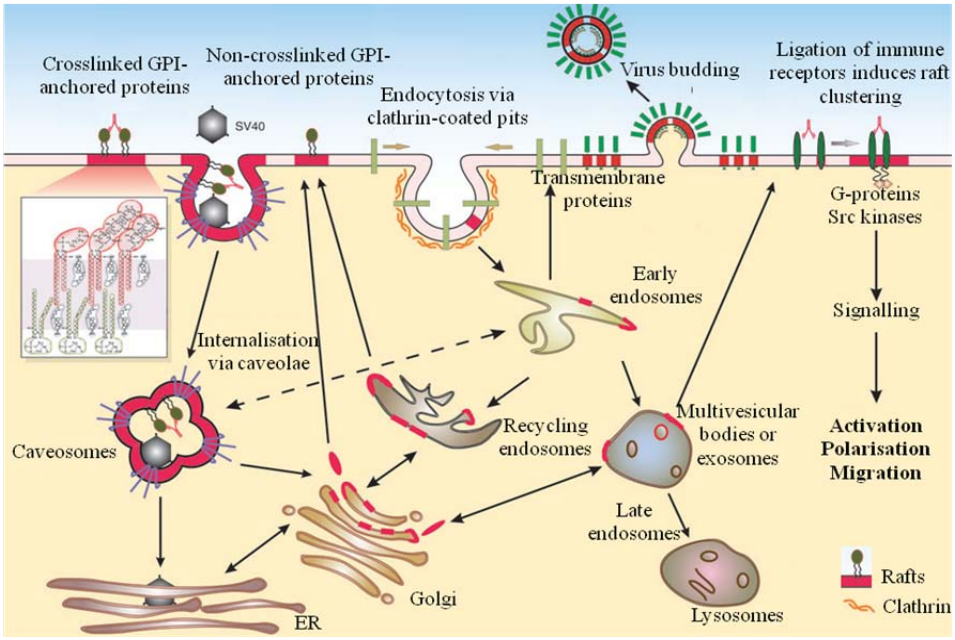

Figure 6. Schematic representation of lipid membrane trafficking, indicating the various steps where specific sorting of different classes of lipids occurs. *Modified from Rajendran & Simons, 2005, Journal of Cell Science (65)*

At the first time, Simons and van Meer supposed by studying polarized epithel cells (Madin-Darby canine kidney, MDCK cells), that the high content of glycosphingolipids in the apical membrane resulted from the differential intracellular sorting of these lipids to specialized apical carrier vesicles in the trans-Golgi network (TGN) (66,67). The apical route is that preferentially transports sphingolipid-cholesterol microdomains carrying GPI-anchored proteins and apical membrane proteins.

Many raft proteins need to be recycled, possibly involving the same signals and mechanisms that affected their delivery to the plasma membrane. Lipid microdomains are also involved in transporting proteins in the endocytic pathways. Endocytosis is complicated and now comprises at least four different, relatively well defined routes of internalization. Clathrin-coated pits may exclude rafts, whereas caveolae and two newly identified clathrin- and caveolae-independent mechanisms seem intimately linked to rafts (Figure 6) (65,68).

Function	Details	References
Trafficking and sorting		
	Apical membrane microdomains	(69)
	Rab GTPase microdomains	(70)
	PI(4,5)P2-actin microdomains	(71)
	AP-3 microdomains	(72)
	Synaptic vesicles/exocytosis microdomains	(73)
	COPII vesicles	(74)
	ER/Lipid droplet surface monolayer membrane microdomains	(75)
Disease related		
	Ciliary prominin microdomains	(76)
	Ceramide microdomains/association with phospholipidosis	(72,77-79)
	Caveolae	(35)
	Virus envelopes	(80)
	Immunological synapse	(81,82)

Table 1 Classification of lipid membrane microdomains

Modified from Schmitz G. and Grandl M., 2008 , Curr Opin Clin Nutr Metab Care (64)

2.5.2 Compartmentalization of receptors and cell signalling

One of the major functions of rafts is the recruitment and concentration of receptors and effectors involved in cellular signalling. Lipid rafts are thought to provide the infrastructure for bringing certain receptors and downstream signalling molecules into proximity, permitting the formation of competent signalling assemblies. There are many instances whereby receptor redistribution into lipid rafts is necessary for certain signalling cascades to proceed.

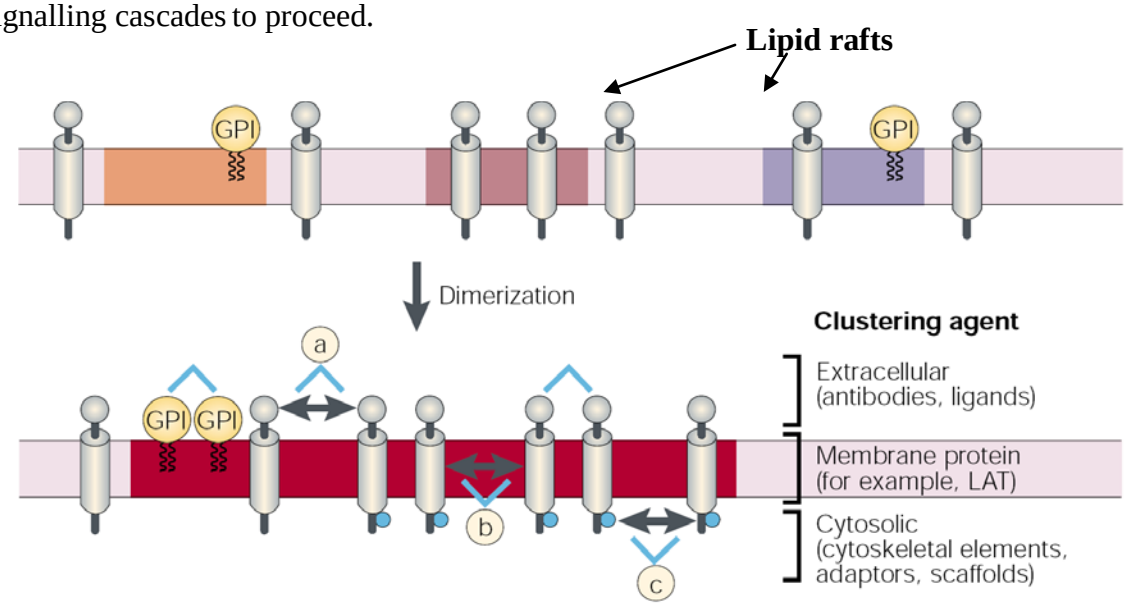

Figure 7. Mechanism of receptor clustering

(a) Rafts are small at the plasma membrane, containing only subset of proteins. (b) Raft size is increased by clustering, forming larger platform in which functionally related proteins can interact. Clustering can be triggered (1) at the extracellular side by ligands, antibodies or lectins, (2) within the plasma membrane by oligomerization, or (3) by cytosolic agents (cytoskeletal elements, adapters, scaffolds). *Modified from Simons K. and Ehehalt R. J. Clin. Invest. (2002)(28)*

Formation of higher-order signalling complexes by clustering of lipid microdomains allows amplification and/or modulation of signals, hereby, lipid rafts are involved in facilitating efficiency and specificity of signalling (Figure 7). Several signalling events have been proposed to associate to lipid rafts. Immune cell receptors, especially T-cell antigen receptor (TCR) (83-90), B-cell antigen receptor (BCR) (91-98), the high affinity IgE receptor FcεRI of mast cells (99-103) or CD14-dependent lipopolysaccharide (LPS)-induced receptor clustering (104-107) are the most extensively investigated examples.

2.5.3.1 Lipopolysaccharide (LPS)-induced CD14-dependent receptor clustering

The recognition of bacterial LPS seems to involve a complex orchestration of protein-protein interactions on the cell surface that finally leads to cellular activation. LPS is the major pathogen-associated molecular pattern (PAMP) in the cell wall of gram-negative bacteria, and is recognized by GPI-anchored CD14 and its co-receptors. The interaction leads to the expression of inflammatory mediators and to the activation of antimicrobial host defense mechanisms (108). Lipid raft integrity is essential for LPS-induced cellular activation, since raft-disrupting drugs, such as nystatin or MBCD, inhibit LPS-induced tumor necrosis factor α (TNF-α) secretion (109).

Several studies were objected to clarify the molecular events that lead to LPS-induced signal transduction. CD14 was the first molecule to be identified as a receptor for LPS (110). Since the LPS receptor CD14 is a GPI-anchor protein without a cytoplasmic tail, it has been suggested that additional signalling receptors co-associate with CD14 in order to initiate signal transduction cascades. Initial data showed that LPS must bind to CD14 and is then transferred to an immobile receptor or a cluster of receptors (104,111). Using fluorescence resonance energy transfer (FRET), Triantafilou et al. identified a structurally heterogeneous complex of receptors concists of heat shock proteins (Hsp) 70 and 90, chemokine receptor 4 (CXCR4), growth differentiation factor 5 (GDF5), CD55 and TLR-4 that is formed after LPS stimulation (Figure 8) (107).

Another group of surface antigens, involved in LPS recognition and signalling, are the β2 integrins CD11b/CD18 (Mac-1) (112,113). It could be demonstrated that anti-Mac-1 antibodies blocks LPS-induced inflammatory cytokines production in murine macrophages (114). CD14 and β2 integrins seem to be both required to the activation of signalling pathways in response to LPS.

Pfeiffer et al. (104) provided further information on CD14-dependent receptor clustering induced by extracellular ligands (lipopolysaccharide, LTA and ceramide), followed by subsequent changes in the dynamics of membrane raft domains. A so-called `basal' receptor cluster has been identified on resting (unstimulated) human monocytes ex vivo, which includes the GPI-anchored receptors CD14 and CD55, the

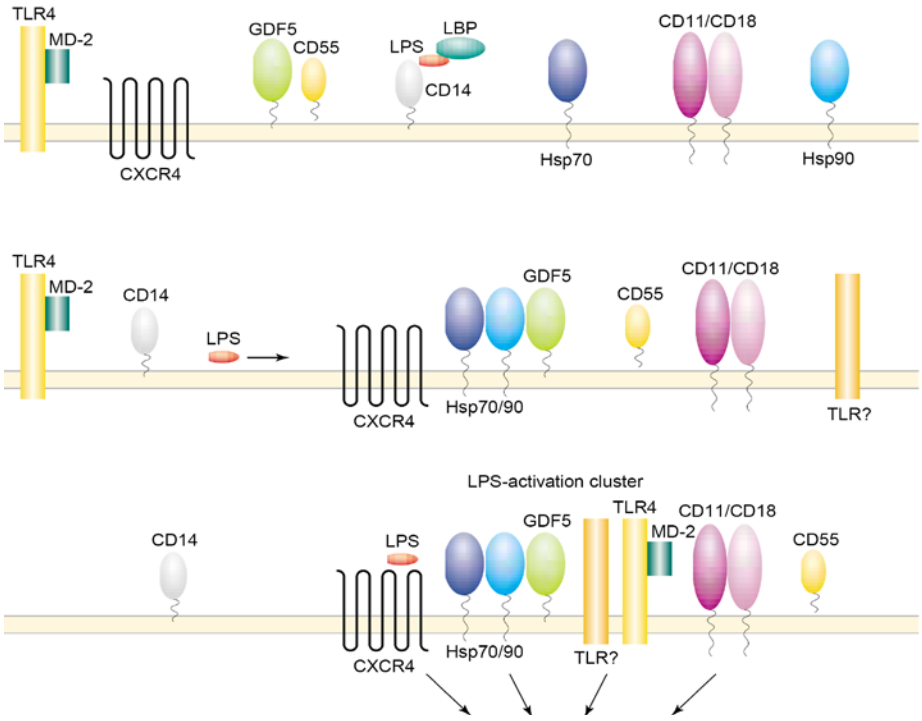

Figure 8. Hypothetical model for the immune recognition of bacteria.

(a) Lipopolysaccharide-binding protein (LBP) binds and catalyses the transfer of LPS to membrane-bound CD14 (mCD14). (b) Signalling molecules are recruited to the site of CD14–LPS ligation. LPS is released from CD14 in the lipid bilayer, and the intercalated LPS binds to a complex of receptors, consist of chemokine receptor 4 (CXCR4), heat shock proteins (Hsps) 70 and 90, growth differentiation factor 5 (GDF5) and possibly CD55. (c) Signal transducing molecules, such as Toll-like receptor 4 (TLR4) complexed with MD-2, Toll-like receptors (TLRs) and/or integrins CD11 or CD18 are further recruited into the activation cluster, triggering multiple signalling cascades. *Modified from Triantafilou et al. 2002, TRENDS in Immunology (107)*

Fcγ receptors CD32 and D64, and the pentaspan molecule CD47 (Figure 9). Activation of monocytes by LPS or lipoteichoic acid (LTA), related to exogenous host defense, leads to coassembly of further receptors, such as members of the toll-like receptors TLR2 and TLR4, the scavenger receptor CD36, the Fcγ receptor CD16, the complement receptor-3 complex (CD11b/CD18) and a member of the tetraspanins, CD81. Interestingly, CD47 is excluded from the receptor cluster in response to LPS/LTA, but in parallel with the pentaspan exclusion, the tetraspanin molecule CD81 associates with the receptor complex (Figure 9).

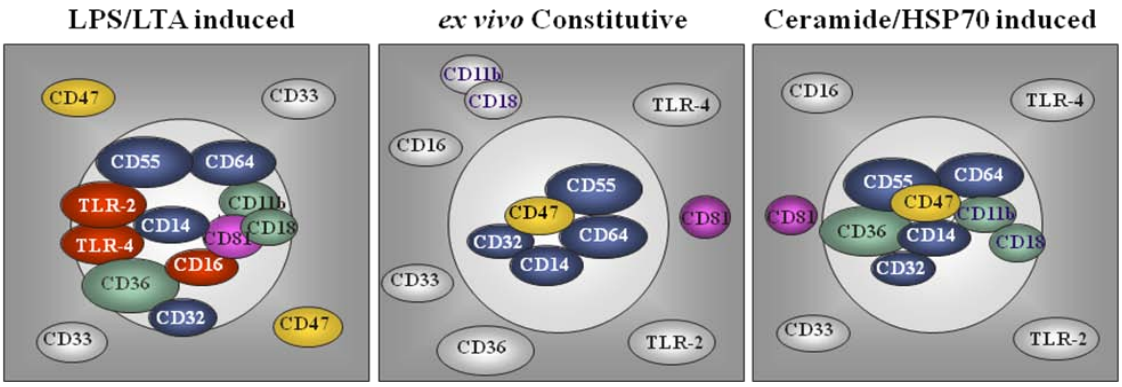

Figure 9. Scheme for formation of integrated CD14-dependent receptor clusters on human monocytes induced by lipopolysaccharide (LPS), lipoteichoic acid (LTA), ceramide or heat shock protein-70 (HSP70) ligand binding. *Modified from Pfeiffer et al., Eur. J. Immunol. (2001) (104)*

2.5.3.2 Atherogenic molecular components-induced CD14-dependent receptor clustering

Although LPS and related molecules serve as the major ligands for CD14, activation of the CD14-dependent anionic phospholipids receptor complex by atherogenic lipoproteins are also involved in certain signalling events in monocytes/macrophages. According to Pfeiffer et al. (104), binding of ceramide, which is released from atherogenic lipoproteins or apoptotic cells, to CD14 induces a receptor cluster including GPI-anchored receptors CD14 and CD55, the Fcγ receptors CD32 and CD64, the scavenger receptor CD36, the complement receptor-3 complex (CD11b/CD18) and the pentaspan molecule CD47C (and probably others), but partially different from a lipopolysaccharide-induced cluster (Figure 9). Formation of this cluster leads to activation of signalling pathways partially distinct from lipopolysaccharide signalling, very likely mainly through CD11b/CD18.

2.5.3 Lipid rafts as sites for host-pathogen interactions

A wide range of pathogens, including viruses, parasites, bacteria and their toxins use membrane microdomains, and especially ceramide membrane microdomains, to enter host cells as an infection strategy. Both cell-surface GPI-anchored proteins and raft lipids are utilized as primary or accessory receptors by invading pathogens (Table 2).

Lipid rafts were initially implicated as a binding site for bacterial toxins. Firstly, the pore-forming toxin aerolysin was found to bind to lipid rafts through GPI-anchored proteins (115). Lipid rafts not only provide a binding sites, but also increase the local toxin concentration, thereby, promote oligomerization, which is a prerequisite for channel formation (116). The function of lipid rafts as concentration devices was later extended to other toxins requiring oligomerization, like perfringolysin O, streptolysin O and listerolysin O (116,117). On the other hand, multimeric toxins were also found to associate with lipid rafts, such as cholera toxin, which offer clusters of receptors. Cholera toxin interacts via its pentameric binding unit the ganglioside GM1 molecules. The binding and uptake of several bacteria was also shown to be mediated by lipid membrane microdomains. For instance, *E.coli* strains expressing the lectin FimH (bacterial adhesin) enter and survive in host cells via GPI-anchored protein CD48 (118,119). *Shigella flexneri* invades epithelial cells by using the raft-associated CD44 receptor at the basolateral side of the cell membranes (120). Uptake of the intracellular pathogens *Mycobacteria* requires high affinity cholesterol binding. Cholesterol also mediates the phagosomal association of coronin-1, a coat protein that prevents degradation of mycobacteria in lysosomes (121).

Viruses also target membrane microdomains during entry into cells, as well as, during assembly of viral components and before budding out of the infected cells. The non-enveloped virus SV-40 binds the GPI-linked class I major histocompatibility complex (MHC) molecule on host cells, which target the virus to caveolae and delivers the virus into the endoplasmic reticulum. Several other viruses, including enterovirus 70 and coxsackievirus B virus use the decay-accelerating factor (DAF/CD55) as a receptor during entry into the host cells. Enveloped viruses also use rafts during internalization

Agents	Raft component	Step requiring lipid rafts	References
Toxins			
Lipopolysaccharide	CD14	Signalling platform	(104,107,109)
Heat labile enterotoxin	GD1	High affinity binding, entry	(122,123)
Aerolysin	GPI-anchored proteins	Oligomerization, signalling	(124)
Perfringolysin O	Cholesterol	Oligomerization, signalling	(125-127)
Streptolysin O	Cholesterol	Oligomerization	(128-130)
Listeriolysin O	Cholesterol	Oligomerization, signalling	(131-133)
Lysenin	Sphingomyelin	Binding, oligomerization	(134)
Vibrio cholerae cytolysin	Cholesterol,	Binding, oligomerization	(135-137)
Cholera toxin	GM1	High affinity binding, entry, signalling platform	(138,139)
Helicobacter pylori toxin VacA toxin	GPI-anchored proteins, sphingomyelin	Binding, oligomerization, internalization	(140-143)
Antrax toxin		Binding, oligomerization, endocytosis	(144-146)
Bacteria			
FimH E. coli	CD48	Raft-mediated internalization	(147-149)
Diffusely adhering E. coli	CD55/DAF	Intracellular survival	(150-153)
Shigella flexneri	CD44	Entry, signalling	(154)
Salmonella typhimurium	EGF receptor	Signalling	(155-157)
Campylobacter jejuni	Cholesterol	Entry	(158)
Mycobacteria	Cholesterol	Entry	(159,160)
L. monocytogenes	E-cadherin/HGF-R	Entry	(161)
Legionella pneumophilia		Entry	(162)
Parasites			
Toxoplasma gondii	CCR5	Penetration, invasion	(163)
Trypanosoma cruzi	Cholesterol, GM1	Entry, invasion	(164-166)
Plasmodium falciparum		Endovacuolation, macromolecular transport	(167-171)
Viruses			
SV40	MHC class I	Caveolae mediated	(172,173)
Enterovirus 70	CD55/DAF	Entry	(174)
Echoviruses	CD55/DAF	Entry	(175,176)
Coxsackievirus B	CD55/DAF	Entry	(177,178)
HIV	CD4, CCR5	Entry, budding/viral fusion	(179-182)
Semliki-forest virus	Cholesterol, sphingolipids	Entry, budding	(183,184)
Ebola, Marburg virus		Entry, budding	(185-187)
Measles virus		Assembly, budding	(188,189)
Influenza virus		Assembly, budding	(190-193)
HSV, EBV		Signalling, budding	(194,195)

Table 2. Pathogens and their components target lipid rafts.

and/or fusion process. For example, the fusion of Semliki-forest virus depends on the presence of cholesterol and sphingolipids in the membranes (196). The initial binding of gp120 subunit of HIV to CD4 promotes clustering of lipid rafts allowing further interactions of HIV with its co-receptor CCR5, which is necessary for the entry process (197,198). Assembling and budding of several viruses have been also reported to involve lipid rafts, including measles and influenza viruses, herpes simplex virus or HIV.

Taken together, membrane microdomains appear to be involved in the re-organization of receptors and intracellular signalling molecules in the cell membrane, permitting interaction of the pathogen with the cell.

2.5.4 Lipid rafts and disease pathogenesis

Regarding the major role of lipid rafts in distinct cellular functions, they have been implicated in the pathogenesis of several human diseases, such as neurological diseases (Alzheimer´s, Parkinson´s and prion disease), infectious and inflammatory diseases (SIRS/sepsis), cardiovascular and metabolic disease, autoimmunity (systemic lupus ertyhematodes [SLE] and rheumatoid arthritis) and lipid storage dieases (Niemann-Pick and Gaucher disease). Table 3 summarizes diseases for which membrane microdomains/rafts and membrane raft proteins are recently implicated.

Diseases	References
Neurodegenerative diseases	
Alzheimer disease	(199-203)
Parkinson disease	(204-206)
Polyneuropathies, demyelinating diseases	(207-210)
Prion disease	(211-213)
Muscular dystrophy	(214,215)
Lysosomal storage disease	
Niemann-Pick disease	(216,217)
Pilzaeus-Merzbacher disease	(218)
Tay-Sachs disease, Morbus Fabry	(219,220)
Immunity	
Autoimmun diseases, chronic inflammation	(221-224)
Asthma and allergic response	(225,226)
B cell response, T cell response	(63,227,228)
Sepsis, septic shock	(229,230)
Metabolic diseases	
Atherosclerosis, cardiovascular disease	(105,231)
Hypertension, hemodynamic regulation	(232)
Diabetes	(233-237)
Others	
Neoplasia	(238-240)
Paroxysmal nocturnal haemoglobinuria	(241)
Osteoarthritis	(242)

Table 3. Diseases for which rafts and raft-associated proteins have been supposed to be responsible.

2.5.4.1 Niemann-Pick type C disease

Niemann-Pick type C (NPC) disease is an autosomal recessively inherited neurodegenerative lysosomal storage disease, due to the defect(s) of the *NPC1* or *NPC2/HE-1* genes (243). NPC disease manifests as a progressive, eventually fatal neurovisceral storage disorder (244). The age of onset and the clinical manifestations are very heterogeneous. Most often, the onset of symptoms occurs in early childhood, leading to fatalities by the teens. Symptoms include hepatosplenomegaly, ataxia, dystonia, seizures, vertical supranuclear gaze palsy (paralysis of down-gaze), and progressive dementia.

Mammalian cells acquire cholesterol primarily from endocytosed low-density lipoproteins (LDL). The Niemann-Pick type C-1 (NPC1) protein is responsible for intracellular trafficking of LDL-derived free unesterified cholesterol. Dysfunctional NPC1 activity leads to development of NPC disease, characterised by altered endosomal sorting and vesicular trafficking, subsequent upon the massive accumulation of cholesterol and glycosphingolipids in late endosomal/lysosomal (LE/L) compartments (Figure 10).

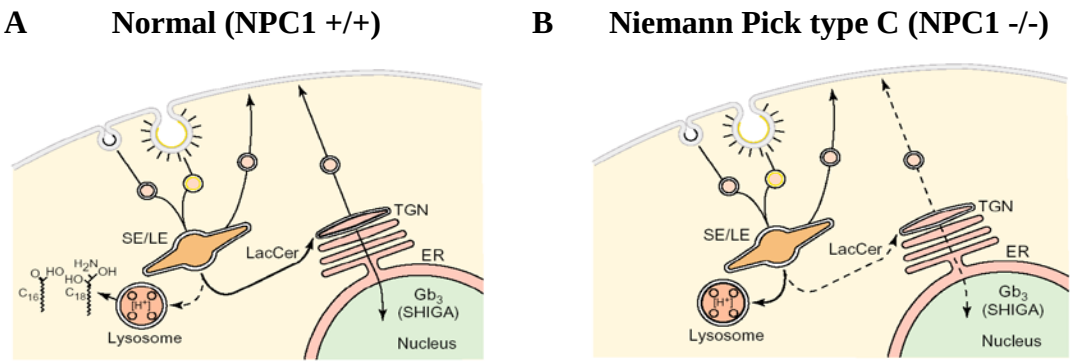

Figure 10. Altered endocytic sorting and membrane trafficking in Niemann-Pick type C disease.
(a) Defective hydrolysis of specific sphingolipids leads to the accumulation of shpingolipids and cholesterol in the endosomal compartment. *Modified Sillence D.J. and Platt F.M. TRENDS in Cell Biology, (2003)(216)*

In normal cells, endocytosed LDLs are delivered to the LE/Ls, where they are hydrolyzed and free cholesterol is released. Homeostasis is achieved when cholesterol is then rapidly transported out of the LE/Ls to the plasma membrane and endoplasmic

reticulum (ER), or first to the *trans*-Golgi network (TGN) and then to the ER (245). In NPC cells, cholesterol uptake and delivery to the late endosomes/lysosomes, and cholesterol ester hydrolysis are normal. However, cholesterol does not exit the endocytic pathway. It has been supposed that in *NPC1* deficient cells accumulation of lipid rafts occurs in the late endosomes and lysosomes, while in normal cells lipid microdomains are only present in early and recycling endosomes (216,246).

In addition to its role in sterol trafficking, some studies suggest that the NPC pathway may be directly involved in trafficking multiple proteins from LE/L compartments. Late endosomes act as sorting organelles to deliver endocytosed molecules to lysosomes for degradation, while at the same time returning other classes of proteins and lipids for transport back to nondegradative compartments (247,248)**.**

2.5.4.2 Membrane microdomains in inflammatory diseases

As discussed previously, membrane microdomains have been shown to mediate internalization of bacteria, viruses and parasites into the host cells, and to regulate the release of inflammatory cytokines from infected cells. Recognition of invasive microbial pathogens is mediated by pattern recognition receptors on the surface of immune cells that recognize PAMS. In gram-negative sepsis, the LPS-binding protein (LBP), CD14 and Toll-like receptor 4 (TLR4) are key molecules for the recognition of LPS. In gram-positive sepsis, components of the bacterial cell wall, peptidoglycan (PGN) and LTA have been shown to activate myeloid cells through an interaction with a receptor complex composed of CD14, TLR2, and perhaps also TLR6 (PGN) or TLR4 (LTA). Gram-positive exotoxins can also act as superantigens, and directly stimulate T lymphocytes by cross-linking the MHC class II of antigen presenting cells to specific chains of the T cell receptor within lipid microdomains (249).

Reactive oxygen species (ROS) can affect the pathogenesis of sepsis by modulating the innate immune signalling cascade within lipid rafts. Oxidative stress alters LPS-induced TLR4 signalling, induces translocation of TLR4 receptor to lipid rafts in macrophages (250,251).

Lipid rafts are also involved in chronic inflammatory diseases, such as SLE. T cells freshly isolated from patients with SLE were found to express higher levels of cholesterol and GM1, constituents of lipid rafts. An increase in lipid raft formation could increase the strength of the T cell signal by reducing the threshold for T cell stimulation (252). Furthermore, lipid rafts from SLE T cells were found to contain higher amounts of CD45 and co-immunoprecipitation experiments revealed that a larger fraction of this pool was associated with Lck. Enhanced localization of CD45 into lipid rafts is a key step for Lck conversion to the active form and a critical step in the activation of T cells (253). There is evidence for a role for lipid rafts in other autoimmune diseases as well. For example, $CD4^{lo}CD40^{+}$ is an autoreactive T-cell subpopulation recently identified to be expanded in autoimmunity (254).

2.5.4.3 Membrane microdomains in cardiovascular diseases

The existence of membrane microdomains in endothelial, vascular smooth muscle cells and macrophages, which are causally related to cholesterol enrichment during atherosclerosis, has been recently implicated. Studies on a correlation between lipid rafts and cardiovascular disease have mainly focused on receptor-mediated signalling in endothelial cells of arteries and the heart muscle. Several mediators of vascular function, including G-protein-coupled receptors, Src family tyrosine kinases, protein phosphatases and nitric oxide synthase, are concentrated within lipid membrane microdomains/caveolae (36,255-257). In particular, binding of angiotensin II to its receptor (angiotensin II receptor) results in the association of the receptor with microdomains in vascular smooth muscle cells (249,258). Nitric oxide (NO), which in contrast with angiotensin II promotes vasodilation and muscle relaxation, is produced in cardiac myocytes by the enzyme nitric oxide synthase (NOS), specifically the endothelial isoform, eNOS, which is localized to rafts in these cells. Furthermore, ion channels, like the potassium channels in the heart, associate with both caveolae and caveolin-free lipid rafts. These channels regulate the membrane potential, and their disruption can lead to hypertension, ischaemia and heart failure (259). These findings further support the function of lipid rafts as signalling platforms.

Atherosclerosis is a chronic inflammation of the vascular wall caused by excessive accumulation of lipid macrophages-derived foam cells (260). The uptake of different LDL species into macrophages requires the binding to the receptor CD36 and its localization to lipid rafts (231). Several recent data suggest that innate pattern recognition receptors also play an important role in clearance of atherogenic lipoproteins (261-265). Minimally modified LDL and its oxidized phospholipids bind to the GPI-anchored CD14 receptor and activate TLR4 receptor pathway on macrophages (266,267). Moreover, binding of ceramide, released from atherogenic lipoproteins or apoptotic cells, to CD14 induces the formation of receptor complex within lipid rafts (104).

Human monocytes/macrophages loaded with atherogenic lipoproteins show an enhanced spreading and increased expression of the Rho family member CDC42, which is reversed by cholesterol depletion of rafts through apolipoprotein A-I, high density

lipoprotein-3 (HDL3) or β-cyclodextrin (268). Furthermore, the cholesterol and phospholipid effux regulator ATP-binding cassette transporter-A1 (ABCA1) has been shown to partially redistribute in membrane microdomains and apolipoprotein A-I mediated cholesterol and phospholipid efflux takes place through lipid microdomains in monocytes/macrophages (269,270). These findings implicate the involvement of lipid rafts in the pathogenesis of this complex disease.

2.6 Methods for studying membrane microdomains/lipid rafts

The ideal method for studying membrane microdomains in cells would provide information about size, abundance, and dynamics of rafts, including residence time of constituent proteins and lipids in rafts. At present, no technique can provide all this informations.

Large body of studies on lipid rafts have been performed on the basis of biophysical properties of microdomains and include the following methodological principles:

(1) Isolation of low-density detergent-resistant membranes (DRMs) based on the relative insolubility of membrane rafts to non-ionic detergents (24,50,271).

(2) Depletion of lipid constituents of rafts disrupts these microdomains, and this is also extensively used method to investigate lipid rafts function.

(3) Visualization of membrane microdomains by different microscopic techniques (272,273).

(4) Recently, Gombos *et al.* have presented a novel flow cytometric test to assess constitutive or induced DRM-association of lymphocyte membrane proteins using mild detergent treatment (274).

Recently, many new approaches have emerged that depend on the distinct diffusion characteristics or enhanced proximity between rafts constitutents (275), for instance stimulated emission depletion nanomicroscopy (276), single molecule tracing (277,278), fluorescence resonance energy transfer (FRET), fluorescence lifetime imaging and fluorescence correlation spectroscopy (279,280), allow direct raft visualization and analysis of raft size and dynamics.

2.6.1 Detergent resistance

Historically, lipid rafts have been defined as being insolubile in cold non-ionic detergents (typically 1% TX-100) (23). Detergent-resistant microdomains float into buoyant fractons of sucrose gradients during ultracentrifugation (24,26,35). These domains have been referred to as rafts, DRMs, detergent-insoluble glycolipid fractions

(DIGs) (35), glycolipid-enriched membranes (GEMs) (281) or Triton-insoluble floating fractions (TIFFs) (282).

The terms DRMs and DIGs reflect the observation that these domains are resistant to non-ionic detergents. This property is resulted of the tightly packed acyl chains in lipid rafts, since the lipid-lipid interactions are more stable than lipid-detergent interactions. These complexes float on a sucrose density gradient during centrifugation and hence easily isolated as lipid raft fractions or the “floating fractions”. The low buoyant density of the detergent-insoluble domains is concerned to in the name TIFF. TX-100 insolubility has proven a useful feature for the analysis of raft domains and several evidences indicate the validity of this criterion. DRMs appear to be derived from lipid rafts, they are rich in cholesterol and sphingolipid, and contain GPI-anchored proteins and signalling molecules. Using model membranes with artificial lipid compositions similar to cell-derived DRMs, it was demonstrated that detergent insolubility correlated well with the presence of liquid-ordered phase of lipids (24,27,48) .

The DRM isolation method has received criticism by some investigators because the sometimes inconsistent results (54). This procedure does not allow purifying all the raft associated proteins as some raft proteins may be connected to cytoskeleton and hence could not be isolated on a floating gradient preparation. Moreover, association of a protein with the raft may be so weak that it is solubilized by the detergent.

The cold detergent procedure for preparing membranes can still provide a convenient tool, and continues to be used.

2.6.2 Manipulation of raft constituents

Cholesterol dependence also defines rafts. Cholesterol, the major lipid of the membrane, is a critical component that controls lipid phase separation and stabilizes lipid rafts. One approach to identify the physiological role of lipid rafts is to deplete the lipid content of rafts (e.g. cholesterol or sphingolipid), thus to reduce or eliminate the ordered domain formation of lipid rafts (283).

Cholesterol contributes to the tight packing of lipids in liquid-ordered domains by filling interstitial spaces between lipid molecules, and the formation of l_o domains is seen only within certain ranges of cholesterol concentration (24,44). Several studies reported the influence of cholesterol on the physical and functional properties of sphingolipid microdomains by using different method to lower cellular cholesterol content such as inducing of cholesterol efflux by HDL_3 or apolipoprotein A-I (apoA-I) (44,284,285). Apolipoprotein A-I (apoA-I) is also regarded as a raft disrupting agent because of its ability to deplete cholesterol and choline-phospholipids from lipid rafts and its subsequent modulation of apoA-I/ABCA 1 dependent lipid efflux. Landry et al. showed that ABCA1 disrupts lipid microdomains/rafts and redistributes cholesterol/sphingomyelin to non-raft domains. They also found that ABCA1 also disrupts caveolae formation. There has been much recent and ongoing research into apoA-I mimetic peptides as therapeutic agents for atherosclerosis and inflammatory lipid disorders (286).

Statins, 3-hydroxy-3-methylglutaryl CoA reductase inhibitors, are among the most widely prescribed drugs in the world and are used to treat elevated levels of cholesterol and heart disease. Statins, through their ability to block cholesterol biosynthesis by inhibiting HMGCoA reductase, provide another way to disrupt membrane microdomains/rafts (287-289). Currently, it is believed that statins inhibit lipid raft formation and prenylation of signaling molecules, thereby disrupting cellular signaling networks. Furthermore, statins have been shown to reduce inflammation in response to lipopolysaccharide.

Cellular cholesterol content can be also decreased by using the following methods, such as sequestration by antibiotics (e.g. nystatin, filipin) or pore forming agents (saponin, O-streptolysin), or depletion of cholesterol by cyclodextrins (283,290).

2.6.3 Visualizing lipid rafts

Morphological approaches have been developed to study the in situ localisation of raft-associated lipids and proteins in intact cells as well as in membrane models (291,292). The most conventional technique is staining of a given raft-associated antigen with specific antibody, and then with a tracer (usually gold- or fluorochrome-) conjugated secondary antibody, and finally examination of the staining pattern either by electron microscopy (e.g. immunogold) or by (high-resolution) fluorescence microscopy or confocal laser scanning microscopy. A further preferred method is the direct visualization of certain lipid components. Probes for lipids are generally classified into two categories: fluorescent lipid analogues and lipid-binding molecules (including proteins such as antibodies and toxins). By using specific toxins, such as theta toxin or streptolysin-O which stains cholesterol, B subunit of cholera toxin labeling ganglioside GM1, or lysenin for visualization of sphingomyelin (293-295). For instance, the co-localisation of several raft proteins with the ganglioside GM1, a raft marker, has been demonstrated in various cell types by confocal microscopy.

More recently, some fluorescent lipid probes (analogues of naturally occurring membrane lipids) have been described with preferential partition into raft microdomains, such as 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine (DMPE) (296) or BODIPY-labelled lipids (297). So far the major experimental evidences for lipid sorting (based on differential partitioning) into ordered domains have largely been derived from studies examining the intracellular trafficking of short chain BODIPY-labeled sphingolipids. The advantage of this lipid analogue is that, due to their polarity, they diffuse into cells when added into culture medium. BODIPY-labeled lipids are attractive for trafficking studies because the BODIPY moiety exhibits a shift in its fluorescence from green to red wavelengths with increasing concentrations in membranes (297). This property can be employed to estimate the concentration of BODIPY-labeled lipids in cellular membranes (298).

Modern microscopy techniques such as atomic force microscopy and fluorescence resonance energy transfer provided the first evidence for the existence of rafts *in vivo*.

These techniques allowed the evaluation of the size of membrane rafts as small patches approximately 50–70 nm in diameter (299-301).

2.6.4. Flow cytometric approach(es) based on detergent (in)solubility

Several evidences support that TX-100 insolubility is a valuable method to study lipid microdomains, although, there are certain limitations (55,302). A raft protein can be connected to the cytoskeleton for example, so it does not float after detergent extraction. Furthermore, the raft association of a given protein can be so weak, that the protein is completely extracted by the detergent. Another major problem with detergent extraction and gradient centrifugation is that the initial subcellular localization of DRMs is unknown. These difficulties are avoidable using flow cytometry for characterization of the detergent insolubility of membrane antigens.

Several previous efforts have been invested to study detergent resistance of cellular plasma membranes by using flow cytometry (303-306). In the early studies, a relative high concentrations (1-2%) of detergent(s) was/were used, that had permeabilized and destroyed the cells, thus resulting inconsistent and not always reproducible data.

The cell lysis can be excluded applying moderate concentration (0.01-0.1%) of TX-100, thus nearly intact cells can be examined, which offers studying only the plasma membrane. On the other hand, proteins linked to the cytoskeleton can also be detected with this approach.

The earlier studies were performed mainly on tumor cell lines, in which the constitution of plasma membranes is not comparable with plasma membranes of freshly isolated circulating blood cells.

Recently, Gombos *et al.* have presented a flow cytometric test to investigate constitutive or induced DRM-association of lymphocyte membrane proteins: it provides kinetic data about detergent solubility for surface antigens under resting conditions (i.e. untreated cells) and upon cholesterol depletion of plasma membranes (i.e. disruption of rafts by methyl β-cyclodextrin /MBCD/) also (274).

Aim of the thesis

The main object of the thesis was to characterize peripheral blood monocytic membrane lipid microdomains based on their detergent resistance. Our goal was to optimize a newly developed flow cytometric assay for rapid analysis of constitutive and induced DRM-association of plasma membrane proteins from freshly obtained whole blood samples. Further, we tested this analytical tool to screen disease specific alterations of membrane lipid microdomains in human peripheral blood monocytes. The detergent insolubility of cell surface antigens, previously demonstrated to play a crucial role in inflammatory response and lipid homeostasis, such as GPI-anchored proteins (CD14, CD55), Fcγ-receptors (CD16, CD32, CD64), scavenger receptors (CD36, CD91, CD163), constituents of the integrin/complement receptor 3 complex (CD11a, CD11b, CD18) and the pentaspanin/tetraspanin family (CD47, CD81), were analysed in detail.

The proposed project addresses the following issues:

I. To establish a flow cytometric detergent solubilisation assay for whole blood monocytes
II. To investigate the effect of *in vitro* stimulation with lipopolysaccharide (LPS) on CD14-dependent receptor clustering in human monocytes
III. To characterize the CD14-dependent receptor clustering *ex vivo* on peripheral blood monocytes in inflammatory diseases (SIRS/sepsis and CAD/myocardial infarction)
IV. To investigate the effect of disturbed cellular membrane trafficking on DRM partition of monocyte antigens
V. To investigate the effect of *in vitro* ezetimib treatment, a drug influencing cellular lipid homeostasis, on DRM association of monocyte proteins.

4. Material and methods

4.1. Chemicals and Immunoreagents

Medium, solutions and chemicals	
BSA (lipid free)	Sigma, Taufkirchen, Germany
Ezetimib ((3R,4S)-1-(4-fluorphenyl)-3-[(3S)-3-(4-fluorphenyl)-3-hydroxypropyl]-4-(4-hydroxyphenyl) azetidin-2-on)	Aventis Pharma, Frankfurt am Main, Germany
FCS (Fetal calf serum)	Gibco BRL, Berlin, Germany
Formaldehyd	Sigma, Taufkirchen, Germany
fluorotrans transfer membranes	Pall, Dreieich, Germany
Iodixanol (OptiprepT)	Axis-Shield PoC AS, Oslo, Norway
LPS from Salmonella minnesota	Sigma, Taufkirchen, Germany
LPS from Escherichia coli	Sigma, Taufkirchen, Germany
LPS from Francisella tularensis	Bundeswehr Institute of Microbiology, Munich, Germany
Macrophage-SFM Medium, low endotoxin	Invitrogen, Karlsruhe, Germany
M-CSF, human (rec. from E. coli)	R&D Systems, Minneapolis, Minnesota
Methylated β-cyclodextrin (MBCD)	Sigma, Taufkirchen, Germany
PBS (w/o Ca2+, Mg2+)	Gibco BRL, Berlin, Germany
SDS	Sigma, Taufkirchen, Germany
Sodium azide	Sigma, Taufkirchen, Germany
Triton X-100	Sigma, Taufkirchen, Germany
Cell culture dishes	
Falcon 6-well cell+	Becton Dickinson, NJ, USA
10 cm tissue culture dishes	Sarstedt, Inc. Newton, USA
LAB-TEK borosilicate chamber slides	Nalge Nunc Int., Naprville, IL, USA
Ultra low attachment 6-well plates	Costar Corning, Bodenheim, Germany
Instruments	
ECL-Plus Western blotting detection system	Amersham/Pharmacia, Freiburg, Germany
FACS Canto flow cytometer	BD Biosciences, Heidelberg, Germany
Heraeus 6000 Incubator	Heraeus, Hanau, Germany
J2-MC centrifuge	Beckman Instruments Inc., Palo Alto, CA, USA
TCS-4D confocal microscope	Leica Lasertechnik, Omnichrom, Heidelberg, Germany Melles Griot

Analysis Software	
FACSDiva software for FACS Analysis	BD Biosciences, Heidelberg, Germany
Lumi-Imager	Roche Diagnostics, Mannheim, Germany
Metamorph for microscopy image Analysis	Universal Imaging, Corp., PA, USA
ScanWare software package	Analysis Universal Imaging, Corp., PA, USA

Antibodies	Clone	Fluorochrome	Supplier
CD4	IOT4a	FITC	Beckman-Coulter
CD4	IOT4a	FITC	
CD11a	25.3	FITC	
CD11b	Leu-15	PE	
CD13	My7	RD1	
CD16	3G8	FITC	
CD18	7E4	FITC	
CD48	J4.57	FITC	
CD71	YDJ1.2.2	PE	
CD14	MfP9	APC	BD Biosciences, Heidelberg, Germany
CD32	3D3	PE	
CD36	CB38 (NL07)	PE	
CD47	B6H12	PE	
CD55	IA10	PE	
CD59	P282 (H19)	FITC	
CD64	10. 1	FITC	
CD81	JS-81	FITC	
CD82	B-L2	PE	Sanbio, Am Uden, Germany
CD91	A2MR-a2	FITC	
CD163	GHI/61	PE	
Cholera toxin subunit B (recombinant) (GM1) Propidium-iodide		Alexa Fluor 488	Molecular Probes Invitrogen, Karlsruhe, Germany
Theta-Toxin (perfringolysin O)		FITC	Biotechnology workgroup of the Center of Excellence for Fluorescent Bioanalysis (KFB), Regensburg, Germany
DMPE-TMR (1,2-dimyristoyl-sn-glycero-3-phospho-ethanolamine-tetramethyl-rhodamine)			Avanti Polar Lipids, Inc., Alabama, USA

Abbreviations: APC: allophycocyanin, FITC: fluorescein isothiocyanate, PerCP: peridinin chlorophyll, R-PE: R-phycoerythrin

4.2. Blood samples

4.2.1 Donors and patients

For flow cytometric experiments peripheral EDTA-blood samples were obtained from healthy blood donors and from patients with NPC disease, SIRS/sepsis, CAD/myocardial infarction (307). All probands gave written informed consent.

4.2.2. Isolation and *in vitro* differentiation of human monocytes

For experiments with Ezetimib (microscopy and western blot analysis) leukocyte-enriched apheresates were obtained from healthy, normolipidemic volunteers after informed consent. Suspensions enriched in human peripheral blood leukocytes were isolated by leukapheresis in a Spectra cell-separator, supplemented with the anticoagulant ADCA (contains acid, citrate and dextrose) and diluted with an equal volume of PBS (w/o Ca2+, Mg2+). The diluted apheresate was then subjected to counterflow centrifugation (J2-MC centrifuge with JE-6B Rotor). Elutriated monocytes were cultured either on dishes with Attachment Surfaces or for analysis by flow cytometry on Ultra Low Attachment 6-well plates to guarantee viability of the cells with minimal activation. They were seeded at 10^6 cells/ml in macrophage serum-free medium supplemented with monocyte-colony stimulating factor (M-CSF, 50 ng/ml) (control) and incubated for up to 5 days at 37°C / 5 % CO2 (Heraeus 6000 Incubator) to induce phagocytic differentiation as described previously (308).

4.3. Immunostaining

For flow cytometry analysis 100 µL aliquots of whole blood samples were incubated for 15 minutes on ice with saturating concentrations of fluorochrome-conjugated mAbs (see capital 4.1). Lysis of erythrocytes and washing were performed as described previously (11). Sample preparation and analysis were always carried out within 6 hours of vein puncture.

4.4. *In vitro* modifications of the plasma membrane microdomains

Methylated β-cyclodextrin (MBCD) and complexed cholesterol with MBCD were used for modifications of the plasma membrane cholesterol content. Human peripheral blood samples were incubated with 10 mM MBCD to deplete membrane cholesterol. Reconstitution of cholesterol after depletion was performed with complexes of MBCD (4,6mg/mL) and cholesterol (100 μg/mL) at 37°C for 30 minutes described by Christian *et al.* (309). After incubation with MBCD or complexed cholesterol the cells were washed and labeled as described above. Control samples were incubated with PBS.

4.5. *In vitro* stimulation of cells with LPS

In vitro activation of peripheral blood monocytes was performed as described by Pfeiffer *et al.* (4). Briefly, cells were incubated with 100 ng/mL LPS (S. minnesota, E. coli and F. tularensis) at 37 °C for 15 minutes, then washed with ice-cold PBS in order to stop further activation, and finally labelled with mAbs. Control ('unstimulated') samples were incubated with PBS instead of LPS.

4.6 Flow cytometry of detergent resistance and data analysis

Four-parametric flow cytometry was carried out by a FACSCanto flow cytometer. Mean fluorescence intensity (MFI), forward scatter (FSC) and side scatter (SSC) signals of unstained and labelled peripheral blood cells were recorded before and 5 minutes after TX-treatment. Data were analyzed by FACSDiVa and CellquestPro softwares.

4.7. Visualization of raft microdomains with confocal microscopy

For raft-specific microscopy whole blood samples were treated with 0.05% cold TX-100 (15 min) at 4°C, and then fixed with 4% formaldehyde (15 min), followed by staining with FITC-conjugated mAb against GPI-anchored protein CD55. For visualization of lipid microdomains, monocytes were labelled with 50 μg/mL raft-specific lipid probe 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-tetramethylrhodamine (DMPE-TMR) (Figure 11) as described previously (296,310).

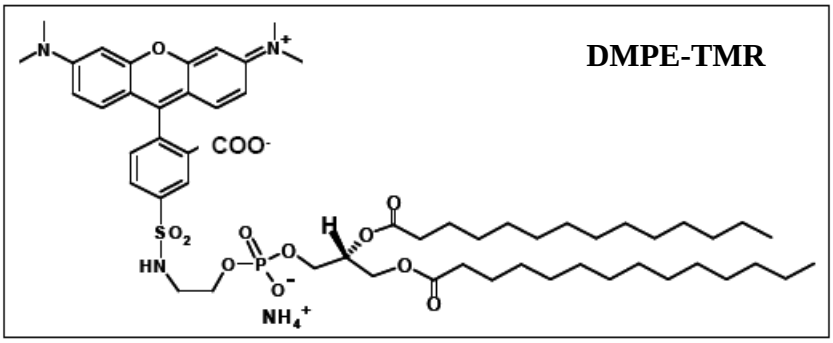

Figure 11. Structure of a saturated lipid probe molecule DMPE (1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine)-TMT (tetramethylrodamine)

For microscopy with ezetimib monocytes were cultured on sodium borosilicate coverglasses, fixed with 4% paraformaldehyde permeabilized by 0.25% Triton X-100 and labeled according to standard procedures. In addition to the CD13-FITC staining, plasma membrane was also visualized by mouse antihuman annexin II IgG1 (clone 5, BD Biosciences), and Texas Red-conjugated horse anti-mouse IgG (Vector Laboratories/Alexis, Gruenberg, Germany).

Confocal images were acquired with an inverted Leica TCS-SP5 confocal microscope equipped with a ArKr 75 mV mixed-ion laser. Acquisition was performed with the Scan Ware software package of the instrument and analysis was carried out using the software Metamorph 6.2.

4.8. Detergent lysis and flotation gradient

Cells were harvested, crude membrane fraction was isolated and TX-100 rafts were prepared. In detail, cells were washed twice with PBS, scrabbed on ice with buffer A (TNE, 50 mM Tris, 150 mM NaCl, 5 mM EDTA, supplemented with protease-inhibitor set III, Calbiochem, Bad Soden, Germany) and spun down at 1200 rpm 7 min, 4°C. The pellet was resuspended in buffer A and homogenized. From this material a crude membrane-fraction was prepared by centrifugation at 100000 *g*, 1 h, 4°C. The supernatant represents the cytosolic fraction and the pellet the crude membrane fraction (MF). MF was lysed in 300 µL buffer A containing 1% TX-100 on ice for 30 min.

The detergent lysate was adjusted to 40% Iodixanol (OptiprepTM) and therefrom 600 µL were given under a density gradient of 0, 20, 25, 30, and 35% Iodixanol (each 600 ml). After centrifugation in Beckman SW55Ti-rotor (160000 *g*, 4 h at 4°C) six 600 µL

fractions were collected from the top of the gradient (Figure 12). Fraction 2 and 3 were considered as the raft fractions and the fractions 4-6 as the non-raft fractions.

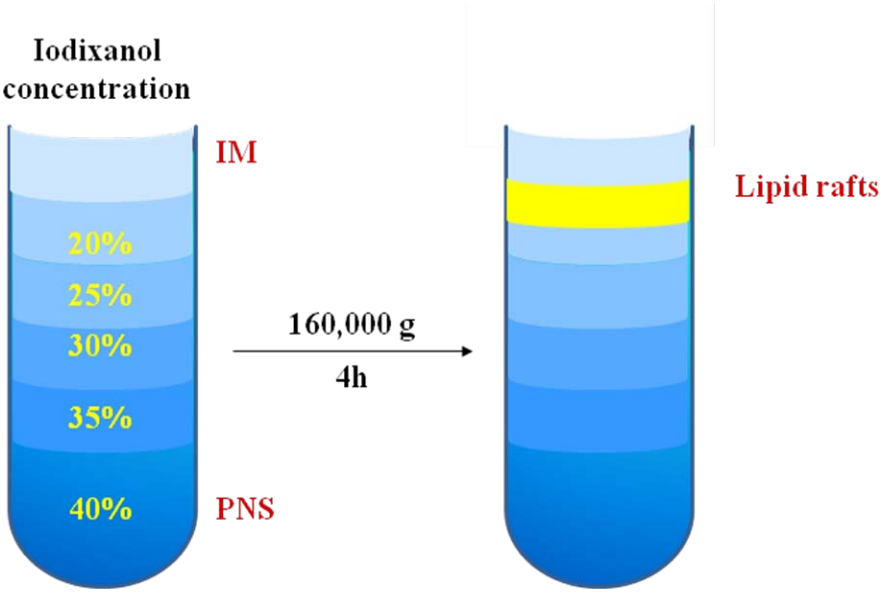

Figure 12. Isolation of lipid rafts by flotation from a postnuclear supernatant (PNS) in a discontinuous iodixanol gradient. IM =Isolation Medium.

4.9. Western blot analysis

Cells were harvested and washed with PBS, frozen (-20°C). Proteins from sucrose flotation gradients were concentrated using acetone precipitation, and equal amounts from each fraction were analyzed by SDS-PAGE using 4–12% gels. After electrophoresis, proteins were transferred to fluorotrans transfer membranes (Pall, Dreieich, Germany). Membranes were then blocked by incubation for 1 h in PBS containing 5% nonfat milk powder and 0.1% Tween 20. For detection of individual proteins, membranes were incubated for 1 h with the corresponding antibodies in a dilution of 1 : 1000 in PBS with 1% nonfat milk powder and 0.1% Tween 20. The following antibodies were used: rabbit polyclonal anti-human CD36, goat polyclonal anti-human CD14; goat polyclonal anti-human CD11b; goat polyclonal anti-human CD55; goat polyclonal anti-human CD32 and goat polyclonal anti-human CD13 (all from Santa Cruz Biotechnology, Santa Cruz, USA) and mouse polyclonal CD16 (Serotec, Eching, Germany). Antibody-positive bands were visualized by peroxidase-conjugated anti-rabbit or anti-goat IgG's (1:20 000). The membranes were developed

with the ECL-Plus Western blotting detection system according to the manufacturer's instructions and analyzed with a Lumi-Imager.

4.10. Fluorescence Resonance Energy Transfer (FRET) by flow cytometry

A FACSCanto flow cytometer and FACSDiVa software were used for the measurements. Measurements were performed without compensation and 30,000 leukocytes were acquired.

The principle of FRET first described by Förster in the late 1940s allows the measurement of distances between surface molecules (Figure 13). The energy transfer parameter (ET_p), which is proportional to FRET efficiency (ET), was calculated according to **(1)** where A is acceptor, D is donor, FL2 is mean fluorescence in channel 2 (488 ⟶530 nm) (donor, R-PE), FL3 is mean fluorescence in channel 3 (488 ⟶ 585 nm), FL4 is mean fluorescence in channel 4 (633 ⟶670 nm, acceptor, Cy5) (each value obtained after autofluorescence subtraction):

(1) ET_p = FL3(D,A) - FL2(D,A)/a - FL4(DA)/b FL3(D,A)

a = FL2(A)/FL3(A)

b = FL4(D)/FL3(D)

FRET efficiency (ET) was calculated from quenching according to **(2)**:

(2) ET = FL2(D) - FL2(D,A) FL2(D)

According to the publication by Szöllösi et al. an ET = 5% was defined as the threshold level for significant transfer efficiency in our experiments (311).

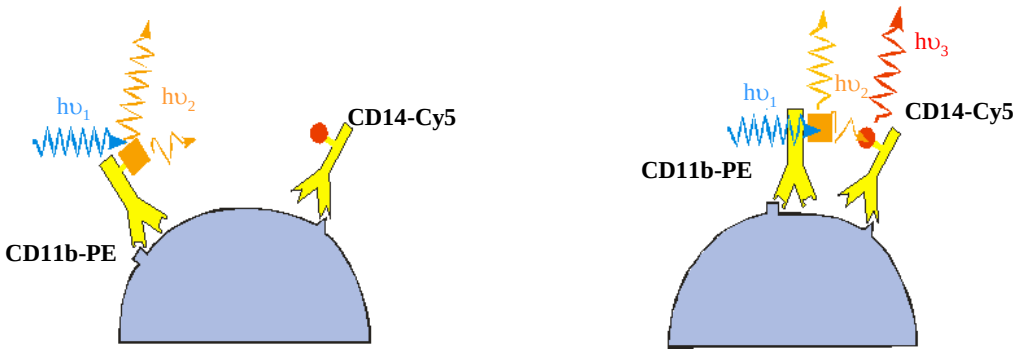

Figure 13. Principle of FRET

A fluorescent donor molecule is excited by a light source, the energy is nonradiatively transferred to an acceptor molecule. The efficiency of this energy transfer depends upon the distance between acceptor and

donor, the degree of spectral overlap between the emission spectra of the donor and absorption of spectra of the acceptor and the relative parallel orientation of donor and acceptor dipoles.

4.11. Statistical analysis

Data are given as mean ± SD. For statistical comparison Mann-Whitney-U oder Wilcoxon signed-ranks test was used. Values for $p < 0.05$ were considered as statistically significant (*).

5. Results

5.1 Establishment and evaluation of a flow cytometric method to assess detergent resistance of plasma membrane proteins in monocytes

The purpose of our initial studies was to establish and optimize a flow cytometric approach for studying monocytic antigens associated to membrane microdomains. The test is on the basis of differential detergent resistance of plasma membrane antigens. Data evaluation is based on comparative detection of their detergent solubility without and with cholesterol depletion of cell membranes, resolved by moderate concentration of cold TX-100 detergent (Figure 14).

Nonionic detergent treatment extracts non-raft associated antigens (detergent-soluble) from the plasma membranes. It causes a rapid drop in the surface expression of a given fluorescent-stained surface receptor, assessed by decrease of the mean fluorescence intensity (MFI). In case of raft-residency, the detergent-insoluble antigens present no significant changes in MFIs.

The TX-100 solubility of antigens is characterized by an index (FCDR: **f**low **c**ytometric **d**etergent **r**esistance), calculated according to the following equation:

$$\mathbf{FCDR = (MFI_{Det} - MFI_{AfDet}) / (MFI_{Max} - MFI_{Af})}$$

where MFI_{Det} is the MFI value of labelled cells treated with TX-100; MFI_{AfDet} is the autofluorescence of unstained and TX-100-treated cells; MFI_{Max} is the MFI value of stained cells without TX-100-treatment (i.e. reflecting the antigen expression); and MFI_{Af} is the autofluorescence of unstained cells without TX-100-treatment, respectively. Thus, FCDR index is a quotient without unit, and it ranges between 0 and 1. According to the generation of the FCDR index it is obvious, that antigens with high FCDR indices are almost insoluble in TX-100 (i.e. likely associated to DRMs/rafts), while others with low FCDR indices are solubilized by TX-100. Hence, these proteins are considered being detergent soluble or non-raft associated proteins.

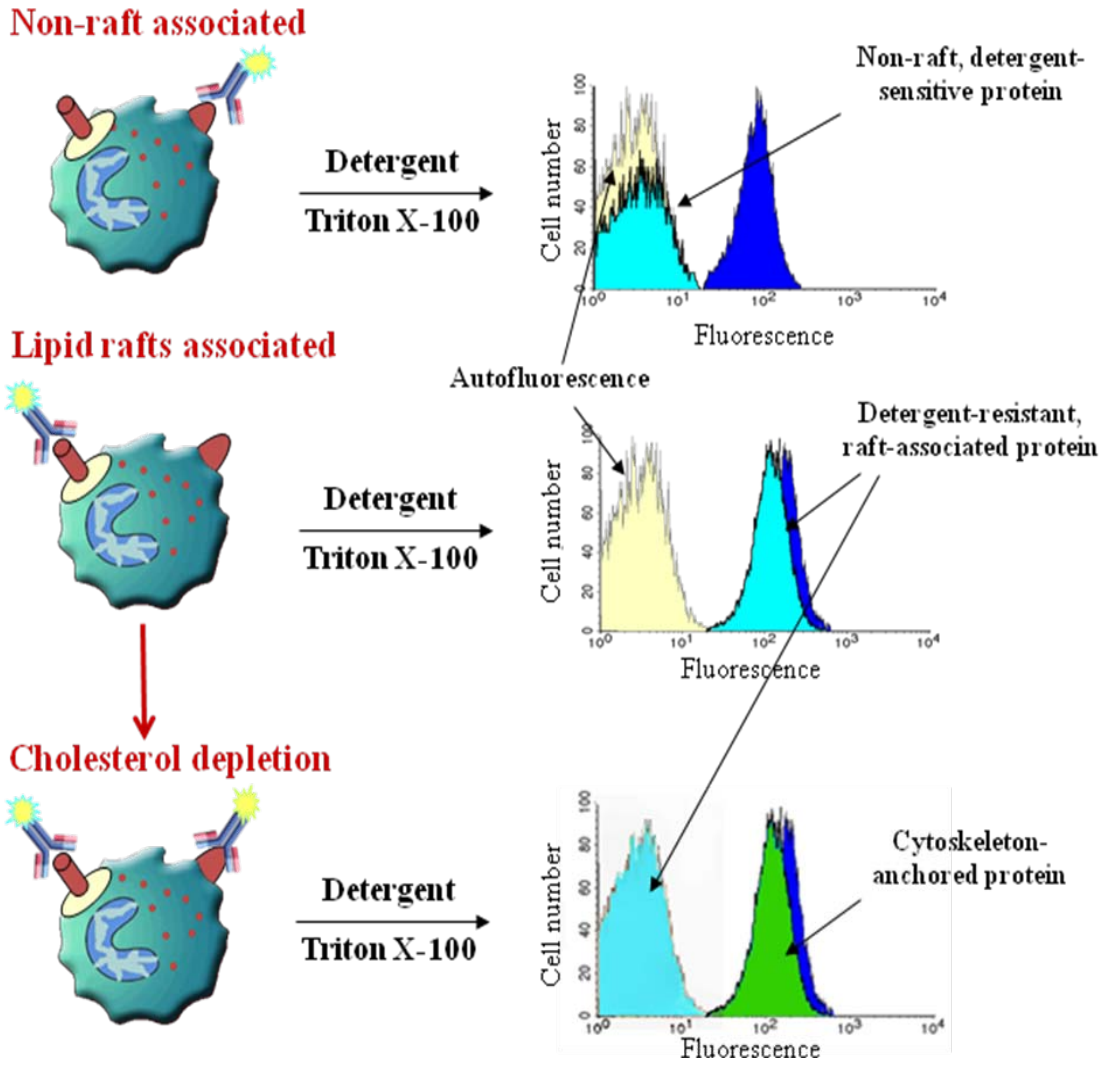

Figure 14. Principle of the FCDR (Flow Cytometric assay of Detergent Resistance) assay

A) Detergent soluble plasma membrane proteins are completely dissolved by low concentrations of TX-100 non-ionic detergent in a short time. B) Proteins associated to lipid rafts or anchored to the cytoskeleton display high resistance to mild detergent treatment. C) The mechanism underlying the observed detergent resistance could be identified by disruption of the raft structure (cholesterol depletion) before detergent solubilization. Decreased detergent resistance of the given antigens after cholesterol depletion indicates the association with lipid rafts. *Modified from Gombos et al., Cytometry (2004) (274)*

5.1.1 TX-100 solubility kinetics of raft-associated and non-raft antigens

In the first experiments, monocyte antigens were screened for detergent solubility/resistance with different concentrations of TX-100 (0.01%, 0.05% and 0.1%) in kinetic acquisition mode to titrate the optimal detergent concentration for human peripheral blood monocytes. Antigens excluded from monocyte rafts such as CD45 or transferrin receptor CD71 were rapidly solubilized by TX-100, leading to a marked reduction of their MFI to the autofluorescence level within 1 minute. By contrast, well-known raft-associated antigens such as the GPI-anchored CD14 and CD55 were resistant to TX-100 treatment, and maintained their MFI during the 5-minute-long measurement (Figure 15).

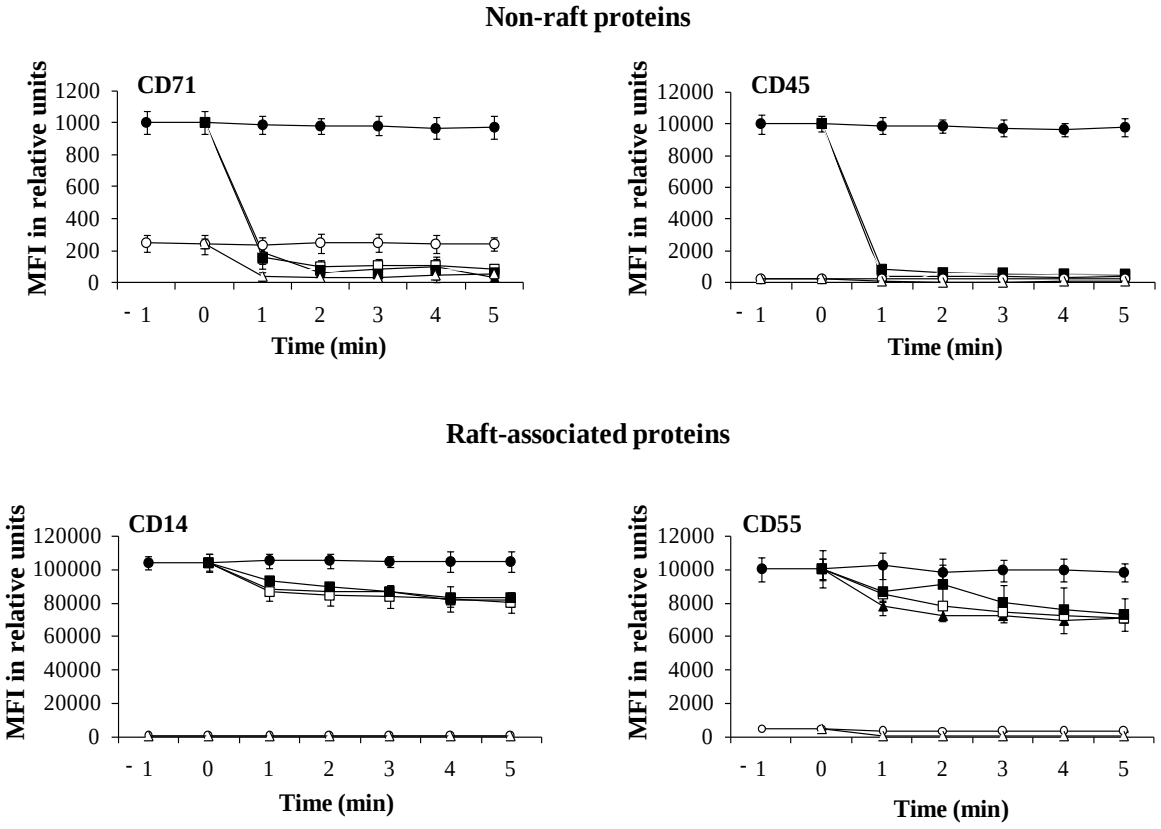

Figure 15. Kinetic analysis of detergent resistance of surface antigens in human monocytes.
Mean fluorescence intensities (MFI) for CD45-Per-CP, CD71-PE, CD14-APC and CD55-FITC on monocytes treated with 0.01% (solid squares), 0.05% (open squares), 0.10% (solid triangles) TX100, and autofluorescence (open triangles) are shown in a time-course. Fluorescence intensities of the given antigens (solid circle) and autofluoresce (open circle) without detergent treatment are displayed, as well. The figure shows the average of three to five independent measurements.

There was no difference in the TX-100 solubility kinetics of antigens using different detergent concentrations (Figure). These data show that 0.01-0.1% of TX-100 treatment is sufficient for extracting non-raft proteins from intact cells within 5 minutes. Following, all of the solubilization experiments were carried out with 0.05% TX-100.

5.1.2 Identification of monocyte population upon TX-100 solubilization

Solubilization of whole blood samples with 0.05% TX-100 at 4 °C slightly changed the light scatter characteristics, as seen by the shift of cell populations toward lower FSC and SSC values. These changes indicate notable cell shrinkage upon detergent extraction (Figure 16A).

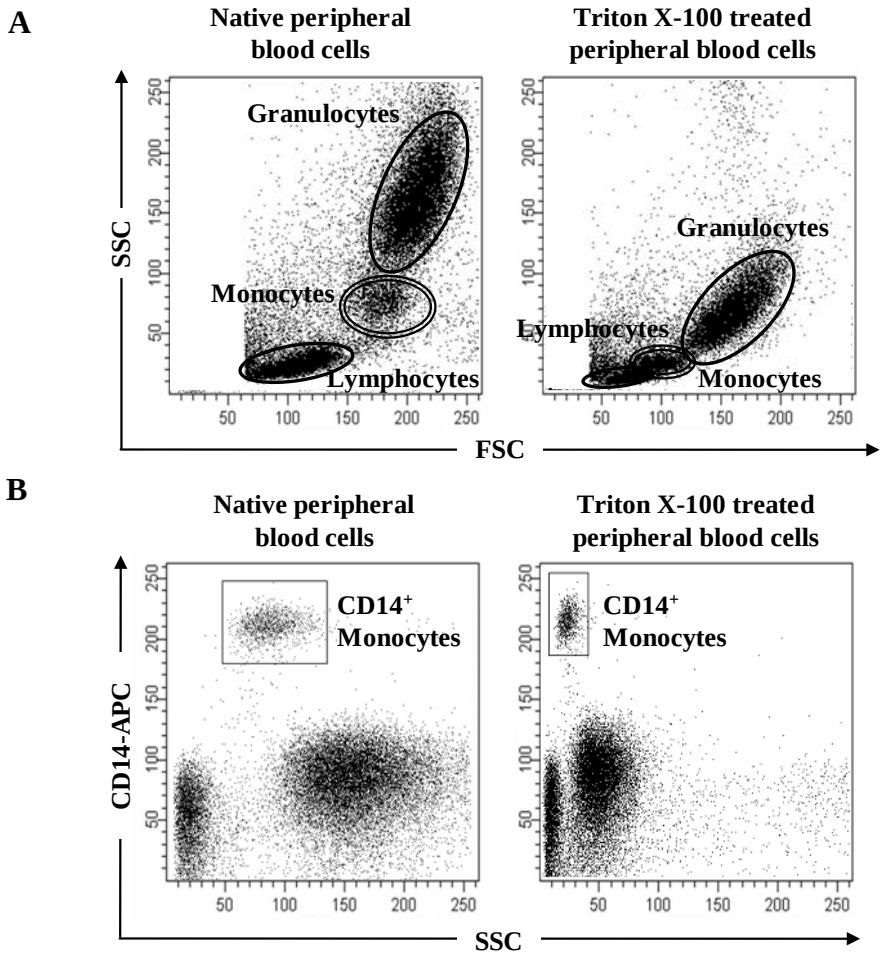

Figure 16. Detection of human peripheral blood monocytes after detergent solubilization by flow cytometry (A) Light scatter plot of untreated and 0.05% TX-100 extracted peripheral blood cells. (B) Detection of peripheral blood monocytes before and after detergent treatment using the GPI-anchored LPS receptor CD14 antigen.

The GPI-anchored LPS receptor CD14 was used to determine the monocyte population before and during detergent treatment (Figure 16B). Dot plots indicate that the detergent treated cell populations can be clearly identified and distinguish from the cell debris. Although, to exclude the possibility of detecting cell fragments, confocal microscopy was carried out on TX-100 treated monocytes. Confocal images confirmed the flow cytometric analysis, thus monocytes after mild TX-100 treatment seem to be morphologically intact with a slightly decreased cell volume (Figure 17). Moreover, we applied a saturated, fluorescent lipid probe DMPE-TMR, which is known to partition into liquid-ordered domains of the membrane. DMPE-TRM stained images revealed that detergent treatment did not significantly affect microdomain structures, hence, a characteristic punctuate staining of lipid microdomains displayed extensive co-localization with the raft-associated GPI-anchored protein CD55.

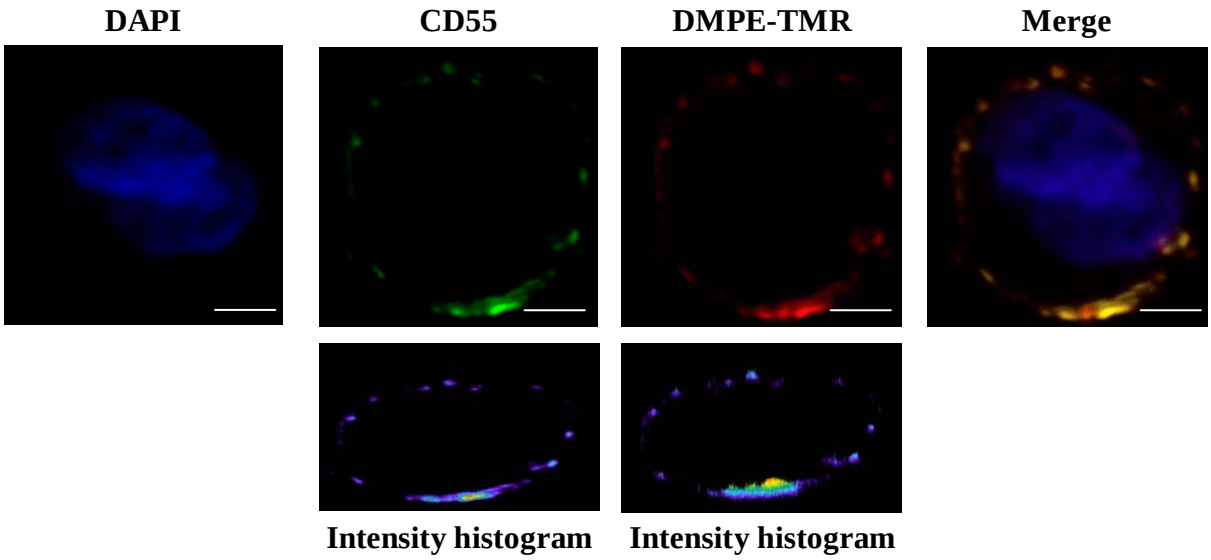

Figure 17. Confocal microscopic images of circulating monocytes after TX-100 treatment.
Monocytes were stained with CD55 FITC-labelled mAb (green), with 50 μg/mL raft specific lipid probe DMPE-TMR (red) and DAPI for nuclear staining. Merge shows an extended focus overlay (yellow). Scale bar = 2,5 μm

5.1.3 Association of surface antigens with DRMs on human monocytes

In the further experiments, monocyte surface antigens, implicated in cell signalling, inflammatory response and/or lipid metabolism were analysed for DRM association from healthy blood donors (Table 4).

Antigens	n*	Detergent resistance to TX-100
Transmembrane proteins		
CD4	4	0.681 ± 0.105
CD13	39	0.860 ± 0.086
CD44	4	0.845 ± 0.052
CD71	14	0.066 ± 0.053
Penta/Tetraspanins		
CD9	18	0.034 ± 0.029
CD47	43	0.415 ± 0.131
CD81	18	0.312 ± 0.066
CD82	4	0.064 ± 0.041
Fcγ-receptors		
CD16	43	0.258 ± 0.084
CD32	43	0.601 ± 0.107
CD64	43	0.634 ± 0.116
Scavenger receptors		
CD36	41	0.351 ± 0.159
CD91[#]	43	0.752 ± 0.084
CD163	40	0.708 ± 0.114
GPI-anchored proteins		
CD14	43	0.871 ± 0.035
CD46	4	0.506 ± 0.087
CD48	23	0.839 ± 0.072
CD55	40	0.768 ± 0.079
CD59	10	0.624 ± 0.087
Integrins		
CD11a	30	0.424 ± 0.096
CD11b	31	0.552 ± 0.108
CD18	40	0.539 ± 0.099
Lipids		
GM1	5	0.674 ± 0.067
Theta toxin	25	0.505 ± 0.108

Table 4. Differential detergent resistance of plasma membrane proteins and lipids in peripheral blood monocytes.

*n: number of experiments, [#]CD91 does not belong to classical scavenger receptors according to Krieger et al (312), but functionally has been reported to behave as a scavenger receptor (313).

A group of antigens, including the GPI-anchored proteins CD14, CD48, CD55, CD59, the Fcγ receptors CD32 and CD64, the scavenger receptors CD91 and CD163, the

complement receptor 3 complex CD11b/CD18 and the transmembrane proteins CD4, CD44, and CD13 showed a high partition in DRMs, as FCDR indices for these antigens were above 0.5 .

By contrast, the transferring receptor CD71, the tetraspanin CD9 and CD82, described before as non-raft residents, were completely solubilized by 0.05% TX-100, as FCDR indices for these antigens were below 0.1.

The Fcγ receptor CD16, the scavenger receptor CD36, the pentaspan CD47, the tetraspan CD81 and the integrin receptor CD11a were not fully extracted by TX-100 and showed an intermediate detergent resistance. Their FCDR indices varied between 0.25 and 0.45, indicating only a partially or a weak association of these receptors to DRMs.

In addition, GM1 ganglioside and cholesterol, lipid components of microdomains were also investigated for detergent resistance. A high FCDR index was found for GM1 ganglioside, supporting its principal association to DRMs. In contrast, characterization of cholesterol´s detergent solubility suggests that cholesterol almost equally distributes between liquid-ordered and disordered membrane phases.

5.2 Effect of *in vitro* raft-modifications on the DRM-association of monocyte antigens - cholesterol depletion and reconstitution

Cholesterol has been shown to be essential for microdomain formation and function (290,314,315). It serves as a spacer between the saturated hydrocarbons chains of densely packed lipids in membrane microdomains/lipid rafts. A widely used approach to study the function of lipid rafts is the disruption of their structural integrity by cholesterol depletion with cyclodextrins like MBCD (283,290). Comparison of detergent solubility of control and cholesterol-depleted membranes can differentiate, whether a given membrane protein is associated to or excluded from membrane microdomains/lipid rafts.

In order to assess the effect of *in vitro* raft-modifications on the detergent resistance of monocyte antigens MBCD-induced cholesterol depletion was analyzed, followed by the reconstitution of membrane cholesterol by MBCD/cholesterol complexes.

Removal of cholesterol with MBCD treatment resulted in increased TX-100 solubility of usually DRM-associated proteins, such as CD14, CD55, CD59 and the aminopeptidase N (CD13), as represented by markedly decrease of FCDR indices (Figures 18).

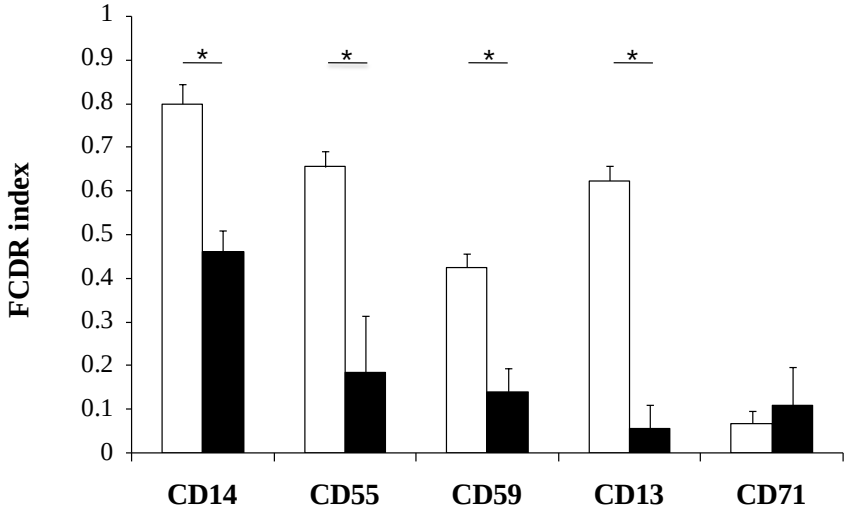

Figure 18. Alteration of the detergent resistance after cholesterol depletion of the plasma membrane.

FCDR indices of untreated (open bars) and cholesterol depleted cells with MBCD (filled bars) are presented as mean ± SD of five independent experiments. $p < 0.05$ (*) was considered as statistically significant.

These changes were reversed by reconstitution of plasma membrane cholesterol of MBCD-depleted cells by providing exogenous complexed cholesterol. As expected, cholesterol reconstitution of the plasma membrane resulted in increased detergent resistance of CD14 and CD55, which were detergent-soluble upon cholesterol depletion (Figure 19). Cholesterol depletion and loading did not influence detergent solubility of the transferrin receptor CD71, indicating its overall exclusion from liquid-ordered membrane domains (Figure 19).

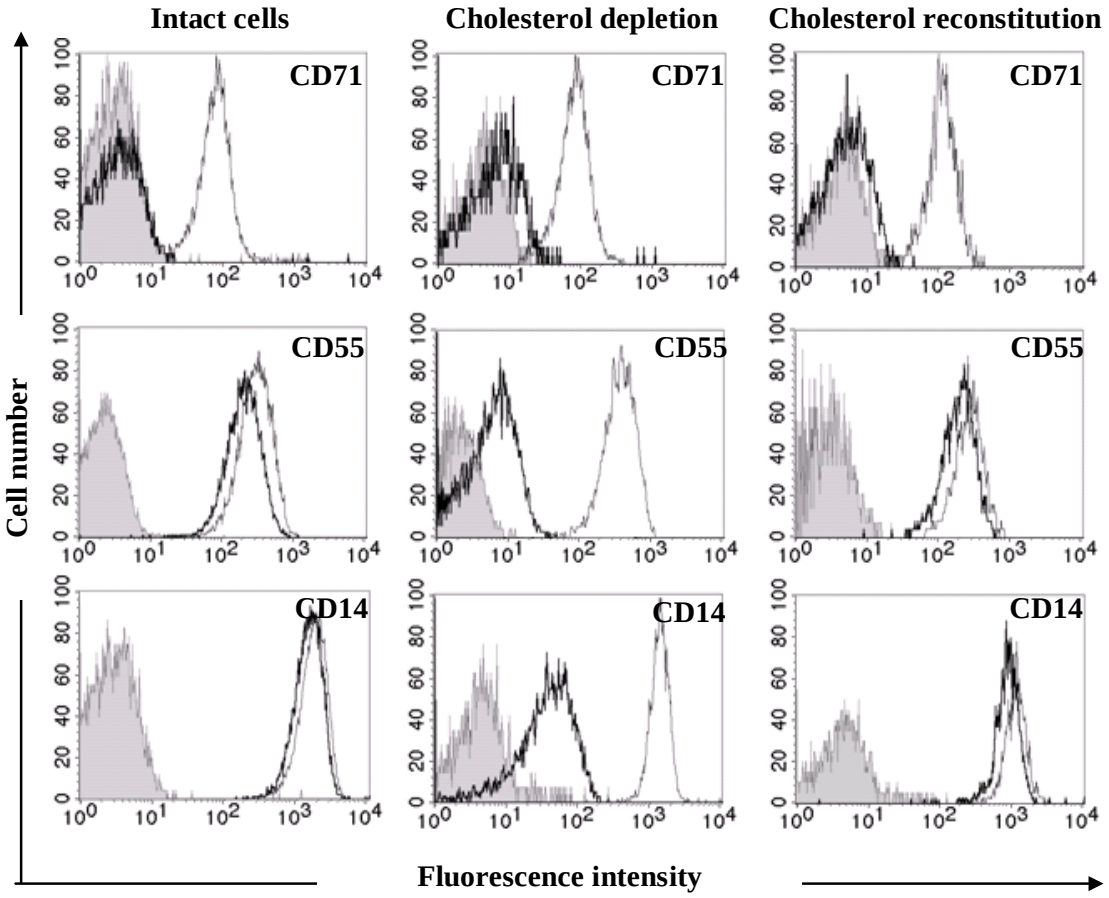

Figure 19. Effect of depletion (MBCD) and reconstitution (MBCD/cholesterol complexes) of membrane cholesterol content.

Mean fluorescence intensities (MFI) of CD71, CD55 and CD14 antigens are demonstrated as histogram-plots before (slight black lines), after TX extraction (thick black lines) and the autofluorescence levels (shaded gray area). **Left:** Different detergent solubility of peripheral monocyte surface antigens. **Middle:** Effect of raft-cholesterol depletion by MBCD. **Right:** Effect of membrane cholesterol reconstitution by MBCD/cholesterol complexes. These findings clearly suggest that (i) MBCD primarily extracts raft-resident cholesterol, and (ii) the MBCD-induced changes of membrane cholesterol are reversible.

5.2. Effect of in vitro LPS stimulation on the DRM-association of monocyte antigens

Several lines of evidence support that the entire bacterial recognition by CD14 and the recruitment of multiple receptors at the site of CD14-LPS ligation takes place within lipid rafts (104,109).

5.2.1 Detergent solubility of antigens under LPS-activated conditions in vitro

In order to investigate whether *in vitro* LPS activation of monocytes may modify detergent insolubility (i.e. raft-association) of given surface antigens the following experiments were carried out. The DRM-association of certain antigens (Table 5) was determined before and after 100 ng/ml of LSP (S. minnesota) stimulation. Particular interest was focused on the tetraspanin CD81 and TLR-4 molecule. These receptors were previously demonstrated to be excluded from rafts in resting monocytes, but were raft-associated after LPS stimulation (104,108).

Antigen	Resting cells	LPS-stimulated cells	Statistics
CD14	0.830 ± 0.019	0.865 ± 0.039	p = 0.019*, n=14
CD55	0.781 ± 0.076	0.813 ± 0.067	p = 0.141, n=8
CD32	0.515 ± 0.075	0.585 ± 0.029	p = 0.028*, n=8
CD64	0.655 ± 0.097	0.705 ± 0.062	p = 0.166, n=4
CD81	0.252 ± 0.049	0.591 ± 0.073	p < 0.001*, n=11
CD11b/CD18	0.419 ± 0.047	0.589 ± 0.045	p < 0.001*, n=7
TLR-4	0.316 ± 0.104	0.601 ±0.101	p = 0.005*, n=7
CD71	0.071 ± 0.013	0.033 ± 0.004	p = 0.013, n=5

Table 5. FCDR values of monocyte antigens before and after in *vitro* LPS stimulation.
Whole blood monocytes were stimulated with 100 ng/ml LPS derived from *S. minnesota* at 37°C for 15 minutes. Data are presented as mean ± S.D. $p < 0.05$ was considered as statistically significant (*). n: number of experiments.

CD81 and TLR-4 exhibited a low FCDR index in unstimulated monocytes, showing that only a small proportion of these receptors are associated with lipid microdomains. The FCDR index exceeded significantly higher level in LPS-treated cells, clearly indicating the activation-induced DRM-association of CD81 and TLR-4. Other

antigens, such as GPI-anchored protein CD14, CD55 and the Fcγ receptor CD32 and CD64 displayed high FCDR indexes in both resting and LPS-induced cells (Table). However, it should be noted that CD14 and CD11b/CD18 presented significantly higher partition in DRMs.

By contrast, CD71 showed low FCDR indexes in both *in vitro* conditions, indicating exclusion of this antigen from rafts irrespective of cellular activation.

5.2.2 Effect of different LPS species on the detergent solubility of monocytes antigens

The LPS of *F. tularensis* is atypical in structure and biological activity. Whereas the LPS of gram-negative bacteria function as potent proinflammatory endotoxins, the LPS of *F. tularensis* is unable to stimulate mononuclear cells to release cytokines or to upregulate surface immunoglobulins on B cells (316). Conversely, it does act as an endotoxin antagonist for mononuclear cells. These observations suggest that *F. tularensis* LPS may not interact with host LPS recognition proteins.

Therefore, the next set of experiments was objected to analyze the in vitro effects of the *F. tularensis* LPS on the CD14-dependent receptor clustering. The following pattern recognition receptors CD11b/CD18 TLR4, CD81 and CD14 were analized on peripheral blood monocytes upon stimulation with F. tularensis LPS and compared with other LPS species of known biological activities belonging to different species: *S. minnesota* and *E. coli*.

We found no difference among the three different bacterial LPS species after 15 minutes stimulation. All three LPS species were able to translocate CD81 or TLR-4 into lipid rafts/DRMs. FCDR indices of the investigated receptors were significantly higher after LPS-stimulation, irrespectively of the LPS species (Figure 20).

Our data obtained by the FCDR assays indicated that similarly to *S.minnesota, E.coli* and *F. tularensis* LPS is also able to induce the co-association of the given pattern-recognistion receptors to CD14 within lipid rafts.

In order to further verify our data, FRET studies were carried out on peripheral monocytes stimulated with different LPS species.

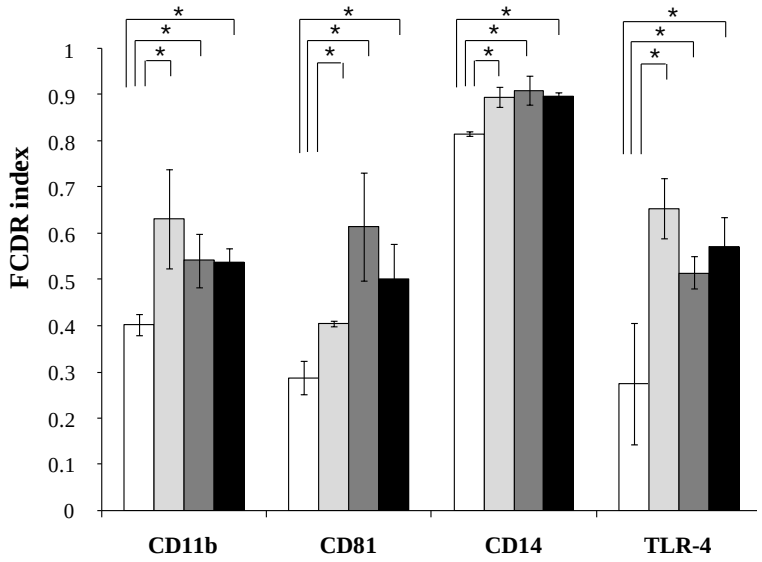

Figure 20. FCDR values of monocyte antigens before and after in *vitro* LPS stimulation.

Whole blood samples were stimulated with 100 ng/ml LSP derived from *E. coli*, *S. Minnesota* and *F. tularensis* at 37°C for 15 minutes or incubated with PBS as a control. Peripheral blood monocytes from healthy controls without stimulation (open bars, n=4) and with E.coli LPS (gray filled bars, n=4), S. Minnesota LPS (hell gray filled bars, n=4) and F. Tularensis (black bars, n=4) were labelled with monoclonal antibodies and treated with 0.05% cold TX-100 for 5 minutes. FCDR indices are presented as mean ± SD.

By performing FRET, we examined whether these receptors co-associate in lipid microdomains. As a positive control, we measured energy transfer between mAbs to different epitopes on CD14 molecules (E% 35.6 ± 6.9). Following, FRET efficiency was measured between CD11b/CD18 and CD14, CD81 and CD14, TLR4 and CD14. On unstimulated monocytes FRET analysis did not show proximity between CD11/CD18, CD81, TLR4 and CD14 molecules. In agreement with our FCDR assay, we found co-assembly of these receptors within lipid rafts, presented by high FRET levels between CD14 and CD11b/CD18, CD81 and TLR-4. Similar high FRET levels were observed after *E. coli* and *S. minnesota* LPS stimulation. Interestingly, stimulation with *F. tularensis* LPS revealed also high FRET efficiency between these pattern recognition receptors (Figure 21).

These data demonstrate that all the examined LPS species induce approximately equally the clustering of the given pattern recognition receptors within lipid microdomains.

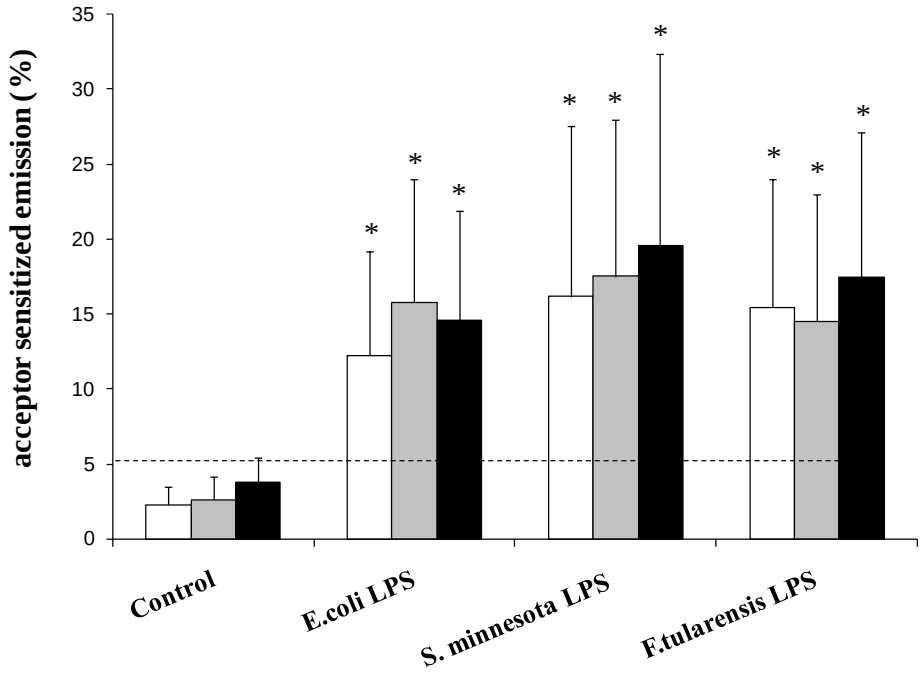

Figure 21. Changes in the molecular co-association of CD11b/18, CD81, TLR-4 and CD14 in response to different LPS species stimulation

Whole blood samples were stimulated with 100 ng/ml LSP derived from *E. coli*, *S. Minnesota* and *F. tularensis* or incubated with PBS as a control. The molecular co-assembly between CD81 (D) and CD14 (A) (open bars, n=4), CD11b/CD18 (D) and CD14 (A) (gray bras, n=4) or TLR4 (D) and CD14 (A) (black bars, n=4) was assessed by flow cytometric fluorescence resonance energy transfer (FRET), and expressed by acceptor sensitized emission (FRET-efficiency in %). The FRET-efficiency > 5% was accepted as significant energy transfer. Donors (D) were stained with R-PE, and the acceptors (A) with Cy5. Data are presented as mean ± S.D., $p<0.01$ (*) was considered as statistically significant.

5.3 Characterization of CD14-dependent receptor clustering on monocytes in inflammatory diseases

Lipid rafts on monocytes/macrophages provide a dynamic microenvironment for an integrated CD14-dependent clustering of a set of receptors involved in inflammation and atherogenesis. Ligand-binding promotes conformational changes and specific coassembly of additional receptors within membrane microdomains (104).

5.3.1 Detergent solubility of monocyte surface antigens in patient with SIRS/sepsis

In order to investigate whether *in vivo* LPS stimulation influences the DRM association of circulating monocyte freshly obtained blood samples from patients with SIRS/sepsis were analysed with the FCDR assay. The detergent insolubility of GPI-anchored proteins (CD14, CD55), Fcγ-receptors (CD16, CD32, CD64), scavenger receptors (CD36, CD91, CD163), constituents of the integrin/complement receptor 3 complex (CD11a, CD11b, CD18) and the pentaspanin/tetraspanin family (CD47, CD81) members were analyzed.

No significant difference in the DRM-association was found for Fcγ-receptors CD16 and CD32, and integrin receptor CD11b from patients with SIRS/sepsis and controls. However, the GPI-anchored protein CD14 and CD55, the Fcγ-receptor CD64, scavenger receptors CD36, CD91, and CD163, and integrins CD18 and CD11a showed significantly higher association to DRMs in SIRS/sepsis patients (Figure 22). Moreover, the tetraspanin CD81 presented a low association with DRMs on monocytes from controls, but segregated in DRMs on cells from SIRS/sepsis patients. By contrast, the pentaspanin CD47 displayed a significantly lower FCDR index in SIRS/sepsis monocytes compared to control cells (Figure 22).

In agreement with Pfeiffer et al., FCDR assay is able to detect LPS-induced specific translocation of CD81 into and dissociation of CD47 molecule from membrane microdomains on whole blood monocytes obtained from patients with SIRS/sepsis.

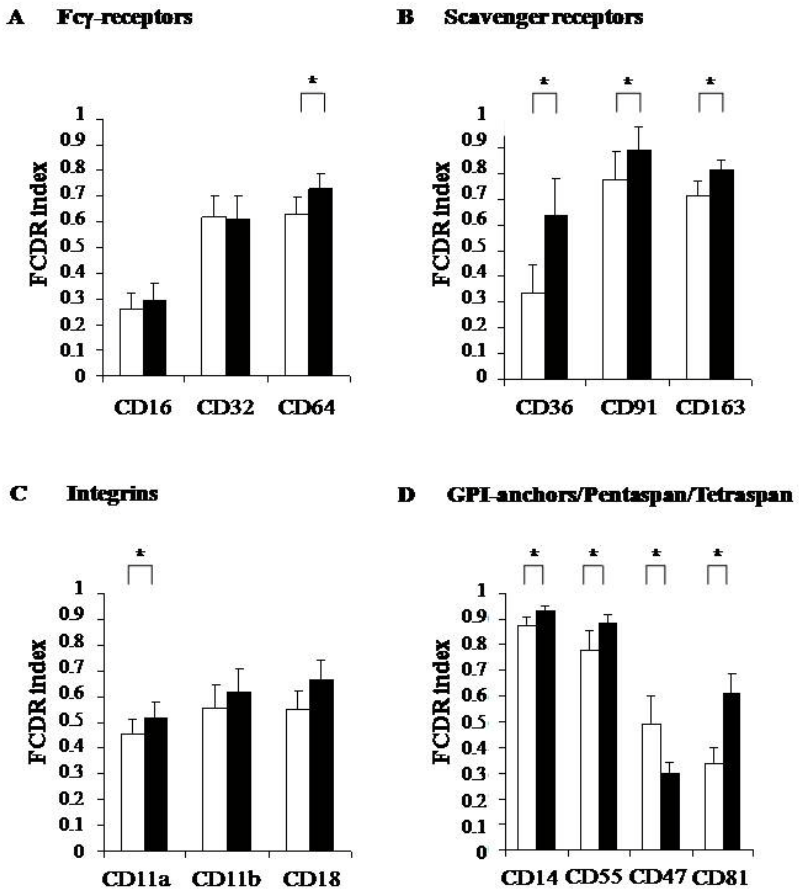

Figure 22. TX-100 solubility of monocyte antigens from sepsis patients.

Ex vivo peripheral blood monocytes from healthy controls (open bars, n=10) and from patients with sepsis/SIRS (filled bars, n=10) were labelled with monoclonal antibodies and treated with 0.05 % cold TX-100 for 5 min. Controls and patients were measured on the same day under the same conditions. $p<0.05$ (*) was considered as statistically significant.

5.3.2 Detergent resistence of monocyte antigens in patients with CAD/myocardial infarction

In order to investigate whether *in vivo* atherogenic lipoproteins influence the DRM-association of circulating monocyte, freshly obtained blood samples from patients with CAD/myocardial infarction were analysed with the FCDR assay. The detergent insolubility of the previously investigated pattern recognition receptors was analyzed.

We found that almost all of the investigated GPI-anchored proteins (CD14, CD55), Fcγ-receptors (CD16, CD32, CD64), scavenger receptors (CD36, CD91, CD163), constituents of the integrin/complement receptor 3 complex (CD11a, CD11b, CD18)

and the pentaspanin family member CD47 molecule presented significantly higher TX-100 resistence from patients with CAD in comparison to healthy blood donors (Figure 23). Interestingly, in contrast to CD81, CD47 revealed an increased detergent resistance, showing that CD47 resides in DRMs of patients with CAD and/or myocardial infarction, while CD81 selectively translocates into DRMs in SIRS/sepsis patients. Moreover, CD16 presented highly increased partition into DRMs only in CAD and/or myocardial infarction patients, but not in patients with SIRS/sepsis (Figure 22 und 23).

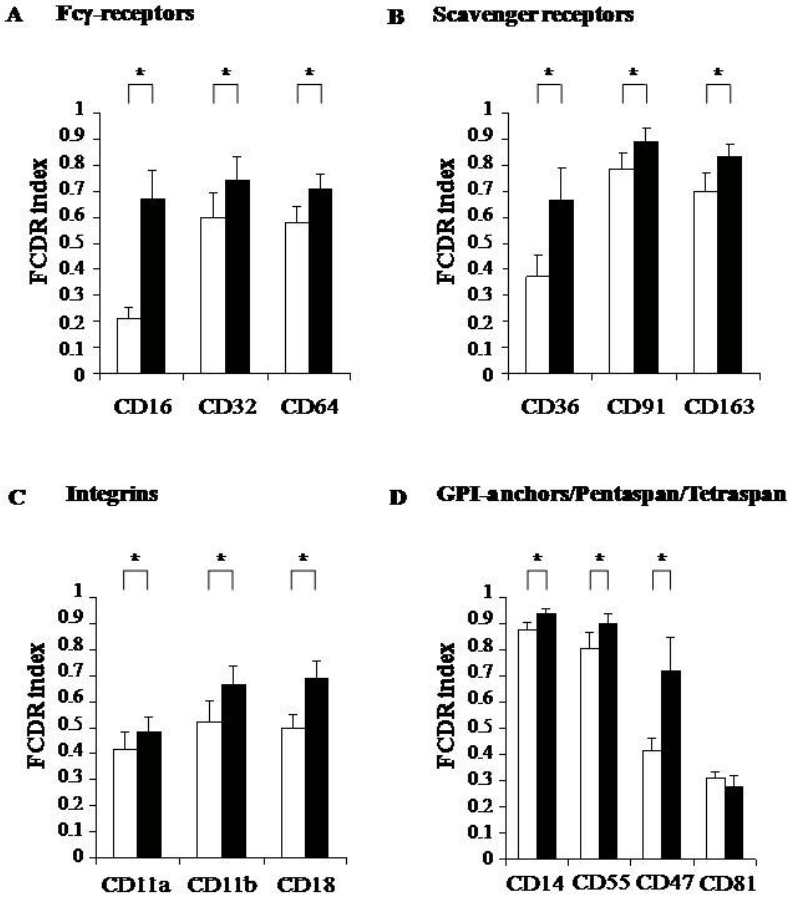

Figure 23. TX-100 solubility of monocyte antigens from CAD patients.

Ex vivo washed peripheral blood monocytes from healthy controls (open bars, n=10) and from patients with CAD (filled bars, n=10) were labelled with monoclonal antibodies and treated with 0.05% cold TX-100 for 5 min. Controls and patients were measured on the same day under the same conditions. FCDR indeces are presented as mean ± S.D. $p<0.01$ (*) was considered as statistically significant.

5.4 Detergent resistance of monocyte antigens in monogenetic disorders of intracellular membrane trafficking

NPC disease is a rare monogenetic disorder affecting cellular cholesterol influx through the endosomal compartment, resulting in the sequestration of glycosphingolipids and cholesterol in the late endosomal/lysosomal compartment (216). Based on this observation, we supposed that increased glycosphingolipid rafts in *NPC1* deficient cells were present not only in the endosomal compartment, but in *NPC1*-defected plasma membranes, as well.

In order to investigate whether altered endocytic trafficking affects DRM association of plasma membrane proteins, *NPC1*-defected (verified by *NPC1* sequencing) circulating monocyte were analyzed.

The detergent insolubility of GPI-anchored proteins (CD14, CD55), Fcγ-receptors (CD16, CD32, CD64), scavenger receptors (CD36and CD91) and the pentaspanin CD47 were tested. We found that each investigated surface antigen showed highly elevated association to DRMs in NPC monocytes as compared to control cells (Figure 24).

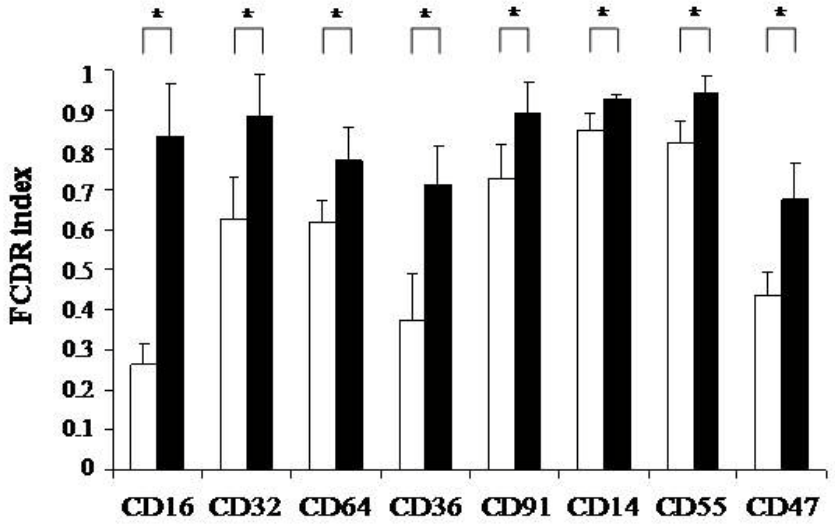

Figure 24. Detergent resistance of monocyte antigens in patients with Niemann-Pick type C disease

Ex vivo peripheral blood monocytes from healthy donors (open bars, n=6) and from patients with genetically confirmed NPC disease (filled bars, n=6) were labelled with monoclonal antibodies and treated with 0.05% cold TX-100 for 5 min. Controls and patients were measured on the same day under the same conditions. FCDR indices are presented as mean ± SD. $p < 0.05$ (*) was considered as statistically significant.

These data suggest that the accumulation of cholesterol and sphingolipids, due to the altered endocytic trafficking results in the enrichment of the specialized microdomains on the plasma membrane.

We also investigated the DRM association of the same proteins in ABCA1-deficient monocytes from one patient with Tangier disease. As shown before, the ABCA1-deficient cells accumulate cholesterol in the trans-Golgi network (TGN)-compartment and fail to release cholesterol to the plasma membrane upon ApoAI/HDL3-induced cholesterol efflux. In contrast to the NPC-deficient cells, ABCA1-deficient monocytes (data not shown) did not show any changes in DRM associations compared to controls.

5.5. Influence of ezetimib, affecting cellular membrane homeostasis, on the detergent-resistance of monocyte antigens

Structural and functional integrity of lipid rafts strictly depends on their lipid content. Lessons from the application of MBCD showed that modification of raft cholesterol content is useful for investigating raft-associated function. It was previously described that cholesterol synthesis inhibitors, such as statins or compactin affect raft structures, likely due to decrease the cholesterol content of plasma membranes (317,318).

Ezetimib was initially described as a selective cholesterol absorption inhibitor, blocking the absorption of dietary and biliary cholesterol at the level of enterocytic brush-border in the small intestine (319). The binding site for ezetimib is CD13, which is a constitutively expressed raft-associated antigen (320). In addition to epithelial cells CD13 is also expressed on the surface of monocytes/macrophages (320,321). Thus, it is tempting to speculate that ezetimib affects structure and/or function of monocytic rafts through CD13.

Therefore, human peripheral monocytes were subjected to short time (15 min) ezetimib incubation, and DRM-association of membrane proteins was investigated. We found, that ezetimib rapidly (within minutes) disrupted raft assembly. Highly detergent-insoluble proteins, such as GPI-anchored CD14, Fcγ receptors CD32 and CD64, moreover, the ezetimib target CD13 exhibited significantly decreased DRM-association after ezetimib treatment (Figure 25) (322).

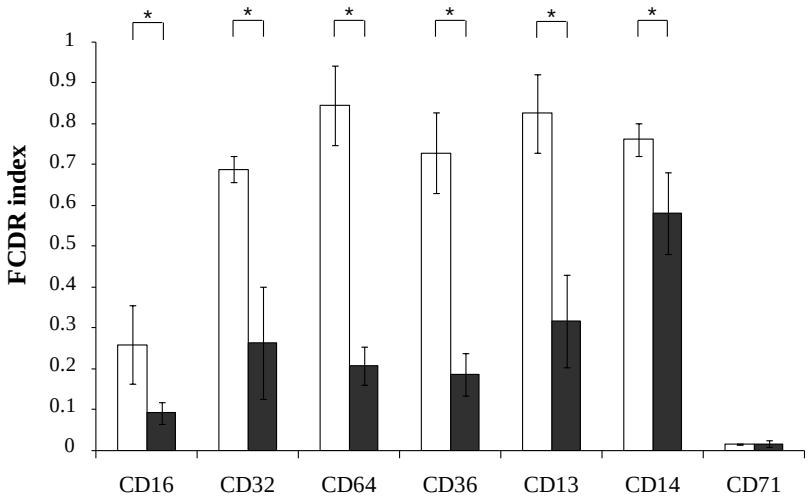

Figure 25. Effect of *in vitro* Ezetimib incubation of the DRM-association of monocyte antigens.

Peripheral blood monocytes from healthy controls (n=5) were treated with 10μM ezetimib for 15 min, then labelled with monoclonal antibodies and treated with 0.05% cold Triton X-100 for 5 min. FCDR indeces are presented as mean ± S.D. Statistical analyses were performed by paired Students *t*-tests. $p < 0.01$ (*) were considered as statistically significant.

Our data indicate that ezetimib modifies lipid raft structure on peripheral blood monocytes, like other cholesterol lowering agents, but the underlying mechanism is still unclear. In order to further characterize and confirm the microdomain disturbing role of ezetimib other methods, including FRET, western blot and confocal microscopy, were applied.

For these experiments, monocyte derived macrophages were differentiated up to 5 days and treated with 10 μM ezetimib. In parallel with the rapid DRM disturbing effect of ezetimib in whole blood monocytes, ezetimib was also able to decrease CD13 in Lubrol-DRM fractions of in vitro differentiated macrophages as detected by immunoblots. Lysis of cells in 1% Lubrol WX followed by sucrose flotation gradient-separation confirms the presence of CD13 in DRMs in control cells, whereas CD13 is elicited in detergent soluble fractions in response to ezetimib (Figure 26A). Parallel to the marked changes in cellular CD13 redistribution, ezetimib also decreases the total cellular content of CD13 (Figure 26B).

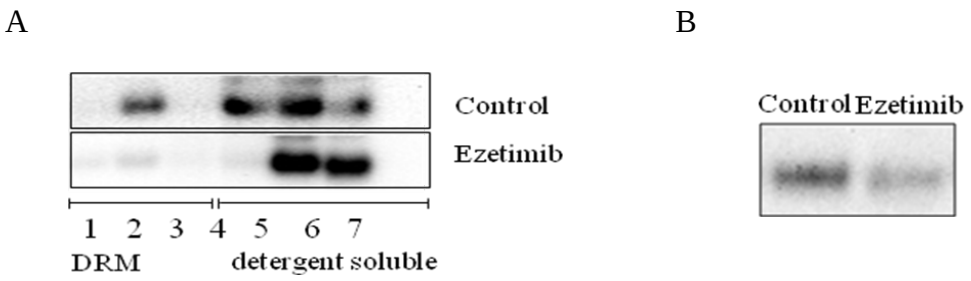

Figure 26. CD13 expression in detergent resistant membrane fractions in human monocytes
Immunoblot of monocytes shows that ezetimib reduces the presence of CD13 in DRM fractions. 1-3: detergent resistant membrane (DRM, i.e. raft), 4-7: detergent soluble fractions (i.e. non-raft). In control cell (treated with DMSO as solvent of Ezetimib) lysates CD13 is clearly detectable in DRM and detergent soluble fractions. By contrast, in Ezetimib-treated cell lysates CD13 is present only in detergent soluble fractions.

Fluorescence co-localization studies with confocal microscopy confirmed the DRM-disturbing role of ezetimib, suggested by flow cytometry and immunoblot. Hence, CD13 and CD64 showed extensive, punctual, co-localization on the cell surface in control cells. Incubation of cells with ezetimib rapidly disturbed the co-localization of these two antigens. CD64 shifted into intracellular structures within 10 minutes, while CD13 remained at the cell surface (Figure 27). Only scattered minor co-localization between CD64 and CD13 was observed in response to ezetimib.

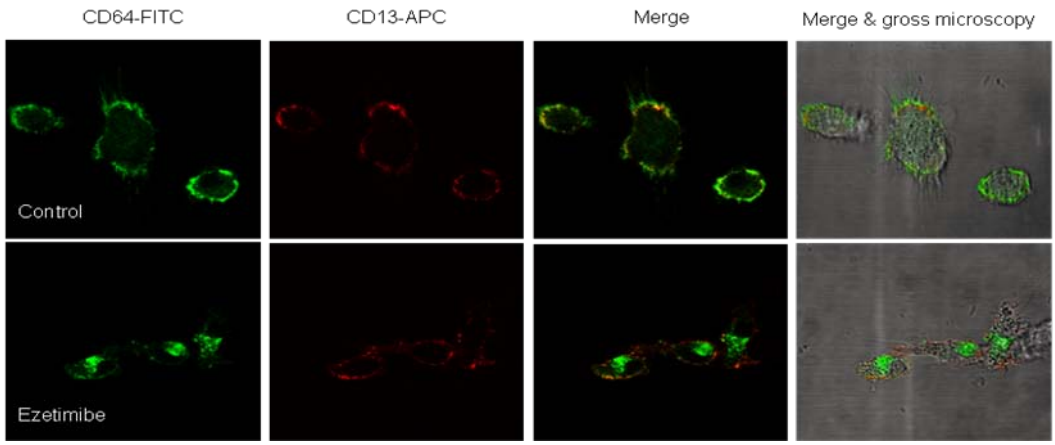

Figure 27. Confocal images of monocyte derived macrophages treated with ezetimib

Representative Confocal images showing punctual surface co-localization (merge) between CD13-APC (red fluorescence) and CD64 (green fluorescence) in human monocyte-derived macrophages (control). The co-localisation is severely disturbed in response to short treatment (10 uM, 37°C, 10 minutes) of cells with ezetimib, indicating a microdomain/DRM disturbing effect of ezetimib. Scale bar: 15 um.

Finally, FRET was performed, which is a highly sensitive indicator for close spatial proximity of molecules. As positive control, we measured a high FRET between CD13 homodimers on surfaces of resting cells. The FRET-signal was slightly decreased in response to incubation of cells with Ezetimib for 10 minutes, but FRET-values remained above the 5% cut-off level, indicating that ezetimib does not influence the homodimerization of CD13. There was also a significant FRET between CD64 and CD13 in untreated monocytes, confirming the co-localization of CD64 with CD13, as coreceptor for CD13. In addition, a significant FRET occurred between CD13 and GPI-anchored protein CD55. The co-assembly of CD13 with CD64 or CD55 detected in

control monocytes was diminished upon incubation of cells with ezetimib (Table 6), confirming the microdomain disturbing role of ezetimib (Orsó E., Wolf Z., Boettcher et al., unpublished data).

Incubation	**FRET-efficiency (in %)**		
	CD13(D) & D13(A)	**CD64 (D) & CD13 (A)**	**CD55 (D) & CD13 (A)**
Vehicle	36.7 ± 5.67 (n=6)	6.9 ± 1.04 (n=4)	5.9 ± 0.66 (n=3)
Ezetimib	25.4 ± 8.81 (n=4)	3.5 ± 0.74 (n=4)	1.9 ± 0.38 (n=3)

Table 6. Changes in the molecular co-association of CD13, CD64 and CD55 in response to ezetimibe
The molecular co-assembly between CD13 homodimers (positive control), or CD64 (D) and CD13 (A), or CD13 (A) and CD55 (D) was assessed by flow cytometric fluorescence resonance energy transfer (FRET), and expressed by acceptor sensitized emission (FRET-efficiency in %). The FRET efficiency > 5% was accepted as significant energy transfer. Monocytes were incubated either with vehicle (DMSO) or Ezetimib (10 μM each) for 10 minutes and labelled with mABs. Donors (D) were stained with R-PE, and the acceptors (A) with Cy5. Data are presented as mean ± S.D., n = number of experiments.

6. Discussion

My thesis evaluated the diagnostic utility of a flow cytometric assay for rapid detection of monocyte surface antigen association to lipid microdomains/DRMs, based on their detergent insolubility on intact cells. Here, we presented that the established detergent based method is applicable to analyse freshly obtained blood samples. Investigation of whole blood cells with this method can provide a simply analytical tool to screen disease specific alterations of membrane lipid microdomains.

6.1 Evaluation of a flow cytometric detergent solubilization assay for whole blood monocytes

Lipid microdomains show relative insolubility in nonionic detergents, classically to TX-100, providing a useful and easy tool for their analysis. In spite of the simplicity and utility of classical DRM isolation, this method implies certain problems, such as complete lysis of the cells by 1-2% detergent extraction, leading to artificial DRM formation from inner cell membranes. Thus, the initial subcellular localization of DRMs can not be defined, and certain raft-associated proteins linked to the cytoskeleton do not float in the DRM fraction, but associate with the pellet fraction (23).

Here, we provide evidence that the above mentioned limitations can be avoided by using only mild detergent extraction (0.01- 0.1 % TX-100), followed by a rapid (1-5 min) flow cytometric detection of the detergent-insoluble fractions of cell surface antigens in human blood monocytes.

Some earlier studies applied flow cytometry to analyze detergent resistance of cell surface proteins, including erythroid cell line differentiation markers (323), lymphocyte membrane proteins (304,305), integrins from hepatocytic cell lines (306). All of these studies applied nonionic detergents (TX-100 or Nonidet-40), however relatively high concentrations (0.5-1%). Gombos et al. developed a flow cytometric assay of differential detergent resistance (termed FCDR assay) to study T-cell line proteins (274). They used relatively low concentrations (0.05-0.1%) of mild detergents for a short period of time (up to 5 minutes). Comparing the fluorescence signals recorded

before and after detergent treatment serves information about the detergent solubility of a given antigens.

Based on this work, we optimized this flow cytometric method to assess human circulating monocytes from freshly obtained whole blood samples. In order to investigate monocytic DRMs under intact conditions, minimal *in vitro* manipulations were carried out: (i) staining of antigens with fluorochrome-conjugated mAbs (causing no cross-linking or signalling) prior to (ii) mild TX-100 treatment (0.01-0.1%). It can not be excluded, however, that binding of mABs to their epitopes may induce receptor clustering, and very small rafts (only a few 10s of nm) fuse forming larger rafts. Therefore, it should be taken into account that using Fab antibodies is preferable to whole immunoglobulin, which may help to avoid crosslinking-induced detergent resistance effects (324).

In the present work, 0.01% TX-100 was used as the lowest concentration of detergent. We found that this concentration is sufficient for specific detection of DRMs in human circulating monocytes, because CD71 was found to be TX-100 soluble. The transferrin receptor CD71 is known to be excluded from lipid rafts, hence, can be used as a negative control (325). However, these data suggest that the appropriate detergent concentration must be determined for each cell and detergent type (324).

Visualization of detergent treated monocytes by confocal microscopy could also confirm that mild, short-duration detergent treatment does not result in the loss of cellular integrity. The cells remained intact particles, enabling them for flow cytometric analysis. In addition, images of saturated lipid probe DMPE-TMR provided clear evidence for retained structural integrity of membrane microdomains/ lipid rafts.

Moreover, the cholesterol sensitivity of detergent resistant membrane confirmed the reliability of this assay on circulating monocytes. Hence, cholesterol plays an essential role in the regulation of raft structure, stability and function (44,326), therefore, detection of raft-integrity disruption through cholesterol depletion is a widely used approach to study the composition and functions of lipid microdomains (283,290).

Finally, this flow cytometric approach offers a several advantages compared with the classical biochemical methods: the test requires low amount of cells (10^6 cells/sample) and short-term analysis (5 min). The assay has a high reproducibility and can be performed on all types of conventional bench-top flow cytometers. The major advantage

of this method is, however, the potential to analyse intact cells minimizing artifacts concerning detergent-induced perturbation/rearrangement of in situ protein assemblies, which is likely occured when the cells are fully lysed with high (typically 1-2%) detergent concentrations.

6.2 Effect of *in vitro* stimulation with lipopolysaccharide on CD14-dependent receptor clustering in human monocytes

The pathways through LPS signals have been investigated extensively. The LPS is delivered to the cell surface by serum LBP and then the LBP-LPS complex binds to receptor CD14, the key molecule of the innate bacterial recognition. CD14 molecule lacks transmembrane and cytoplasmic domains, thus, needs additional signalling receptors to initiate signal transduction cascades. Since CD14 is a GPI-anchor protein, and residues in lipid membrane microdomains/rafts, it is tempting to speculate that the entire bacterial recognition and the recruitment of multiple signalling molecules take place within lipid rafts. Several studies investigated this principle (104,108,327).

Triantafilou et al observed the formation of an activation cluster following LPS stimulation, comprising of TLR-4, CD14, 5hsp 70, hsp 90, CD55, GDF and CXR4 (107). Perera et al. strengthen the concept of activation clusters as they have shown that the interactions of CD14, CD11b/CD18 and TLR-4 are essential for LPS-inducible gene expression (327). Pfeiffer *et al*. also detected the formation of distinct activation cluster in response to LPS, LTA or ceramide stimulation (104).

In my thesis the compartmentalization of given pattern recognition receptors was investigated in response to LPS stimulation. Our data demonstrate that flow cytometric screening test, based on TX-100 insolubility of surface antigens, is useful to detect dynamic changes of microdomain/DRM association in whole blood monocytes. Certain molecules involved in the innate recognition of bacteria, such as GPI-anchored proteins CD14, CD55 and Fcγ-receptors CD32, CD64 were constitutively found in membrane microdomains. In contrast antigens, like pentaspanin CD81 and TLR-4, did not show raft residency, but were recruited into lipid microdomains following LPS stimulation.

In addition, we provided evidence on the LPS recognition of *F. tularensis* with FCDR assay. *F. tula*rensis is known to own an atypical structure and function. Exposition of *F.*

tularensis LPS to host cells elicits no proinflammatory cytokine release from mononuclear cells (316). Our observations suggest that *F. tularensis* LPS interacts with host recognition proteins and initiate the same receptor co-assembly on the monocytic surface similar to enterobacterial LPS, such as *E. coli* or *S. minnes*ota.

6.3 Characterization of CD14-dependent receptor clustering *ex vivo* on peripheral circulating monocytes in inflammatory diseases

Monocytes/macrophages express a broad range of pattern recognition receptors (PRRs) implicated in immune recognition and atherosclerosis, followed by surface changes, signalling and uptake, contributing to homeostasis, host defense and innate effector mechanisms. Several phenotypes of macrophage are found in plaques, including inflammatory macrophages and also foam cells, which develop when cholesteryl esters accumulate in the cytosol of intimal macrophages (4).

CD14 plays a major role in the inflammatory response to bacterial pathogens and in atherogenic lipoprotein clearance. Expansion of monocytes with reduced CD14 expression was observed in inflammation (8) and this subpopulation of monocytes showed a positive correlation with atherogenic lipoprotein profiles (9,11). Moreover, CD14 serves as a receptor for ceramide, which shares some structural and functional similarities with the lipid A moiety of LPS (328). Ceramide is known to accumulate in atherogenic lipoproteins and in membranes of apoptotic cells (79).

Although, CD14 signalling through TLRs has been extensively investigated in exogenous host defense, an involvement of TLRs in the pathogenesis of atherosclerosis was more recently implicated. TLRs are expressed on macrophages and endothelial cells in atherosclerotic plaques. Some data indicated that oxidized LDL (oxLDL) binds TLR4–CD14 complexes and induce proinflammatory cytokine secretion (267,329). Scavenger receptors are another type of PRRs that mediate internalization and lysosomal degradation of modified lipoproteins, LPS, fragments of pathogens and apoptotic bodies (330). The uptake of different LDL species, such as oxidized LDL and enzymically cleaved LDL, into macrophages requires the binding to the receptor CD36 and its localization to lipid rafts (231). Furthermore, differentially modified LDL

internalization induces formation of different raft domains in the macrophage plasma membrane (331).

Other class of monocyte/macrophage innate immune receptors, including complement receptors and Fcγ receptors (CD16, CD32 and CD64), are also involved in the uptake of opsonized lipoproteins and pathogens (330). The activation of the classic complement pathway appers to be a key factor in atherosclerosis, as well as, in the progression of the chronic inflammatory response of Alzheimer´s disease. The multiligand receptor LRP (CD91) mediates the cellular uptake of a great variety of ligands, e.g., chylomicron remnants and protease-protease inhibitor complexes and also interacts with amyloid precursor protein (APP), and regulates its proteolytical processing and the production of the Aß peptide (332).

Recently, Pfeiffer et. al. provided evidence that the binding of lipopolysaccharide and ceramide, a constituent of atherogenic lipoproteins, results in ligand-specific receptor clustering in membrane microdomains. Lipid rafts on monocytes/macrophages provide a dynamic microenvironment for an integrated CD14-dependent clustering of a set of receptors involved in innate immunity and clearance of atherogenic lipoproteins. In resting cells, CD14 was associated with CD55, the Fcγ-receptors CD32 and CD64 and the pentaspan CD47. Ceramide further recruited the complement receptor 3 (CD11b/CD18) and the scavenger receptor CD36 into proximity of CD14. LPS, in addition, induced co-clustering with TLR4, Fcγ-receptor CD16 and the tetraspanin CD81 while CD47 was dissociated. Thus clustering of signalling receptors to a common recognition platform in lipid rafts may provide an interesting mechanism by which different ligands induce distinct cellular processes in systemic inflammation (SIRS or sepsis) and cardiovascular disease.

In the present work detergent insolubility of these ligand-induced receptors was reinvestigated on monocytes from patients with SIRS/sepsis (i.e. *in vivo* LPS activation), or CAD/myocardial infarction (i.e. *in vivo* atherogenic lipoprotein stimulation). *In vivo* LPS stimulation in SIRS/sepsis resulted in an increased detergent insolubility of CD14 and CD55, CD64, CD36, CD91, CD163, CD11a, CD11b/CD18 and CD81 suggesting their stronger association to DRMs due to clustering, while the increased detergent solubility of CD47 indicates its disassembly from lipid

microdomains. A similar phenomenon was observed for *in vivo* atherogenic lipoprotein activation from patients with CAD/myocardial infarction. Almost all of the investigated receptors presented an elevated detergent resistance (e.g. CD14, CD55, CD16, CD32, CD64, CD36, CD91, CD163, CD11a, CD11b, CD18 und CD47), with the exception of the tetraspanin CD81, which translocates to DRMs only upon LPS stimulation. Our results also support the concept of lipid raft heterogeneity, namely rafts exist in small distinct microdomains with a diverse receptor composition and upon ligand activation they selectively fuse to form large signalling platforms (227).

6.4 Investigation of the effect of disturbed cellular membrane trafficking on DRM partition of monocyte antigens

Hence, assuming that cellular cholesterol trafficking disorders, such as NPC and Tangier disease, may influence membrane microdomain size and composition, and the cellular alterations may be present on circulating monocytes. NPC disease is characterised by blockade of late endosomal sorting and vesicular trafficking, with subsequent accumulation of cholesterol and sphingolipids in the NPC-compartment (61,216). It has been shown that in *NPC1* deficient cells membrane microdomains accumulate in late endosomes and lysosomes (216), thus, it is hypothesized that microdomains are assembled in the plasma membranes, too. Indeed, analysis of antigens of defective *NPC1* bearing monocytes revealed a highly elevated DRM association of all investigated cell surface antigens, suggesting the enrichment of lipid microdomains in the plasma membrane. This phenomenon is most likely due to the dysregulated cholesterol and sphingolipid influx, in response to a defective late endosomal cholesterol efflux towards intracellular stores. In contrast, familial HDL deficiency syndrome, such as Tangier is characterized by impaired ApoA-I-mediated cellular cholesterol and phospholipid efflux due to ABCA1-mutation, leading to cholesteryl ester accumulation in the TGN-compartment and impaired plasma membrane cholesterol content (245). Interestingly, ABCA1 deficient monocytes did not show a significant difference in the detergent resistance for surface antigens as compared to controls. These data suggest that the blockade of ABCA1-dependent

cholesterol efflux, preferentially leads to enhanced lipid droplet formation and TGN storage, but does not affect microdomains structure in the plasma membrane.

6.5 Investigation of the effect of ezetimib, a drug influencing cellular lipid homeostasis, on DRM association of monocyte proteins

Ezetimib is a lipid-lowering drug, which selectively inhibits intestinal cholesterol absorption (319). Ezetimib significantly lowers total cholesterol, low-density lipoprotein cholesterol and triglycerides, and increased high-density lipoprotein cholesterol (333-336), however, its greatest effectiveness results from its use in combination with statin therapy (337-340).

CD13/aminopeptidase N an ectoenzyme of the superfamily of zink metalloproteases has been reported as a molecular target of ezetimib in the enterocyte brush border (319). Although another protein, Niemann-Pick C1-like 1 (NPC1L1), was also proposed to be the main target for ezetimibe (341,342). Recently, it has been published that ezetimibe is equally capable of reducing cholesterol uptake in NPC1L1-deficient and wild-type mice (343). Binding of ezetimib to CD13 on enterocyte membranes was shown to be sufficient for the effect of the drug. Moreover, the binding site for ezetimib on CD13 differs from the aminopeptidase activity of this antigen.

CD13 is widely expressed and involved in certain biological processes, such as growth and invasion of tumors (344,345), cellular motility (346), regulation of angiogenesis (347,348), cellular internalization of human coronavirus 229E (349,350) and participates in human cytomegalovirus infection (350-353).

CD13 is a raft microdomain-resident, type II membrane protein, and both of its homodimeric polypeptides have a very short cytoplasmic domain of eight amino acid residues with no described signaling motifs (320). Thus, it is very likely that signal transduction through CD13 requires additional membrane proteins, quite likely localized in rafts. Recently, it has been reported that CD13 functionally interacts with the Fcγ receptor CD64 on monocytic cells (354).

Since CD13 is constitutively expressed in lipid rafts of monocytes/macrophages, it is tempting to speculate that ezetimib influences structure and/or function of rafts in these cells. Indeed, the present study provides evidence that ezetimib modulates the cellular

re-distribution and expression of CD13 in monocyte-derived macrophages. Moreover, the expression of further raft-associated antigens such as the Fcgγ-receptors CD16 and CD64, and the scavenger receptor CD36, extensively implicated in cellular uptake of modified lipoproteins or phagocytosis, is also down-regulated in response to ezetimib (322). The decreased expression of these receptors upon ezetimib administration may lead to an impaired macrophage-differentiation and lipid-uptake, and thus ezetimib quite likely exerts an in vitro anti-atherogenic effect in monocyte-derived macrophages, which involves rafts (322).

CD13 requires co-receptor for subsequent signalling, and CD64, CD16 or CD36 are demonstrated being putative CD13-interacting antigens (354). Ezetimib may exert its cellular effect through disruption of the co-assembly of CD13 with CD64/CD16 or CD36, and in this scenario, the missing co-association of receptors may lead to their disappearance from lipid rafts. The finding that CD13 disappears from DRMs/rafts in response to ezetimib may support this hypothesis. Binding of ezetimib to CD13 quite likely induces a conformational change in the extracellular domain of CD13, masking the specific sequence required for coassociation of CD13 with other receptors. Such a mechanism has recently been proposed for ezetimib in enterocytes (319). Alternatively, shedding of CD13 in response to ezetimib may also explain the loss of its expression.

In summary, ezetimib likely modifies raft assembly in monocyte-derived macrophages through a CD13-dependent dissociation of pre-clustered receptor complexes. It is known that lipoproteins regulate Fcγ receptor expression and Fcγ receptor-mediated phagocytosis. Thus, given the importance of cholesterol uptake by phagocytes in atherosclerosis and the fact that ezetimib blocks low-density lipoprotein (LDL) uptake in macrophages, determining whether CD13 is a target for ezetimib in these cells is an interesting question, which is currently being addressed (355).

7. Conclusion and perspectives

This work established and applied a novel flow cytometric assay for rapid analysis of surface antigens association to lipid microdomains/rafts on circulating monocytes. The method is based on detergent insolubility of lipid microdomains to cold nonionic detergent, such as TX-100.

Constitutive and activation-induced DRM-association of certain antigen was demonstrated upon *in vitro* LPS stimulation. We showed a specific clustering of tetraspanin CD81 and TLR-4 receptors into lipid microdomains in response to LPS activation. Furthermore, a strengthened DRM association of surface antigens upon CD14-dependent receptor clustering were observed, as well as an inversed DRM partition of penta-/tetraspanin family member CD47 and CD81 in SIRS/sepsis and CAD/myocardial infarction patients. Moreover, Fcγ receptor CD16, which is primarily involved in phagocytosis and atherogenic lipoprotein clearance, showed enhanced DRM-association only in CAD/myocardial infarction patients, but not in SIRS/sepsis patients. Our observation regarding ligand-induced receptor clustering also supports the concept of lipid raft heterogeneity, namely rafts exist in small distinct microdomains with a diverse receptor composition and upon activation they selectively fuse to form large signalling platforms.

Our data demonstrate that defective NPC1 bearing monocytes reveal a highly elevated DRM association of all investigated cell surface antigens. These findings suggest the enrichment of lipid microdomains in the plasma membranes, likely due to accumulation of the lipid constituents in the plasma membrane, as well. Interestingly, ABCA1 deficient monocytes do not show a significant difference in the detergent resistance of surface antigens.

Finally, we presented that ezetimib, a lipid lowering drug, rapidly disrupts lipid microdomain assembly on peripheral blood monocytes, possibly through disturbing the association of CD13 with its co-receptors CD36 and CD64.

In conclusion, our data indicate that this rapid flow cytometric assay provides a simply and powerful analytical tool to screen disease specific alterations of membrane microdomains from freshly obtained blood samples. This assay offers some advantages, which make it suitable for routine daily practice: the method requires low amount of

cells (10^6 cells/sample) and short time (5 min) compared with the classical biochemical methods. The assay has a high reproducibility and can be performed on all types of conventional bench-top flow cytometers. The major advantage of this method is that analyzing intact cells by flow cytometry minimizes artifacts concerning detergent-induced perturbation/rearrangement of in situ protein assemblies, which is likely to occur when the cells are fully lysed with high (typically 1-2%) detergent concentrations. Flow cytometry analysis can provide additional information on lipid rafts in plasma membranes and allows a dynamics follow-up of lipid rafts partitioning. Further characterization of protein and lipid composition of detergent resistance membranes may lead to the detection of disease-specific alterations and could contribute to monitor effects of therapeutic agents on membrane constituents. On the other hand, flow cytometry through multi-color staining analysis allows the possibility to investigate parallel different surface antigens on different cell populations without the need to purify them by physical separation methods.

8. Summary

Lipid rafts resemble cholesterol- and glycosphingolipid-enriched, liquid-ordered plasma membrane microdomains, allowing specific interactions that modulate signal transduction, membrane trafficking and pathogen entry. Recently, lipid rafts have been also implicated in a range of monocyte/macrophage functions, including endotoxin-mediated activation, scavenging and recycling of atherogenic lipoproteins, cellular cholesterol influx/efflux and trafficking. Due to their tightly ordered lipid phase, membrane microdomains show a relative resistance to nonionic detergents (classical to Triton X-100) providing an easy analytical tool to study them.

The aim of the thesis was to analyze specific antigens of lipid microdomains in monocytes that establish the raft-associated, ligand induce CD14-dependent specific activation of these cells. The studies established and applied a rapid flow cytometric detergent resistance-based (FCDR) assay to investigate microdomain association of proteins on circulating monocytes from whole blood samples. By using FCDR assay, constitutive and activation-induced detergent resistant membrane (DRM) associations of certain antigen were demonstrated upon *in vitro* LPS stimulation. Characteristic alterations and different patterns of CD14-dependent receptor co-assembly within microdomains could be detected *ex vivo* in different disease states, such as inflammatory response (SIRS/sepsis and CAD/myocardial infarction) or disorder affecting cellular cholesterol trafficking (Niemann-Pick type C disease). Moreover, we provided evidence that this assay is capable to detect effects of therapeutic agents affecting membrane microdomains structure and/or function, such as ezetimib a lipid lowering drug.

Our results demonstrate that flow cytometric analysis of short time in situ detergent extraction provides a powerful tool for rapid examination of blood monocyte DRMs. This may contribute to screen patients with potential microdomain abnormalities and/or monitor effects of therapeutic agents on membrane constituents. In addition, multi-color analysis allows the possibility to explore parallel different surface antigens on different cell populations without the need to purify them by physical separation.

9. Összefoglalás

A lipid tutajok koleszterinben és glikoszfingolipidben gazdag, "rendezett-folyékony" struktúrájú mikrodoménjei a plazmamembránnak, amelyek nagyfokú laterális mobilizációjuk révén modulálják a jelátviteli illetve membrántranszport folyamatokat és a különböző patogének inváziójá̀t. Újonnan felvetődött a lipid tutajok szabályozó szerepe számos monocyta funkcióval kapcsolatban, beleértve az endotoxin-közvetített aktiválást, a sejtek koleszterin transzportjának irányítását, valamint az atherogén lipoproteinek felvételének és raktározásának szabályozását. A legelterjedtebb módszer a mikrodomének tanulmányozására - molekuláris kölcsönhatásaikból adódóan - a nemionos detergensekkel (például Triton X-100) szembeni rezisztencia.

A munkám célja a monocyták specifikus antigénjeinek vizsgálatát tűzte ki célul, amelyek a lipid tutajokhoz társuló, ligand indukált CD14-függő sejtaktivációt képezik. A kísérleteink során egy a detergens rezisztencián alapuló áramlási citometriás módszert alkalmaztunk perifériás monocyták fehérjéinek mikrodomén asszociáltságának vizsgálatára friss vérmintákon. A módszer által konstitutív és aktiváció-indukálta detergens rezisztens membrán (DRM) asszociációkat tudtunk kimutatni *in vitro* LPS stimuláció hatására. CD14-függő receptorkomplex fehérjéinek specifikus és különböző mintázatú DRM-asszociációit lehetett továbbá kimutatni *ex vivo* különböző betegségekben, gyulladásos folyamatokban (SIRS/szepszis, koronária betegség/szívinfarktus) és lipidek transzport zavarával járó kórképben (Niemann-Pick C típusú betegség). Ezentúl, ez a vizsgálati módszer képes terápiás szerek mikrodomének szerkezetét és/vagy funkcióját érintő hatásainak detektálására, mint például az ezetimib lipidcsökkentő gyógyszer.

Eredményeink kimutatták, hogy a detergens resisztencián alapuló áramlási citometriás eljárás hatékony módszer perifériás monocyták DRM asszociációjának gyors elemzésére. Ez a módszer hozzájárulhat lehetséges mikrodomén specifikus elváltozások szűréséhez és/vagy gyógyszerek mikrodomén funkcióját befolyásoló hatásának felderítéséhez. Továbbá, a multiparaméteres áramlási citometria használata lehetővé teszi különböző sejtfelszíni antigének párhuzamos vizsgálatát különböző sejtpopulációkon izolálás nélkül.

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11. Acknowledgements

I wish to thank all the colleagues and friends who have helped me during these years.

Especially I would like to acknowledge the following people:

First and foremost I earnestly express my appreciation to Professor Gerd Schmitz who provided me the opportunity to work in his lab. I wish to express my gratitude to my supervisors Professor Gerd Schmitz and Dr. Evelyn Orsó for their excellent continuous support and amazing expertise in science. I sincerely thanks to Evelyn for introducing me to the wonderful world of flow cytometry.

I would also like to express my deepest appreciation for the support I received from Professor László Romics. I extend my thanks to Professzor József Mandl and his co-workers, especially to Dr. Ágota Vér and Pálné Nigovicz Zsuzsa for their readiness to help at all times.

I warmly thank all the employees of the Institute for Clinical Chemistry and Laboratory Medicine of the University of Regensburg, especially to the team of the flow lab. My special thanks to Ella and Anna for your excellent technical help throughout these years. I also want to acknowledge to Professor Tobias Werner for your assistance with the confocal microscope.

Special thanks to my lab mate Katie, Köbi, Gyuri and Peter! Our time in Regensburg will never be forgotten! A big thanks all the other students Margot, Marion and Sasha, as well. I take this opportunity to express my sincere thanks to Katie, whose support and care I will always need. Her friendship is one of the best things that happened to me.

Last but not least, I would like to dedicate this work to my parents and my sister. Without their support this work would not have been possible.

Bernie, “mein Schatz”, for simply being there for me at all times.

For all help and for all support THANK YOU!

11. List of own publication

Publications related to the thesis

1. ***Zsuzsanna Wolf****, Evelyn Orsó, Tobias Werner, Alfred Böttcher, Gerd Schmitz.* A flow cytometric screening for detergent resistant surface antigens in monocytes
Cytometry A. 2006 Mar;69(3):192-5

2. *Evelyn Orsó, Tobias Werner,* ***Zsuzsanna Wolf****, Sascha Bandulik, Werner Kramer and, Gerd Schmitz.* Ezetimibe influences the expression of raft-associated antigens in human monocytes
Cytometry A. 2006 Mar;69(3):206-8

3. ***Zsuzsanna Wolf****, Evelyn Orsó, Tobias Werner, Hans H. Klünemann and Gerd Schmitz.* Human blood monocyte cholesterol homeostasis correlates with the presence of detergent resistant membrane microdomains
Cytometry A. 2007 Jul;71(7):486-94

Publication not related to thesis

4. *Ferencz V, Kari B, Gaal J, Meszaros S,* ***Wolf Z,*** *Hegedus D, Horvath A, Folhoffer A, Horvath C, Szalay F.* Bone disorders in experimentally induced liver disease in growing rats
World J Gastroenterol. 2005 Dec 7;11(45):7169-73

Abstracts to cite

1. ***Wolf Zsuzsanna,*** *Ferencz Viktória, Mészáros Szilvia, Szalay Ferenc, Horváth Csab*a. A csont ásványianyag-tartalmának vizsgálata kísérletes májcirrhosisban, patkánymodellen.
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