UNIVERSITY OF TROMSØ UIT

FACULTY OF HEALTH SCIENCES
DEPARTMENT OF MEDICAL BIOLOGY
VASCULAR BIOLOGY RESEARCH GROUP

Pattern recognition receptors in liver sinusoidal endothelial cells: Roles in the innate immune system and scavenger functions

Jaione Simón-Santamaría

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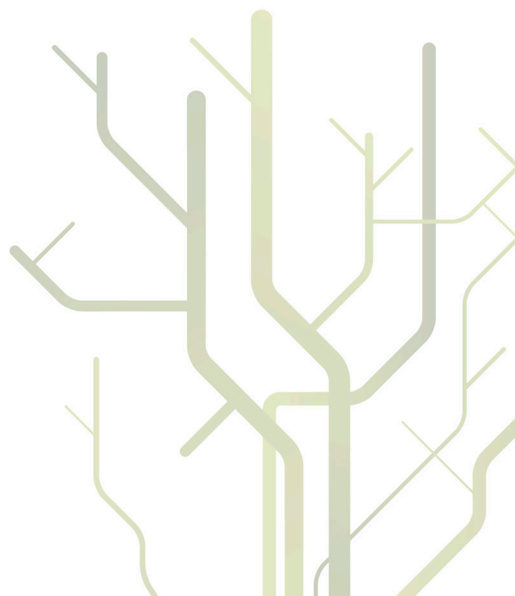

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Paper I

Montserrat Martin-Armas, Jaione Simon-Santamaria, Ingvild Pettersen, Ugo Moens, Bård Smedsrød, Baldur Sveinbjørnsson. Toll like receptor 9 (TLR9) is present in murine liver sinusoidal endothelial cells (LSECs) and mediates the effect of CpG-oligonucleotides. *Journal of Hepatology*, 44 (5): 939-946, 2006.

Paper II

Kjetil Elvevold*, Jaione Simon-Santamaria*, Hege Hasvold, Peter McCourt, Bård Smedsrød, Karen Kristine Sørensen. Liver sinusoidal endothelial cells depend on mannose receptor-mediated recruitment of lysosomal enzymes for normal degradation capacity. *Hepatology*, 48: 2007-2015, 2008.

*Shared first authorship

Paper III

Jaione Simon-Santamaria, Ivana Malovic, Alessandra Warren, Ana Oteiza, David Le Couteur, Bård Smedsrød, Peter McCourt, Karen Kristine Sørensen. Age-related changes in scavenger-receptor mediated endocytosis in rat liver sinusoidal endothelial cells. *Journal of Gerontology: Biological Sciences,* 65(9): 951-960, 2010.

ABBREVIATIONS

AGE: advanced glycation end product
APC: antigen presenting cell
CD: cluster of differentiation
DAMP: damage associated molecular pattern
FcγR: Fc gamma receptor
FITC: fluorescein isothiocyanate
FSA: formaldehyde treated serum albumin
HDL: high density lipoprotein
ICC: immunocytochemistry
IgG: immunoglobulin G
IL: interleukin
KC: Kupffer cell
LDL: low density lipoprotein
LPS: lipopolysaccharide
LSEC: liver sinusoidal endothelial cell
M6PR: mannose-6-phosphate receptor
MR: mannose receptor
MyD88: myeloid differentiation protein 88
NFκB: nuclear factor kappa B
NK: natural killer
ODN: oligodeoxynucleotide
PAMP: pathogen associated molecular pattern
PC: parenchymal cell
PICP: C-terminal propeptide of type I procollagen
PIIICP: C-terminal propeptide of type III procollagen
PRR: pattern recognition receptor
RT-PCR: reverse transcription polymerase chain reaction
SC: stellate cell
SR: scavenger receptor
TGF: transforming growth factor
TLR: toll-like receptor
TNF: tumor necrosis factor
vWF: Von Willebrand factor

GENERAL BACKGROUND

Galen (AD 129 – 199/217), a Greek physician, anatomist and philosopher, and probably the best known physician of the Roman ancient times, thought that the liver was the main organ of the human body, arguing that it emerged first of all the organs in the formation of a fetus. He also thought that circulation was a double system of distribution, where the venous blood was created in the liver and the arterial blood in the heart, from where the blood was distributed to the rest of the body and was later regenerated in liver or heart (1). Although many of Galen's theories have been proven wrong long time ago blood is in fact produced in liver during fetal life and the liver is indeed a vital organ with an extraordinary variety of functions.

The liver may be viewed as the major chemical plant of the body and plays both the role as a producer and a garbage disposer. The producing activity includes synthesis of the major plasma proteins (e.g. albumin, proteins involved in coagulation, complement, and acute-phase reactions), as well as other pivotal molecules such as glycogen, cholesterol and urea (2). The liver also plays a central role in detoxification and drug metabolism (3), and is an important host defence organ. The phagocytic function of the numerous liver macrophages (Kupffer cells) is well described (2, 4-6). Another important, yet less well studied host defence function of the liver is the efficient removal from blood of unwanted self and foreign soluble macromolecules, such as waste products from connective tissue turnover, and various microbial constituents (7, 8). This function is mostly carried out by the endothelial cells that line the numerous capillaries (i.e. sinusoids) of the liver lobules. These liver sinusoidal endothelial cells (LSECs) are characterized by a very thin and perforated (fenestrated) cytoplasm, the expression of several high affinity endocytosis receptors, and a well developed endocytic apparatus (7-10).

Despite the effective LSEC endocytosis of foreign material and endogenous waste products (reviewed in (8)) and other deleterious substances such as oxidized low density lipoproteins (LDLs) (11) via receptors (i.e. the mannose receptor (MR), scavenger receptors (SRs)) that are also regarded as major players in innate immunity (i.e. so-called pattern recognition receptors; PRRs)

the role of the LSEC as part of the immune system is still unclear, and information about the important scavenger function of these cells is not included in widely used textbooks in immunology, cell biology, and physiology (12-14).

The main focus of this thesis work was directed to gain more information about the expression and functions of PRRs in LSECs, and their possible roles in LSEC biology and host defence, as well as the effect of aging on the LSEC scavenger function.

The microenvironment of the liver sinusoidal endothelial cell (LSEC)

Approximately 80% of the blood that perfuses the liver enters via the hepatic portal vein that drains the intestine, pancreas and spleen. This blood is poorly oxygenated and rich in nutrients and may also contain toxins, bacteria and virus from the gut. In the liver lobules, the venous blood from the terminal branches of the portal vein mixes with well-oxygenated blood from the hepatic arterial branches and travels through the numerous sinusoids of the liver lobules before entering the central venules and hepatic veins, from where it drains into the inferior vena cava (15).

The organization of the sinusoids varies within the hepatic lobule; near the portal venules and hepatic arterioles, the sinusoids are arranged in interconnecting polygonal networks, whereas further away from the portal venules the sinusoids become organized more as parallel vessels that terminate in central venules (15)(Fig.1). The liver sinusoids are narrow vessels (diameter approximately 5-7 μm (16)). Here the traffic between blood and the liver parenchymal cells (PCs; hepatocytes) occurs through the fenestrated endothelium and the subendothelial space of Disse.

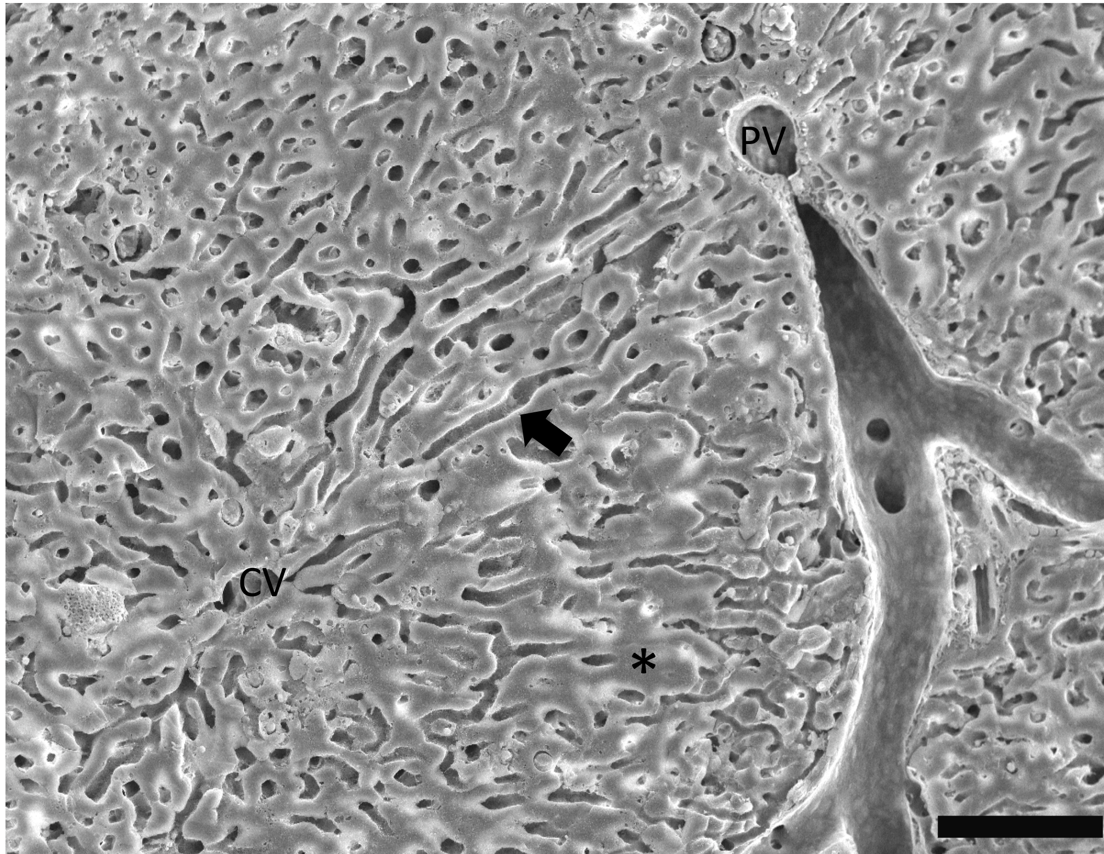

Fig. 1: Scanning electron microscopy (SEM) image of a rat liver

SEM image of a rat liver showing the structure of the classic liver lobule with a portal venule (PV), central venule (CV), numerous sinusoids (arrow) and parenchyma (*). Blood flows from the PV to the CV through the sinusoids Bar is 100 μm. (Micrograph kindly provided by Dr. Karen Sørensen).

The PCs make up most of the liver cell volume (approximately 80%; measured in male Sprague-Dawley rats (17)) and represents the hepatic "chemical reactor" where most of the intermediary metabolic processes and synthetic reactions take place (2).

The space of Disse is located between the endothelium and the PCs and is filled up with microvilli from the PCs and a loose matrix consisting of proteoglycans (e.g. heparan sulphate) and some proteins, including collagens type I, III, IV, laminin, and fibronectin (examined in human and mouse livers) (18-20). Stellate cells (SCs), which are the pericytes of the sinusoidal vessels, are also located in the space of Disse, whereas Kupffer cells (KCs) and different types of resident lymphocytes are normally located at the luminal aspect of the sinusoidal lining. The various populations of non-parenchymal

cells (NPCs) found in connection with the liver sinusoids (Fig. 2) will be presented in greater detail in the next paragraphs.

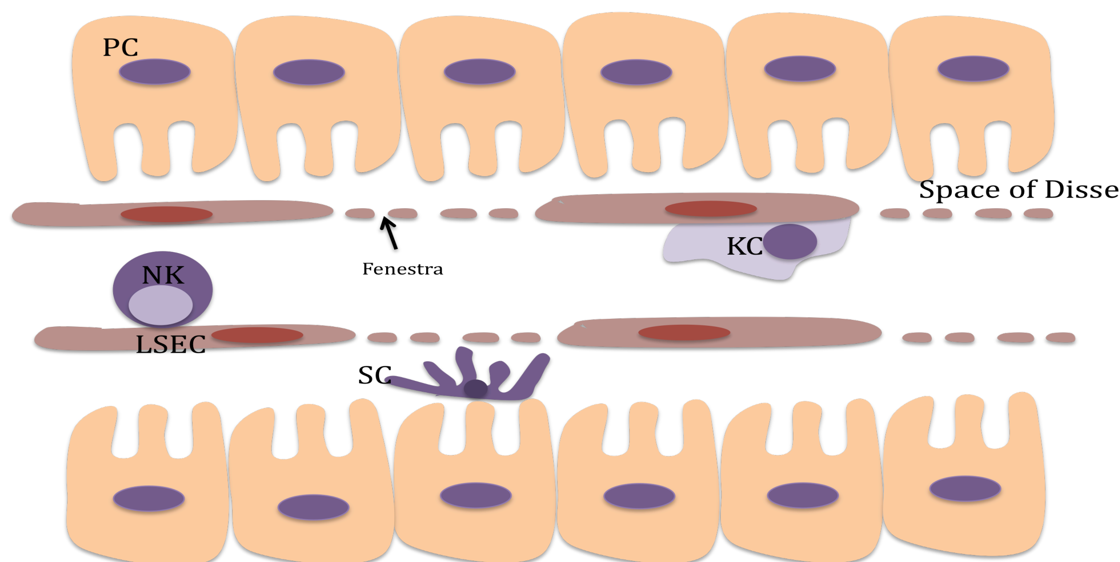

Fig.2: Localization of the main cell types of the liver sinusoid

Schematic drawing of the localization of parenchymal cells (hepatocytes; PC), liver sinusoidal endothelial cells (LSEC), Kupffer cells (KC), stellate cells (SC) and lymphocytes (i.e. natural killer cells; NK).

The Kupffer cells (KCs) make up the largest population of macrophages in the body (approximately 20 % of total macrophages in young male C57BL/6 mice) (21) and constitute approximately 30% of the NPCs (measured in male Sprague-Dawley rats) (17). However, the relative and absolute numbers may vary between species and age groups (22). The KCs are located towards the sinusoidal lumen, either on top or in between the LSECs (23). Their most studied function is the removal of blood borne particulate material by phagocytosis (e.g. bacteria and bacterial components, red blood cells, complement components, immune complexes, and collagen fragments interacting with immune competent cells) (24-27). Many of these substances come directly from the gut and the KCs act like an effective filter cleaning the blood before it enters the general circulation.

KCs express several receptors involved in phagocytosis, and several of these such as SRs are regarded as PRRs of the innate immune system. PRRs are

defined as a group of proteins that recognise special molecular patterns present in pathogens (pathogen-associated molecular patterns, PAMPs), e.g. lipopolysaccharide (LPS) from the wall of gram negative bacteria, unmethylated-CpG DNA from bacteria or virus, and flagellin from flagellated bacteria (28, 29). The PRRs can also recognize molecules from the host that are able to elicit an immune response, so-called alarmins or damage-associated molecular patterns (DAMPs) (30, 31), such as heat-shock proteins (32) or hyaluronan fragments (33).

The PRRs on KCs include many Toll-like receptors (TLRs 1 to 9) (34-36); this type of receptors will be presented more in detail later in the thesis. In addition, KCs also express several SRs, including SR class AI/II, which recognizes acetylated and oxidized LDLs, LPS and lipoteichoic acid (part of the gram positive bacteria cell wall) (37, 38), MARCO (in mouse) that recognizes *Staphylococcus aureus* inactivated bacteria and acetylated LDL (39) and SR-BI recognizing high density lipoproteins (HDL) (40). They also express Fcγ-receptors (FcγRs) that recognize IgG-immune complexes (41), complement receptors 1, 3 and 4 (CR1, CR3 and CR4) (42), and a galactose receptor, identical to the asialoglycoprotein receptor expressed by PCs (43, 44).

Of note, an important PRR, the MR, which is expressed on many extrahepatic macrophages, has been reported to be absent in human KCs (45) and to be expressed to a much lower extent in mouse and rat KCs than in LSECs (46, 47).

The KCs can also act as antigen presenting cells (APCs) and induce T-cell mediated responses, and like other macrophages they produce and release a wide range of molecules involved in host defence reactions (4); e.g. activation via TLRs leads to production and release of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and interferon-γ (36, 48).

Stellate cells (SCs; also named Ito cells, or vitamin-A storing cells (49)) are specialized pericytes that are located in the space of Disse and extend their

processes to wrap around the endothelial wall. These SC protrusions react to chemotactic signals to generate a contractile force (50) that may regulate the diameter of the sinusoid lumen. This cell type represents approximately 1.4% of the liver cell volume (measured in Sprague-Dawley rats) (17). They are the main site of vitamin-A storage in the body (2, 23), and are thought to represent the main source of extracellular matrix components in the sinusoidal wall (e.g. collagen type I, III, IV, V and VI, hyaluronan, heparan sulphate, and fibronectin), as well as extracellular matrix degrading enzymes (metalloproteinases) (23, 49). The SCs also produce several growth factors and cytokines for instance transforming growth factors (TGFs), IL-6, IL-10 and platelet derived growth factor (49), and the cells have been intensely studied in connection with the development of liver fibrosis (51).

The liver also contain several resident lymphocyte populations, which include *NK cells* (also called pit cells), *NK T cells, naïve T-cells and B-cells* (reviewed in (52)). These cells are located in the lumen of the sinusoid and extravasate to reach the parenchyma in inflammation (53, 54). Recently it was reported that LSECs present chemokines (CXCL12 and CXCL9) to $CD4^+$ T-cells that enhance their transmigration (53). NK cells are the best studied of the resident liver lymphocytes, and constitute approximately 10% (in mice) to 30-50% (in rat, human) of the lymphocytes in healthy liver (5). They are cytotoxic granular lymphocytes that destroy tumor cells, viruses, intracellular bacteria, and parasites (55-57). Dendritic cells are also reported in liver, mostly located in the vicinity of central veins and portal tracts and not in direct contact with the sinusoids (58, 59).

The different liver cells need to interact in order to function correctly, and communication occurs both through juxtacrine and paracrine signalling (60, 61). For example it has been shown that LSECs require vascular endothelial growth factor (VEGF) produced by PCs (and/or SCs) to maintain their fenestrae (61). The PCs may also make direct contacts with cells at the luminal side of the endothelium, e.g. lymphocytes and KCs, through the LSEC fenestrae (2, 62).

LSEC localization, morphology and functions

Localization and morphology

Forty years ago, Eddie Wisse's electron microscopy studies of perfusion fixed liver specimens from rat showed for the first time that a distinct type of endothelial cells lined the liver sinusoids. The cells had numerous open pores (fenestrae), and as opposed to most other types of endothelia, the cells did not rest on a basal lamina. The cells further contained many bristle-coated (now named clathrin-coated) micropinocytic vesicles and fewer mitochondria than most other cell types (10, 63) (Fig.3).

Since these pioneering observations, fenestrated LSECs without a continuous basal lamina have been identified in several mammalian species (64-68), as well as in chicken (68) and bony fish (69). In general, the sinusoidal endothelium is very thin (150-175 nm in young humans (70)) and the fenestrae (approximately 100-200 nm in diameter) are arranged in groups named sieve plates. The fenestrae diameter and number per cell area vary between species, strains and age-groups (reviewed in (71)), as well as with the location within the liver lobule (66). Thus, the diameter of fenestrae in centrilobular regions are wider than in periportal regions (e.g. 174.6 ±1.0 vs. 147.2 ± 0.9 nm, respectively, reported in rat, (66)).

Although the LSECs represent only a small fraction of the total volume of liver cells (2.8% of the liver cells or 45% of the NPCs in Sprague-Dawley rats (17)) they have been reported to contribute to around 45% of the total mass of pinocytic vesicles in liver, and contain around 17% of the lysosomal volume of young adult rat liver (17). The cells contain numerous clathrin-coated pits and vesicles, a well-developed endocytic machinery (9, 72), and the specific activities of several lysosomal enzymes are higher in LSECs than in PCs and KC (73), suggesting that these endothelial cells are geared to effective degradation of endocytosed material.

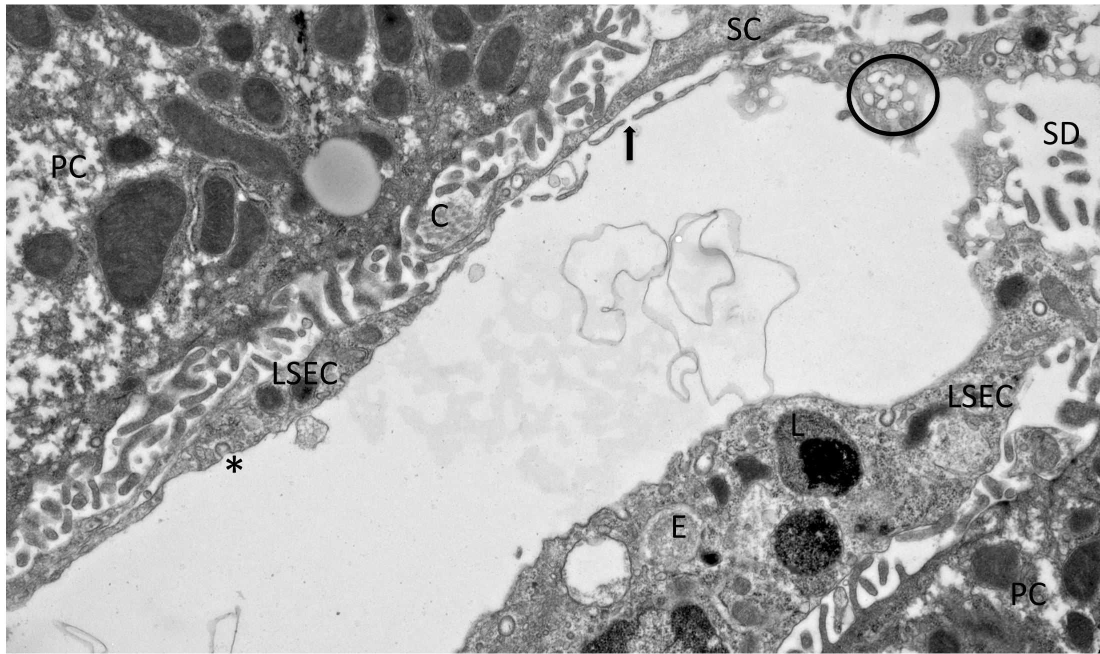

Fig. 3: Transmission electron microscopy (TEM) image of rat liver sinusoid

TEM image of a rat liver sinusoid showing parts of two parenchymal cells (PC), two sinusoidal endothelial cells (LSEC), and a stellate cell (SC). Some of the characteristical features of LSECs are labelled; fenestrae organized in sieve plates (inside the circle, which encloses a sieve plate in a tangentially cut part of an LSEC), thin endothelium with fenestrae (arrow points to a fenestra in a transverse section of an LSEC), coated pits (*), lysosomes (L), and endosomes (E).

Sieve function

The concept of the liver sieve was created at the same time as the LSECs were discovered (10, 74, 75). The presence of real holes in the endothelial cells, without a basal lamina forming a diaphragm underneath the cells, should allow a "free" traffic of substance between the blood sinusoids and the subendothelial space of Disse and the PCs.

It is now well recognized that the fenestrations permit the passage of a wide range of solutes and substrates, such as albumin and other plasma proteins, chylomicron remnants and lipoproteins, into the space of Disse (76), but excluding bigger particles like chylomicrons and blood cells. In addition, blood cells are thought to massage the plasma fluid through the fenestrations by virtue of the fact that their diameter is greater than that of a typical liver sinusoid (liver sinusoid: 5-7 μm; red blood cell: 7.3 μm) (16, 66); either by "forced sieving" (believed to be caused by the red blood cells passing through the sinusoids) and/or by "endothelial massage" (due to the fact that white

blood cells are bigger than the sinusoidal diameter and less plastic than the erythrocytes) (66).

The ultrafiltration of fluid through the fenestrae is thought to be especially important for the hepatic metabolism of lipoproteins (77, 78). Le Couteur et al have suggested that there is a link between the defenestration commonly associated with aging and impaired clearance of cholesterol rich chylomicron remnants in elderly people, increasing the risk for development of atherosclerosis (78).

Fenestrae are dynamic structures, whose diameter and number vary in response to a variety of hormones (e.g. acetylcholine, adrenaline, noradrenaline, serotonin), drugs (e.g. cocaine, nicotine, ethanol), and toxins (e.g. LPS), or even to changes in the underlying extracellular matrix (reviewed in (67)). During disease conditions fenestrae are often lost; e.g. in rat endotoxin shock resulted in a 40% reduction in LSEC porosity (both size and number of fenestrae were affected) (79); mice infected with mouse hepatitis virus type 3 showed a decrease in the number of fenestrae (80), and development of liver fibrosis leads to a progressive loss of fenestrae accompanied by development of a basal lamina (81). LSEC defenestration is also observed in experimental melanoma and lung cancer liver metastasis mouse models (82).

Studies of fenestrae dynamics have been hampered by the fact that their diameter is smaller than the resolution limit for light microscopy, excluding imaging of these structures in live cells. However, recent advances in light microscopy techniques such as three-dimensional structured illumination microscopy (83) to enhance image resolution appear to be very promising tools to reveal new structural and functional information about fenestrations and sieve plates.

Scavenger function

Over the last 30 years increasing knowledge has accumulated about the role of LSECs as scavenger cells, a task they share with cells of the mononuclear phagocyte system, such as the liver KCs (7).

This central function of the LSECs was discovered by the beginning of the 1980s. In 1981 Fraser et al. reported that radio-labeled hyaluronan injected intravenously into rabbit disappeared from blood at great speed and was taken up almost exclusively by the non-parenchymal fraction of the liver cells (84). Two years later Eriksson et al. reported that the LSECs were responsible for this uptake (85). Today a wide range of macromolecules (Table I), including various connective tissue molecules, modified plasma proteins and lipoproteins, and microbial constituents like unmethylated CpG are known to be cleared from the circulation mainly by the LSECs (7, 8, 11, 86-89).

To perform their scavenger function, LSECs carry a set of endocytosis receptors enabling the cells to clear all major categories of biological macromolecules that are not supposed to circulate. These receptors include SRs (38, 90-92), in particular stabilin-1 and stabilin-2 (92-94), the MR (95) and the FcγRIIb2 (96, 97). In addition, LSECs express other receptors that may be involved in endocytosis (reviwed in (98)). However the importance of these other receptors for the scavenger function of LSECs is yet to be shown.

Interestingly, blood clearance and organ distribution studies of SR and MR ligands in species representing different vertebrate classes have shown that the LSECs of amphibia, reptiles, and birds exert similar scavenger function as LSECs in mammals. In phylogenetically older vertebrates, distinct populations of specialized endothelial cells with a corresponding scavenger function as the mammalian LSECs are carried in either heart (endocardium) (99-102) or in kidney (venous sinusoids) (103-105) of bony fishes or in specialized gill vessels of cartilagenous fishes and jawless chordates (7). Due to the fact that these endothelial cells are located in different organs, depending on the vertebrate class, a common name cannot be assigned to the cells based on organ location. Therefore the term “scavenger endothelial cell” has been introduced reflecting the very active scavenging activity performed by these cells. Of note, the clearance function of the scavenger endothelial cells and macrophages is complementary: scavenger endothelial cells (including LSECs) normally perform clathrin-mediated endocytosis of colloids and soluble substances, and are usually not phagocytic, whereas macrophages

are professional phagocytes and responsible for clearance of particulate material (7, 106).

Table 1: Ligands that are endocytosed by the mammalian LSEC

Endogenous ligands	**Receptor**	**Reference**
Hyaluronan	Stabilin-2[a]	(92, 107, 108)
Chondroitin sulphate	Stabilin-2[a]	(109, 110)
Nidogen	SR	(111)
Heparin	n.d[b]	(112)
Serglycin	SR	(113)
N-terminal propeptides of types I and III procollagen	SR, stabilin-2	(92, 114, 115)
Collagen alpha chains (types I, II, III, IV, V, XI)	MR[c]	(86, 116, 117)
C-terminal propeptide of type I procollagen	MR	(117)
Tissue plasminogen activator	MR	(118) (115)
Lysosomal enzymes	MR	(87, 119, 120)[e]
Salivary amylase	MR	(121)
Soluble immune complexes	FcγRIIb2	(122)
Modified host molecules	**Receptor**	**Reference**
FSA	SR, stabilin-1, stabilin-2	(11, 123)
AGE-albumin	SR, stabilin-2 (stabilin-1[d])	(124, 125)
Oxidized LDL	Stabilin-1, stabilin-2	(126)
Agalacto-orosomucoid	MR	(127)
Ahexosamino-orosomucoid	MR	(127)
Exogenous ligands	**Receptor**	**Reference**
LPS	TLR4	(128)
CpG oligodeoxynucleotides	SR	(89)[f]
Invertase	MR	(129)
Mannan	MR	(130)
Ovalbumin	MR	(95)
Ricin	MR	(46)

SR, Scavenger receptor; n.d, not determined; MR, Mannose receptor; FSA, formaldehyde-treated albumin; AGE, Advanced-glycation end product; Ox-LDL, Oxidized low-density lipoprotein; LPS, lipopolysaccharide.

[a]Until 1999 it was thought that the LSEC carried a distinct hyaluronan receptor mediating endocytosis of hyaluronan and chondroitin sulphate. However, the purification and characterisation of this receptor (72, 92), revealed that it also mediated uptake of typical SR ligands and in 2002 the receptor got the official name stabilin-2 (94).

[b]Oie et al. (112) reported that the uptake of heparin was via an unidentified receptor, distinct from stabilin-2.

.[c]Uptake of collagen α-chains was previously thought to occur via a specific collagen receptor. This receptor was purified in 2007 (86) and found to be identical to the MR, which has distinct binding sites for mannose and collagen α-chains.

[d]AGE-albumin affinity to stabilin-1 is tested in transfected cell lines only (131, 132).

[e]Reference 87: Paper II in this thesis. [f]Reference 89: Paper I in this thesis.

Endocytosis mechanisms in the LSEC

Endocytosis is defined as the uptake of material into a cell by an invagination of the plasma membrane and its internalization in a membrane-bounded vesicle, and can be divided into pinocytosis (uptake of soluble material) and phagocytosis (uptake of particles) (133). Pinocytosis can be further divided into several categories of uptake modalities, the most common being clathrin-mediated endocytosis and caveolin-mediated endocytosis. Other types are macropinocytosis (bigger area of the cell membrane that ruffles, gets invaginated and closes again) or bulk-fluid endocytosis (not receptor-mediated, involving small invaginations of cell membrane (134)). In the present thesis the term receptor-mediated endocytosis refers to clathrin-mediated endocytosis.

Many studies have shown that the LSEC is a cell type specialized for effective endocytosis; the are richly equipped with lysosomes and pinocytic vesicles (10, 17), and show high expression of proteins involved in clathrin-mediated endocytosis such as clathrin, α-adaptin, β-adaptin, Rab4, Rab5, Rab7 and rabaptin5 (9), as well as high specific activities of lysosomal enzymes (73, 135). A recent study showed a unique net-like distribution of clathrin heavy chains, and tubulin, the building blocks of microtubules, in LSEC; this network partially colocalized with endosomal markers (136). The intracellular transport of endocytosed material was altered by nocodazole (disrupts microtubules), but did not influence ligand internalization or the recycling endocytosis

pathway, suggesting that intact and functional microtubule networks are not required for internalization and recycling (136).

The LSEC uptake of soluble macromolecules depends mainly on clathrin-mediated endocytosis (137). The internalization of ligand is rapid: the half-life for the surface pool of ligand-MR complexes has been measured to be only 10-15 sec in freshly isolated rat LSEC cultures (95). Receptor-ligand complexes are brought to early endosomes via coated vesicles and primary endosomes (138). The ligands usually dissociate from the MR and SR receptors in the early endosomes, and the receptors recycle back to the plasma membrane (93, 95, 139, 140). Ligand-receptor complexes may also be recycled back to the cell surface, however the role of this recycling is not clear (95, 139). Interestingly, unlike the fate of ligands taken up via the MR and SRs, which are mostly uncoupled from their receptors in the early endosomes, immune-complexes internalised via the FcγRIIb2 in LSECs are to a large extent returned to the cell surface (96, 141). This leads to a slow net internalization of cell-surface bound ligand with a half time of internalization measured to be about 15 min as compared to < 1 min by the MRs and SRs (95, 141, 142).

The traffic from early to late endosomes in LSECs is also a rapid process. By using antibodies to early endosome antigen 1 (EEA1; an early-endosomal protein involved in endocytic membrane fusion (143)) Hellevik et al. showed by immune electron microscopy that the endocytosed ligand (fluorescein isothiocyanate (FITC) labelled denatured collagen) left the early endosomes 20 min after internalization in rat LSECs in vitro (144). The traffic of denatured FITC-collagen from late endosomes to lysosomes was markedly slower and only 53% of the ligand was measured in the lysosomes after 16 hours (144).

This process goes markedly faster in vivo, and e.g. the presence of ^{125}I-labelled degradation products of ^{125}I-labelled FSA (an SR ligand), was measured in blood 10 to 12 min after intravenous injection in mice (87). Intravenous injection of ^{125}I-tyramine-cellobiose labelled ovalbumin (an MR ligand) in rats followed by subcellular fractionation of whole livers and immune electron microscopy of liver tissue showed ligand uptake in early endosomes

after 6 min and in lysosomes after 24 min (145, 146), whereas traffic of FSA to lysosomal compartments (analysed by subcellular fractionation of livers) was even faster, and took from 9 to 12 min (142).

In LSECs, protein degradation starts already in late endosomes (144, 147-149) and continues in lysosomes. Lysosomes were originally discovered in 1955 by De Duve and his collaborators as organelles enriched in acidic hydrolases and potentially harmful to the cell (150). In addition to their crucial role in the endocytic pathway, lysosomes also are the terminal destination for cellular material subjected to autophagy and for secretory material targeted for destruction (reviewed in (151-154).

Endo-lysosomal degradation is carried out by a number of acid hydrolases capable of digesting most endocytosed macromolecules. As mentioned before within the liver, the LSECs are the cell population with the highest specific activity of several lysosomal enzymes (73, 87, 135).

Implications of endocytosis

Besides waste clearance and uptake of nutrients, endocytosis is needed for several cellular processes, such as post-translational maturation of peptide hormones and antigen presentation (148) and signal transduction (155).

In LSECs two important physiological functions may converge in the endocytic pathway; clearance of endogenous waste and host defence. The SRs and MRs, the two major types of endocytic clearance receptors of these cells (7, 8, 86, 87) are also regarded as PRRs in the innate immune system (reviewed in (156, 157)).

Interestingly, some of the metabolites that are produced by lysosomal degradation (i.e. glutamate and lactate) are reutilized. Glutamine is an important metabolic fuel for dividing cells, is hydrolized into ammonia and glutamate and is also the major nontoxic shuttle of ammonia in the urea cycle (158). Recently, studies in liver cell bioreactors showed that LSECs are important collaborators in the liver ammonia metabolism, producing more ammonia than the PCs (LSEC bioreactors produced 22.2 nM/hour/10^6 cells into the growth media while PC bioreactors produced 3.3 nM/hour/10^6 cells).

LSECs also released more glutamate than PCs (LSEC bioreactors: 32.0 nM/hour/10^6 cells; PC-bioreactors: <7.0 nM/hour/10^6 cells). Furthermore, the metabolites released by the LSECs were used by the PCs; and it was suggested that one function of the high catabolic activity of the LSEC is to secrete high-energy metabolites to be used for mitochondrial ATP production by the PCs (159). A similar type of collaboration between highly endocytically active endothelial cells and metabolically active parenchymal cells has been described in the Atlantic cod (*Gadus morhua*) (160). The cod endocardial endothelial cells are specialized scavenger endothelial cells, analogous to the mammalian LSECs (7, 101, 102), secreting high-energy metabolites (acetate) that may be taken up by the underlying cardiomyocytes and used as an ATP-source (160).

However, degradation does not necessarily need to be the ultimate fate of the endocytosed products. In paper II, we hypothesized that the LSEC recruits lysosomal enzymes via MR-mediated endocytosis to maintain its high specific lysosomal enzyme activity and degradation capacity.

LSEC endocytosis receptors

Scavenger receptors

In 1979 Brown, Goldstein and co-workers discovered a receptor, known today as the scavenger receptor type A (SR-A) (161) when they observed that acetylated LDL was taken up much more efficiently than native LDL by mouse peritoneal macrophages. Today the term SR denotes a structurally heterogeneous family of receptors that share the common property of recognizing a broad range of polyanionic molecules, including different types of modified LDLs (reviewed in (90, 162, 163)). Many different cell types express SRs, including monocytes and macrophages, smooth muscle cells and endothelial cells (90, 163-165). Macrophage uptake of oxidized LDL via SRs is thought to play a key role in the formation of foam cells in the arterial wall during atherogenesis (162, 166).

Due to the broad range of ligands recognized by these receptors, which includes surface constituents of Gram-positive and Gram-negative bacteria (167-169), and bacterial DNA oligonucleotides and plasmids (170), the SRs are considered part of the innate immune system where they function as PRRs (28, 164).

The LSEC has been reported to express SRs belonging to class A (SR-A (38, 91)), class B (SR-B1 and CD36 (91)) and class H (stabilin-1 and stabilin-2 (92, 94)).

<u>SR class A:</u> SR-A was the first SR discovered in the LSEC (38, 171) and is a type II transmembrane glycoprotein expressed on the plasma membrane which contains a scavenger receptor cysteine-rich (SRCR) domain and a collagenous domain in the extracellular part of the molecule (172, 173), and comes in two splice variants; SR-AI/II. The receptor is primarily expressed in macrophages but is also found in activated smooth muscle cells and endothelial cells (38, 174). It has been shown that SR-AI/II–deficient mice, compared to wild-type controls, are more susceptible to experimental infections with *Staphylococcus aureus*, *Listeria monocytogenes* and herpes simplex virus type-1 (175, 176). Disruption of the SR-AI/II gene in apolipoprotein E knockout ($ApoE^{-/-}$) mice, which have high plasma values of cholesterol and develop severe atherosclerosis, lead to reduced formation of atheromatous plaques, indicating the involvement of this receptor in the development of atherosclerosis (176).

The rate of blood clearance of intravenously administered acetylated or oxidized LDLs was the same in SR-A deficient and wild-type mice (176-178). Furthermore, there was no difference in the endocytosis (rate and capacity) of soluble SR-ligands (i.e. acetylated LDL, advanced glycation end product (AGE)-albumin, FSA) in LSEC cultures established from SR-AI/II knockout and wild-type mice (179, 180). These observations strongly suggest that receptors other than SR-A are involved in LSEC SR-mediated endocytosis.

<u>SR class B:</u> CD36 is a transmembrane glycoprotein expressed on monocytes, macrophages, dendritic cells, adipocytes, smooth muscle cells, capillary endothelial cells, and platelets (reviewed in (181)). It is suggested to

be involved in the development of atherosclerosis, platelet activation and thrombus formation (156, 182). SR-BI/II (two different splice variants) is expressed on monocytes, macrophages, adipocytes, liver cells, and in steroid-producing tissues (183). In the liver, SR-BI is highly expressed in the PCs where it mediates selective HDL uptake by a mechanism distinct from the classical LDL receptor-mediated pathway (183). An important role for SR-BI in the entrance of hepatitis C virus (HCV) has been suggested, but is debated (163). Studies in male Wistar rats also showed expression of this receptor in LSECs and KCs but the relative expression of SR-BI at mRNA and protein levels was much lower in the NPCs than in the PCs (91). CD36 expression was also reported in LSECs by the same authors (91) but no quantitative comparison was done between the different liver cell types on protein level. CD36 expression is also reported in human LSECs by immune electron microscopy and immunohistochemistry at light microscopy level (184, 185). However, recently Li et al. reported no protein expression of CD36 in male Sprague Dawley rat LSECs, and the authors suggested that there may be species or strain differences in the expression of this protein (126).

The most important SR on the LSECs has been suggested to be stabilin-2 (92, 124), probably together with stabilin-1 (11, 93).

SR class H: This class comprises **stabilin-1** and the homologous protein **stabilin-2** (94). Due to their isolation by different research groups at about the same time the nomenclature has been confusing: stabilin-1 is also known as FEEL-1 (fasciclin, epidermal growth factor (EGF)-like, laminin-type EGF-like, and link domain-containing scavenger receptor-1) (132) and Clever-1 (common lymphatic endothelial and vascular endothelial receptor-1) (186), whereas stabilin-2 synonyms are FEEL-2 (132), HARE (hyaluronan receptor for endocytosis) (108) and the LSEC hyaluronan/SR receptor (92).

Both stabilins are large type I transmembrane proteins with a similar overall structure. Stabilin-1 has an N-terminal extracellular region containing 7 fasciclin domains, 16 EGF-like domains, 2 laminin-type EGF-like domains and 1 X-link domain, a transmembrane region and a short cytoplasmic domain. The main structural difference between stabilin-1 and -2 is that stabilin-2

contains 15 instead of 16 EGF-like domains. Studies in mouse, rat, pig and human have shown that the two stabilins are expressed in sinusoidal endothelia of spleen, liver, lymph nodes, and bone marrow (45, 92, 166, 187, 188). Stabilin-1 and -2 were also recently reported in bovine choriocapillaris endothelial cells (189). In addition, stabilin-1 is found in alternatively activated macrophages (94, 190), and in newly formed blood vessels (191), indicating a role of this receptor in angiogenesis.

During embryonic development of mouse liver, stabilin-2 is expressed in all liver vascular endothelia early on and then becomes restricted to the liver sinusoids at embryonic day 19.5 (192). In the mature LSEC stabilin-2 is highly expressed on the cell surface and is also associated with pinocytic vesicles, whereas stabilin-1 appears to have a predominantly intracellular distribution but is also seen at the cell surface (11, 93). Both proteins are associated with clathrin, adaptor protein-2 and early endosomes, and cycle between the cell surface and endosomes (93, 140).

Stabilin ligands: Hyaluronan is taken up via stabilin-2 only, by binding to the X-link domain, whereas stabilin-1 has a non-functional link domain. Other extracellular matrix components taken up by stabilin-2 include N-terminal propeptides of types I and III procollagen and chondroitin sulphate (8, 92, 193).

Both stabilins mediate the LSEC endocytosis of AGE-albumin (93), FSA and oxidized LDL (11) in LSECs. Studies in macrophages have shown that stabilin-1 also binds the glycoprotein SPARC (secreted protein acidic and rich in cysteine), a soluble non-structural component of extracellular matrix that plays a role in tissue remodeling, angiogenesis and wound healing (194), and SI-CLP (stabilin-interacting chitinase like protein) which is a chitinase-like cytokine sorted into late endosomes by stabilin-1 in macrophages (195). Stabilin-1 is also suggested to play a role in adhesion of lymphocytes and malignant cells to lymphatic endothelium and to support the migration of peripheral blood mononuclear cells and leukocytes through vascular and lymphatic endothelium (186, 196). The stabilins have further been reported to

mediate phagocytosis of apoptotic cell bodies in alternatively activated macrophages and stabilin transfected cell lines (197, 198).

Endocytosis studies using primary cultures of LSECs have clearly shown that the stabilins mediate rapid internalization of various waste macromolecules that are too large to be filtered through the kidney glomeruli. If not rapidly eliminated from blood, it has been suggested that these molecules may accumulate in various tissues increasing the risk for vascular complications (199-201). Recently a large and thorough study was published about the effects of stabilin deficiency in mice (20). Single deletions in either Stab1 or Stab2 showed very few phenotypic effects including a slight increase in the collagen content in liver, mostly in the stabilin-1 knockout mice, and increased serum levels of hyaluronan in the stabilin-2 deficient animals. However, stabilin-1/-2 double knockout mice showed a significantly reduced life span compared to single knockouts and wild-type controls and developed mild liver fibrosis, as well as severe fibrosis in the kidney glomeruli. The authors suggested that proper hepatic clearance of potentially noxious agents from blood via stabilin-1 and stabilin-2 is necessary to maintain tissue homeostasis not only in the liver but also in distant organs. The same paper also introduced a new putative ligand for both receptors, namely growth differentiation factor 15 (GDF-15), which is a member of the TGF-β family, and the first cytokine found to be cleared by the LSECs.

The mannose receptor

The MR was first recognized in the late 1970s as a receptor involved in the clearance of endogenous glycoproteins with mannose in the terminal position of their carbohydrate side chains (202). The receptor displays three different ligand binding regions: i) an outer cysteine-rich amino-terminal domain, which recognizes specific sulphated sugars (203); ii) a fibronectin type II repeat which binds collagens (204, 205), and iii) a series of eight adjoining carbohydrate recognition domains (also named C-type lectin-like domains), that bind glycoproteins and glycolipids exposing D-mannose, L-fucose and/or *N*-acetyl-D-glucosamine in terminal position of their sugar side chains (206, 207).

The MR is expressed in most tissue macrophages, immature dendritic cells, mesangial cells in the kidney, tracheal smooth muscle cells and retinal pigment epithelium (reviewed in (208, 209)). In the liver this receptor is expressed predominantly – if not only – in the LSECs (45-47).

The carbohydrates recognized by the MR are abundant on the surface of many bacteria, fungi, and some viruses, including the gp120 of human immunodeficiency virus (HIV) (210), lipoarabinomannan from *Mycobacterium tuberculosis* (211), capsular polysaccharides from *Streptococcus pneumonia* (212), and β-glucans in the cell wall of the fungal pathogens, *Pneumocystis carinii* (213) and *Candida albicans* (214). Several studies have therefore suggested a role for the MR in host defence ((204, 209, 213, 215). However, two studies in MR knockout ($MR^{-/-}$) mice failed to show enhanced susceptibility to *P. carinii* and *C. albicans* infections (209, 215, 216); whereas an infection study with *Cryptococcus neoformans* showed a decreased life span and inability of the $MR^{-/-}$ mice to elicit a $CD4^{+}$ T-cell response, suggesting that the receptor plays a nonredundant role in priming mannoprotein mediated $CD4^{+}$ T-cell responses in vivo (217). Interestingly, shedding of soluble MRs by metalloproteinase-mediated cleavage of membrane bound MRs has been reported as a response to *P.carinii* and *C.albicans* infections (218).

Whether MR is involved in antigen processing and presentation through the major histocompatibility complexes types I or II (MHC I and MHC II) is currently under discussion. In particular, there are contradictory reports as to whether the receptor-antigen complex travels through the endocytic pathway and is presented to the MHC proteins or if the MR just mediates the uptake of antigens to be presented but is not directly involved with the MHC proteins (208, 209).

The MR, however, plays an important role in homeostasis of several glycoproteins (219) that are released to the body fluids during normal and pathophysiological tissue turnover. Ligands for the LSEC MR include tissue plasminogen activator (118, 220), neutrophil granulocyte-derived myeloperoxidase (221), salivary amylase (121), denatured collagen (86), and

C-terminal procollagen propeptide of type I and type III procollagen (PICP and PIIICP)(117) and lysosomal enzymes (87, 219). When injected into the circulation of laboratory animals, these soluble MR ligands are taken up almost exclusively in the LSECs.

Recently it was shown that the clearance of denatured collagen occurs via the MR and not by a unique collagen-α-chain receptor as thought previously (86). Unlike mannosylated glycoproteins, denatured collagen binds to the fibronectin-like domain of the MR (205), and there is no cross-competitive inhibition of ligands for the different domains on the MR. This probably explains why it was believed for more than 20 years that LSECs carry a distinct collagen α-chain receptor in addition to the MR and SRs (8, 116, 222).

The Fc gamma receptor IIb2

Fc gamma receptors (FcγRs) recognize the Fc domain of immunoglobulin G (IgG) present on immune complexes. Four major classes have been identified (FcγRI-IV), each of them with several isoforms and widely expressed in cells of hematopoietic origin (reviewed in (223, 224)). LSECs express one of these receptors, the FcγRIIb2 (96). The receptor plays an important role in removing soluble IgG-immune complexes (97, 225-232). This receptor is also expressed on murine follicular dendritic cells (233), and in endothelial cells in human placental villi (234). It is worth noting that the LSEC is the only cell type in liver expressing FcγRIIb2 (96), making this receptor an ideal marker to distinguish LSECs from all other types of liver cells.

Other endocytosis receptors in LSECs

L-Sign (liver/lymph node-specific ICAM-3 grabbing non-integrin) also known as DC-SIGNR, CD209L or CLEC4M, is involved in recognition and uptake of virus, including HIV (235), HCV (236), and severe acute respiratory syndrome coronavirus (SARS-CoV) (237). The receptor is expressed in endothelial cells of the liver sinusoids, lymph nodes, placenta and lung (235, 238).

LSECtin (liver and lymph node sinusoidal endothelial cell C-type lectin) is another protein from the same family as L-Sign. This receptor is expressed predominantly by sinusoidal endothelial cells of human liver and lymph (239)

but also on peripheral blood and thymic dendritic cells (240) and KCs (241, 242). LSECtin has been suggested as an important receptor in the regulation of immune responses in liver where it has been shown to interact with L-Sign in response to HCV (243). LSECtin may also inactivate T-cell responses in this organ (241). The role of L-Sign and LSECtin in LSEC endocytosis is largely unknown.

LRP-1 (Low-density lipoprotein receptor-related protein-1) is mainly expressed in PCs and macrophages but has also been reported in neurons, activated astrocytes, and fibroblasts (244). It recognizes a wide range of ligands, including lipoprotein particles containing ApoE (245, 246), urokinase-type plasminogen activators, amyloid precursor protein (247)), and the tissue factor pathway inhibitor (247-249). Recently, Øie et al reported the finding of a functional LRP-1 in LSECs (250), however only around 10% of the LRP-1 activity in the liver was due to the LSECs.

LYVE-1 (lymphatic vessel endothelial hyaluronan receptor-1) is a hyaluronan binding protein. The receptor is expressed in lymphatic vascular endothelium and sinusoidal endothelia of lymph nodes, liver and spleen (251-253), and is also reported in macrophages in malignant tumors (254), during the development of mouse kidneys (255), in human placenta (256) an in embryonic blood vessels (257). Initially, the role assigned to this receptor was the clearance of hyaluronan from lymph (253), but the receptor is also suggested to play an important role in development, wound healing and tumorigenesis (254, 255, 257-259). In liver the expression is restricted to LSECs (252, 260) and the LSEC expression has been found to be reduced in chronic inflammation, cancer and cirrhosis (252) (261). Of note, stabilin-2 is the major endocytic hyaluronan receptor in LSEC (20), and the relative contribution (if any) of LYVE-1 is unknown.

Role of the LSEC in host defense

The immune system can be grossly divided in two parts: i) the innate immune system, and ii) the adaptive immune system.

Innate immunity is an evolutionarily ancient part of the host defense mechanisms, and is present in both invertebrates and vertebrates. It includes a variety of mechanisms; mechanical and chemical barriers (e.g. epithelial barriers, mucus, antimicrobial peptides and acids), the complement system, and cellular responses such as phagocytosis and production of oxygen radicals (5). Several cell types are involved in innate immune responses; the most studied are macrophages, neutrophil granulocytes, dendritic cells, and NK cells.

Adaptive immunity on the other hand has evolved quite recently in the evolution and is present only in vertebrates (28). Adaptive immunity is mediated by lymphocytes (T-cells and B-cells), and involves great variability and rearrangement of gene segments in response to antigens. The adaptive immune system can provide immunological memory of infection. In vertebrates, there is a great deal of cooperation between the innate and adaptive immune system, and cells of the innate immune system (e.g. macrophages and dendritic cells) are also involved in adaptive immune responses as APCs and as effector cells (28).

The liver receives blood from the systemic circulation and the intestine, and the liver cells, primarily those lining the sinusoids are therefore exposed to many microbial antigens/products (mostly derived from intestinal microorganisms) (262). The liver has created a special immunological environment that allows it to not react to the great amounts of harmless microorganism from the gut, raising the hypothesis of liver tolerance. At the same time the liver responds to certain pathogens when it is required (263).

The LSEC was until recently not regarded as a cell type involved in immunity, however, research over the last 2 decades has revealed that this cell type may play important roles both in innate and adaptive immunity, which will be discussed in the following chapters.

In 1994, Matzinger proposed an alternative model to the classical idea of “self non-self” recognition; this model was called the “Danger Model” (264) proposing that APCs are activated by danger/alarm signals from cells injured

by exposure to pathogens, toxins and even to mechanical damage (264, 265). Cells that die via necrosis release their content to the exterior, and any intracellular product from these cells could potentially be a danger signal when released (reviewed in (265, 266)). The fact that LSECs are effective scavengers of potentially dangerous endogenous waste, as well as foreign material suggests a role of LSECs in innate immunity.

Expression of pattern recognition receptors (PRRs) in LSECs

SRs and MRs recognize self waste molecules (some of these molecules are able to elicit an immune response and are therefore called alarmins or DAMPs (30, 31)) as well as a number of common structures carried by microbes (PAMPs). These receptors are therefore considered to be PRRs, known to represent a central part of the innate arm of the immune system (267). Studies on human alveolar macrophages have demonstrated that MR-mediated signalling leads to IL-8 production when the MR is activated together with TLR2 (268). The role of SR-A and SR-B as PRRs has been frequently dealt with in the literature, but so far very little is known about the role of stabilins as PRRs (163).

TLRs are another important group of PRRs. The TLRs activate the innate immune system in response to molecules expressed by pathogens (viruses, bacteria, fungi and protozoa) (reviewed in (269, 270)) and to host molecules such as heat-shock proteins (271) and DNA (272). TLRs are expressed on various immune cells, including macrophages and monocytes (273, 274), dendritic cells (275), B cells (275) and subsets of T cells (276), and they are also reported in cells that have not been described as “classical” immune cells such as pulmonary epithelial cells (277), fibroblasts (278, 279) and endothelial cells in skin (280), liver (36, 89, 128), and human umbilical vein

The TLRs are considered as link players between the innate and adaptive immune systems because upon activation they promote the selection of bacterial antigens for optimal presentation on MHC class II and/or production of co-stimulatory molecules and cytokines necessary for activation and differentiation of T-cells (28). So far, human and mouse share several functional TLRs1-9. Mice also have TLRs11-13, whereas TLR10 is selectively

expressed in humans, however, the biological agonists of this receptor have not been found (281). Recently a paper was published where knocking down TLR13 in mouse embryonic fibroblasts by RNA silencing increased the susceptibility for infection with vesicular stomatitis virus (282).

Table 2 presents biological agonists (immunostimulatory ligands) recognized by TLRs.

Table 2: Biological agonists for TLRs

TLR	Agonist	Reference
TLR1/TLR2	Triacyl lipopeptides (Bacteria and mycobacteria)	(283)
TLR2	Peptidoglycans (Gram+ bacteria)	(284)
	Porins (*Neisseria* sp.)	(285)
	Lipoarabinomannan (*Mycobacteria* sp.)	(286)
	Phospholipomannan (*Candida albicans*)	(287)
	Glucuronoxylomannan (*Cryptococcus neoformans*)	(288)
	T-GPI-mucin (*Trypanosoma* sp.)	(289)
TLR3	dsRNA (virus)	(290)
TLR4	LPS (Gram- bacteria)	(291)
	Mannan (*Candida albicans*)	(292)
	Glucuronoxylomannan (*Cryptococcus neoformans*)	(288)
	Glycoinositolphospholipids (*Trypanosoma* sp.)	(293)
	Envelope proteins (HERV, RSV, MMTV)	(294) (295, 296)
	Heat-shock protein 70, hyaluronan fragment and fibrinogen (host molecules)	(271, 297, 298)
TLR5	Flagellin (flagellated bacteria)	(299)
TLR6/TLR2	LTA (Group B *Streptococcus* sp.)	(300)
	Zymosan (*Saccharomyces cerevisiae*)	(301)
TLR7/TLR8	ssRNA (RNA viruses)	(302)
TLR9	Unmethylated CpG-DNA (Bacteria)	(303)
	DNA (HSV-1, MCMV)	(304, 305)
	Host DNA	(272)
TLR11	Profilin-like molecule (*Toxoplasma gondii*)	(306)
TLR13	Unknown (Vesicular stomatitis virus)	(282)

T-GPI-mucin, glycosylphosphatidylinositol-mucin; dsRNA, double stranded RNA; ssRNA, single stranded RNA; LPS, lipopolysacharide; HERV, human endogenous retrovirus; RSV, respiratory syncytial virus; MMTV, Mouse mammary tumor virus; LTA, lipoteichoic acid; HSV-1, herpes simplex virus-1; MCMV, mouse cytomegalovirus.

Before paper I of this thesis was published (89), only TLR4 had been reported in LSECs (128).

Human TLR4 was first sequenced in 1998 together with four other TLRs (307), and has been found to be expressed in most organs, with the highest expression in spleen (308). The receptor is expressed on the cell surface and binds LPS, in particular its lipid portion (lipid A) (309). TLR4 is responsible for most of the pathogenic phenomena associated with Gram-negative bacterial infection such as endotoxin shock (310). The mechanism by which TLR4 is activated is well studied, especially in macrophages (reviewed in (281)). The receptor cycles between the Golgi apparatus and the plasma membrane before the activation by LPS (311). LPS binds to TLR4, forming a complex also with the LPS binding protein (LBP), CD14 and MD2 at the plasma membrane and travels to the endosomes (312). This initiates the binding of myeloid differentiation protein 88 (MyD88) with the MyD88-adapter like or TIR domain-containing molecule (Mal/TIRAP) (313, 314). The binding results in the activation of transcription factor NFκB, which translocates to the nucleus and induce the production of proinflammatory cytokines. There is also other another pathway, where TLR4 moves into the early endosome and causes the binding of TRIF-related adaptor molecule (TRAM) and TIR-domain-containing adapter-inducing interferon-β (TRIF). This results in the activation of the interferon regulatory factor-3 (IRF3) pathway (315) and the production of type I interferons (316), which are important antiviral and antibacterial cytokines.

In Paper I of this thesis we report that TLR9 is also expressed in LSECs (discussed further in the General discussion), and a recent study (36) showed that murine LSECs produced TNF-α and IL-6 when treated with agonists for TLR2, TLR3, TLR4 , TLR6/2, TLR8 and TLR9 .

Role of the LSEC in adaptive immunity

The role of LSECs in adaptive immunity is not clear and not many studies have been performed.

One of the first studies that were performed on isolated LSECs indicated the expression of an Fc receptor in the cells (assessed by the formation of rosettes with red blood cells), however the presence of Ia antigen (today referred to as MHC II) or a CR3 was not detected. However the main focus of this paper was on KCs as antigen presenting cells (317).

Nowadays the discussion is focused on whether LSECs acts as APCs, analogous to dendritic cells. Knolle and co-workers reported that LSECs, like dendritic cells express molecules that are necessary for antigen presentation, including MHC I and II and the co-stimulatory molecules CD40, CD80 and CD86 (318). The same group claimed that LSECs could take up antigen (ovalbumin) via the MR and stimulate $CD4^{+}$ T-cell responses (319). This finding has been contradicted by Katz *et al* (320) who used another cell separation technique to make purified murine LSEC cultures (KCs and DCs were removed from the cultures by specific antibodies), and found that LSECs, in contrast to dendritic cells, had low or absent expression of MHC II, CD86, and CD11c. They found that LSECs could not induce proliferation of $CD4^{+}$ or $CD8^{+}$ T cells, and therefore concluded that LSECs alone are insufficient to activate naive T cells (320). Yet another later paper reported that primary murine LSECs (immunoselected using their expression of CD105, or endoglin) do express MHC class II and CD86, but not CD11c (321).

The conflicts associated with the expression of MHC II in LSECs is further underlined by several earlier studies that failed to demonstrate this molecule on rat or human LSECs (106, 141, 322). Recently a master student in our group found that LSECs from mice did express MHC II as evidenced by immunoblotting and immunofluorescence on freshly isolated cells in serum free medium (323).

The question is therefore still open as to whether LSECs (or subpopulations of LSECs) can act as true APCs. It is clear, however, that the LSECs have some important features in common with dendritic cells, which are linked to innate immunity such as high endocytic activity, expression of MR, SRs, FcγRIIb2 and TLRs.

LSEC function in aging

Several age-related changes have been documented in the liver, including reduced organ volume, accumulation of lipofuscin in PCs, diminished hepatobiliary functions, a shift in the expression of a variety of proteins (71, 324), and impaired metabolic drug clearance (325).

However, it was not until recently that age-related changes in the hepatic sinusoid were reported (326). Earlier studies in rat models have suggested few or no age-related morphological changes in the sinusoid (327). In 2001, Le Couteur et al reported a significant defenestration of the sinusoidal endothelium as well as increased endothelial thickness and partial depositions of basal lamina and collagen in the space of Disse in old F344 rats (326), and similar changes are now reported in C57BL/6 mice (22, 328), baboons (*Papio hamadryas*) (329), and humans (70). The term “pseudocapillarization” (78, 326) was launched to differentiate the typical age-related sinusoidal morphology from the capillarization that occurs in liver fibrosis. Capillarization describes the transdifferentiation of the sinusoidal endothelium towards a continuous vascular endothelium resting on a basal lamina. However, it has been debated whether the pseudocapillarization is really different from the early stages in liver capillarization (60).

Age-related changes in the hepatic sinusoid have been recently reviewed (71). In this respect results from studies on KCs are conflicting. Both an increase in the number of KCs (330) and a decrease in the volume density of these cells (331) have been reported, as well as an increased (22, 330), unchanged (332, 333) or decreased phagocytic and/or endocytic activity (334, 335). Changes in SCs with aging have been more consistently reported in different species. These include an increase in the fat and in vitamin A content (22, 328), indicating that the cells are not activated as seen in fibrosis (49).

The age-related changes in the LSEC ultrastructure are associated with altered but inconsistent expression of several cellular proteins. For example, the endothelial marker von Willebrand factor (vWf) (a glycoprotein involved in

hemostasis, and found in blood plasma, platelet α-granules, endothelial cells, and subendothelial connective tissue (336)) is not normally expressed in LSECs of healthy young liver sinusoids, but is upregulated in LSEC in old individuals (70, 326, 329, 337). Increased vWf expression with aging is also reported in endothelium in other vascular beds in humans (338). LSEC expression of caveolin-1 (a fundamental component of caveolae) was also found to be reduced at old age (337), and the authors suggested a link between this finding and the decrease in fenestrae associated with old age. However, mice deficient in caveolin-1 were found to have normal LSEC fenestration (339), contradicting this notion. Increased ICAM-1 expression (intercellular adhesion molecule-1, a glycoprotein involved in leukocyte adhesion (340)) was found in old C57BL/6 mice (22). In this study, increased leukocyte adhesion to the sinusoidal endothelium in old animals was also observed, indicating low-grade inflammation and endothelial dysfunction in the old liver.

Recently the term "Inflamm-aging" (by Claudio Franceschi) (341) was launched to highlight the phenomenon that aging is accompanied by a low-grade chronic, and systemic up-regulation of the inflammatory response and that the underlying inflammatory changes are common to most age-associated diseases. Increased numbers of immune cell clusters in the liver parenchyma and increased level of inflammatory cytokines gene expression (342) have been reported in the livers of old C57BL/6 mice. The authors suggested that the liver microenvironment of old animals allowed the formation of ectopic accumulations of lymphoid cells called “tertiary lymphoid organs” in chronically inflammed tissue (342).

Little is known about the effect of aging on endocytosis per se, and only a few studies have addressed endocytosis in LSECs with aging (22, 333-335, 343). The in vivo capacity for uptake of ^{125}I-colloidal (heat-aggregated) albumin in rat LSEC was reported not to be influenced by age (333). Others have reported a 53% reduction of the in vivo LSEC uptake of ^{3}H-azoaniline-albumin in 22–24 months old rats compared with 6–8 months old rats (334), and an 80% reduction of in vivo LSEC uptake of ^{35}S-sulfanilate-azo-albumin in 28 months old rats compared with 12 month rats (335). In the latter study, uptake

of ^{35}S-sulfanilate-azo-albumin in LSEC after intravenous injections was also examined in 4 month old animals. Interestingly, the LSEC endocytosis of this molecule peaked at 12 months of age, whereas the uptake in 4 and 28 months old rats was similar. Recently, in vivo microscopy was used to detect uptake of two fluorescently labeled SR ligands, AGE-albumin and FSA, in the LSECs following injections into the mesentery vein of four different age-groups of C57BL/6 mice representing prepubertal (3.5 weeks), young adult (3 months), middle-aged (14-15 months) and old animals (27 months) (22). The results indicated a gradual reduction in the endocytic function from young adult to old age, with the most pronounced reduction in centrilobular sinusoids.
Two studies in rat report lysosomal enzymes activity in liver as a function of age; one showed increased levels of several lysosomal enzymes in both KCs and LSECs in old animals (344), whereas another study showed no clear general trend, with varying results for different enzymes (345).

The possible pathophysiological effects of the structural and functional changes observed in the old liver sinusoid are not known. However, endothelial capillarization and pseudocapillarization have been suggested to impede the transfer of substrates, such as drugs and albumin, between blood and PCs (71, 76, 346). There is also some evidence that the altered liver sieve in cirrhosis and aging might contribute to hypoxia in PCs, thus providing an alternative mechanism for the apparent differential age-related reduction of oxygen-dependent phase I metabolic pathways (76). It has further been suggested that pseudocapillarization may contribute to age-related decreased liver clearance of chylomicron remnants, explaining partially the increased risk for development of atherosclerosis with age (78).

AIMS OF THE STUDY

Effective removal of unwanted self and non-self molecules from blood is essential to keep the organism healthy. LSECs have been shown to effectively clear such macromolecules via receptor-mediated endocytosis. The cells express several high affinity endocytosis receptors, including the mannose receptor (MR), and several scavenger receptors (SRs), which are known also for their role as pattern recognition receptors (PRRs) in host defence. The present study aimed to further examine the expression and function of PRRs in LSECs, the role of LSEC endocytosis in initiating innate immune responses, and how aging may affect the important scavenger function of these cells. Three sub-projects were designed to reach this aim:

- As bacterial DNA have been found to be removed from blood very efficiently by the liver (347), we wanted to 1) examine the role of LSECs in blood clearance of unmethylated CpG oligodeoxynucleotides (CpG-ODNs, a TLR9 agonist), 2) examine if LSECs express TLR9, and 3) examine if CpG-ODN uptake in LSECs elicit an immune response by interacting with TLR9.

- Due to the high endocytic activity of the LSECs, the cells need great amounts of lysosomal enzymes for efficient degradation of internalised material. The MR, besides recognizing mannose residues on microorganisms, also mediates endocytosis of lysosomal enzymes. Using an MR knockout mouse model we wanted to study whether the cells recruit lysosomal enzymes via this receptor and the effect of MR deficiency on LSECs catabolism.

- Little is known about the LSEC endocytic activity at old age. We therefore wanted to study this by comparing endocytosis in cells from young and old individuals and examine how the endocytic capacity of the cells correlate with age-related morphological changes (e.g. defenestration).

SUMMARY OF PAPERS

Paper I - Toll like receptor 9 (TLR9) is present in murine liver sinusoidal endothelial cells (LSECs) and mediates the effect of CpG-oligonucleotides

Background and aim: Liver sinusoidal endothelial cells (LSECs) may represent an important interface between host and pathogens. They are known to be the main site of clearance of DNA ODNs from the circulation. Bacterial DNA and synthetic ODNs containing unmethylated CpG motifs activate cells of the innate immune system through interaction with TLR9. The aim of the study was to investigate if and how CpG-ODNs activate LSECs.

Methods: A preparation of synthetic unmethylated CpG ODNs was used as TLR9 agonist. ^{125}I-FITC-labelled CpG (0.1mg/kg) was injected into the tail vein of male Balb-c mice, and blood and organs were collected for anatomical distribution studies. FITC-CpGs (4 mg/kg) was injected intravenously to study the hepatic distribution by fluorescence microscopy. TLR9 expression was examined by immunolabelling of frozen liver sections, immunocytochemistry (ICC) of isolated LSECs and RT-PCR of LSEC mRNA. Expression of MyD88 was examined by ICC and RT-PCR, and NFκB activation was studies by ICC. Production of cytokines (IL-1β and IL-6) was examined by ELISA. Endocytosis studies were performed in freshly isolated mouse LSEC cultures.

Results: Liver was the main site of uptake of intravenously injected ^{125}I-FITC-CpGs. The circulatory $t_{1/2}$ of the ligand was 4 min, indicating en effective uptake mechanism in this organ. FITC-CpG specific fluorescence was observed along the liver sinusoids, indicating uptake in LSECs. Immunolabeling for TLR9 also showed positive staining along the sinusoids. Expression of TLR9 in LSECs was confirmed by RT-PCR and ICC of isolated cells. Uptake of non-labelled CpGs in LSEC cultures resulted in activation of the transcription factor NFκB and secretion of IL-1β and IL-6. Cytokine production was inhibited by incubation of LSEC cultures with monensin and chloroquine, which blocks endocytosis receptor recycling and intracellular transport of endocytosed ligands, respectively, indicating that CpG was first taken up by receptor-mediated endocytosis and transported through the

endocytic pathway before it could bind to endosomally located TLR9. Incubating the CpG-treated cells with AGE-albumin, a well-known SR ligand, also abrogated the LSECs production of cytokines, strengthening this idea.

Conclusions: The study showed for the first time the presence of a functional TLR9 in LSECs, which emphasizes the importance of these cells in the innate immune system of the liver. Uptake of CpGs via receptor-mediated endocytosis was necessary for TLR9-signalling.

Paper II - Liver sinusoidal endothelial cells depend on mannose receptor-mediated recruitment of lysosomal enzymes for normal degradation capacity

Background and aims: Liver sinusoidal endothelial cells (LSECs) are largely responsible for the removal of circulating lysosomal enzymes via MR-mediated endocytosis. We hypothesized that LSECs rely on this uptake to maintain their extraordinarily high degradation capacity for other endocytosed material.

Methods: An MR knockout ($MR^{-/-}$) mouse model was used to test this hypothesis. ^{125}I-cathepsin-D or ^{125}I-FSA were injected intravenously in $MR^{-/-}$ mice and wild-type control mice and blood samples and tissues collected for examination of circulatory half-life and tissue distribution of ligands. Endocytosis experiments with radioiodinated or fluorescently labeled ligands were performed in purified LSEC cultures or NPCs from $MR^{-/-}$ and wild-type mice. The activity of five lysosomal enzymes, including cathepsin-D, was measured in LSECs and liver PCs, and cathepsin-D protein expression was examined by immunoblotting.

Results: Circulatory half-life studies of ^{125}I-cathepsin-D in $MR^{-/-}$ and wild-type mice showed a total dependence on the MR for effective clearance of cathepsin-D. Endocytosis studies in LSEC cultures confirmed this finding. ^{125}I-FSA, a ligand for the LSEC SRs, was used to study catabolism of endocytosed material in $MR^{-/-}$ and wild-type mice. When injected intravenously, the plasma clearance, liver uptake, and the starting point for release of degradation products to blood, were similar in both experimental groups, indicating normal endocytosis and intracellular transport of SR ligands in $MR^{-/-}$ mice. However, the rate of FSA catabolism in the liver of the MR deficient animals was reduced to approximately 50% of wild-type values. A similar reduction in intracellular degradation was recorded in LSEC cultures from $MR^{-/-}$ mice compared to wild-type controls. It was also found, in accordance with the previous result, that $MR^{-/-}$ LSECs had markedly and significantly reduced enzyme activities for four out of five lysosomal enzymes tested, i.e. cathepsin-D, α-mannosidase, β-hexosaminidase and arylsulfatase,

but not acid phosphatase, compared to wild-type controls. Immunoblot analysis showed that the content of pro-cathepsin-D relative to total cathepsin-D in wild-type LSECs was less than one-fifth of that in PCs, indicating lower endogenous lysosomal enzyme production in the LSECs.

Conclusion: We conclude that LSEC depend on MR-mediated recruitment of lysosomal enzymes from their surroundings to keep up their high catabolism of endocytosed material.

Paper III - Age-related changes in scavenger-receptor mediated endocytosis in rat liver sinusoidal endothelial cells

Background and aims: Liver sinusoidal endothelial cells (LSECs) play an important role in systemic waste clearance by effective endocytosis of many blood-borne macromolecules. However, little is known about how this function is affected by aging and how age-related morphological changes (e.g. defenestration) affect the endocytic capacity. We therefore aimed to study these questions.

Methods: Endocytosis of ^{125}I-labelled FSA was examined in freshly isolated LSECs from young and old F344/BN F1 hybrid rats. LSEC protein expression of stabilin-1 and stabilin-2 was examined by SDS-PAGE and western blotting of cell solubilisates, and the LSEC fenestration was measured by scanning electron microscopy (SEM). Ultrastructural changes in the old liver sinusoid, and the stabilin expression along the sinusoids was studied by transmission electron microscopy (TEM) and immunofluorescence microscopy of liver biopsies from young and old rats.

Results: At low ligand concentrations (0.1 μg/ml) LSECs from both age groups showed similar uptake of FSA, but at all higher concentrations (1-128μg/ml) cells from the old group showed a significant reduction in endocytic capacity (~30% reduction at high ligand doses). LSECs protein expression of the two major SRs for FSA endocytosis, stabilin-1 and stabilin-2, and their staining patterns along liver sinusoids, were similar at young and old age, suggesting that other parts of the LSECs endocytic machinery are affected by aging. The old rats showed significantly increased thickness and reduced fenestrae numbers of the LSECs in vivo, but no increase in collagen or basal lamina deposits in the subendothelial space, as reported in inbred rats and other species. These findings suggest that endothelial changes precedes matrix formation in the development of age-related pseudocapillarization. When comparing the maximum endocytic capacity for FSA in vitro with the porosity (% fenestrated area/cell area) of LSECs isolated from the same

animal, we found no correlations between endocytic activity and cell porosity in either of the age groups. This indicates that reduced fenestration does not necessarily lead to reduced endocytosis although both parameters can be affected by aging.

Conclusion**:** We report a significantly reduced LSEC endocytic capacity at old age. This could be of importance for old individuals subjected to increased levels of circulatory waste products.

GENERAL DISCUSSION

LSECs express a functional TLR9: CpG-mediated TLR9 signaling is dependent on scavenger receptor mediated endocytosis

As described in the General Background, LSECs have been suggested to play a role in host defence beyond the mere scavenging and catabolism of soluble macromolecules. In paper I, we present evidence that LSECs express a functional TLR9, and that signalling via this receptor is dependent on SR-mediated endocytosis of the TLR9 agonist, unmethylated CpG ODN (89).

Unmethylated CpG ODN was chosen as a ligand due to the immune stimulatory effects of its CpG domains. While vertebrate CpG dinucleotides are often methylated and not very frequent, viral and bacterial CpG dinucleotides are non-methylated and occur with a much higher frequency than in vertebrate DNA (348). The availability of synthetic CpG ODNs has opened up for the possibility to study if and how specific CpG sequence motifs, sugar, base or backbone modifications as well as secondary and tertiary structures may affect the immune modulatory effects of the CpG ODNs via TLRs (349). Before we started our study, the CpGs uptake in liver had been reported (350), but possible effects on LSECs had not been not examined.

TLR9, first described in 2000 (351), binds DNA and responds to bacterial and viral unmethylated CpG DNA by signalling via NFκB (352, 353). Studies in macrophages and dendritic cells have shown that TLR9 is normally located in the endoplasmic reticulum, and it is suggested that the internalization of CpG DNA induces the translocation of TLR9 to early endosomes where it binds the ligand and triggers the subsequent activation of downstream mediators (354). The endoplasmic reticulum UNC93B1 membrane spanning protein is recognized as a key player in TLR9 trafficking through its ability to bind strongly to the transmembrane regions of the nucleotide sensing TLR (355). However, the exact mechanism by which these proteins mediate the effects needed for proper TLR9 relocation and signalling remains unknown. It has been suggested that the complex regulation of TLR9 signalling helps to separate the recognition of microbial and self nucleic acids and thus protect

the host from an inappropriate immune response (356, 357). After binding to its ligand, TLR9 activates MyD88 that is essential for initiating the TLR9 signalling. MyD88 starts a cascade of phosphorylation resulting in the translocation of NFκB to the nucleous where induces the transcription of proinflammatory cytokine genes, including TNF-α, IL-6 and IL-12. TLR9 in macrophages and dendritic cells is also involved in antiviral responses by activating the interferon regulatory factor-7 (IRF-7), which then translocates to the nucleus to mediate the transcription of interferon-α-genes (reviewed in (356, 357)).

In Paper I (89) we found that CpG ODN was taken up by the LSECs both in vivo and in vitro. We also showed that the cells express TLR9, as well as the downstream MyD88 and NFκB that are required elements in the signaling cascade to produce an inflammatory response. Incubation of LSEC cultures with CpG ODNs (5μg/ml) lead to NFκB translocation to the nucleus, as well as the production of IL-1β and IL-6. Taken together this indicates that the LSEC is an active player of the innate immune system.

The liver uptake of CpGs should be taken into account when working with CpG ODNs as an adjuvant in vaccines, cancer therapies and others applications (358). The way of administration of drugs and vaccines that are ligands for LSECs should be chosen so as to surpass these cells and thereby obtain the desired effect in the target organ and not an unwanted response in the liver.

Another novel finding in this study was that a well-known ligand for the LSEC SRs (i.e. AGE-albumin) could abrogate the response to treatment of LSECs with CpG ODNs, indicating that SRs were involved in this uptake. The main SR receptor for AGE-albumin in LSECs is stabilin-2, probably together with stabilin-1 (93, 124). It is known from a previous report using SR-A knockout mice that the liver uptake of CpG-ODN can proceed independently of SR-A (359), but because of the redundancy of SRs in the LSEC it is difficult to rule out the involvement of a receptor based on single-gene knockout mouse models. Another study in mice, using both SR-AI/II and MARCO gene knockout models, showed that both SR-AI/II and MARCO mediated

macrophage uptake of CpGs but that only uptake via MARCO triggered the production of cytokines (i.e. IL-12) via the TLR9 pathway (360). In LSECs the SRs stabilin-1 and stabilin-2 are highly expressed, and have been suggested as the main SRs in these cells (11, 92, 93, 179). Although we did not study the exact SR involved in the LSEC CpG uptake in Paper I, unpublished observations by Dr. Peter McCourt (Department of Medical Biology, University of Tromsø; personal communication) showed a high uptake of FITC-CpGs in HEK-293 cells transfected with either stabilin-2 or stabilin-1, suggesting that cells expressing these receptors (such as LSECs) take up CpGs via stabilins.

Possible outcome of TLR9-signalling in LSEC

The results of paper I were obtained in vitro, and the possible effects of the cytokines produced by the LSECs on other cells in the liver were not studied. However, both KCs (361), PCs (362) and SCs (363) respond to inflammatory cytokines. Signalling in liver is complex and only rudimentary understood, probably because the net effect of any cytokine depends on several factors such as timing, local environment and the presence of competing elements (i.e. soluble antagonist) (364). From previous studies, it is well known that IL-1β acts mostly pro-inflammatory (reviewed in (365)) whereas IL-6 has both pro-inflammatory and anti-inflammatory properties. Although IL-6 is a potent inducer of the acute-phase protein response in liver, it may also down-regulate the synthesis of IL-1β (364, 366, 367) and induce the synthesis of Interleukin receptor antagonist, IL-Ra, a soluble form of IL1 that competes for the IL-1 receptor in target cells (368). IL-1β responses are also strongly controlled by the serum levels of IL-Ra, which is measured to be around 700pg/ml in healthy humans (369). Therefore the amounts of IL-1β produced to elicit an immune response need to be high enough to overcome this competition.

Immune stimulatory molecules may also influence LSEC endocytosis. The endocytosis via SRs and MRs in rat LSECs was enhanced by TNF-α and Il-1β stimulation in vitro (370). This could enhance the LSECs ability to act as a sink for potential dangerous macromolecules, preventing them from reaching other organs.

Excessive activation of TLR signaling may cause tissue damage. Therefore, TLR signalling is strictly negatively regulated. To my knowledge, this has not been studied in LSECs. Also in other cell systems the literature on negative regulation of TLR9 is not overwhelming. Recently an ubiquitin-protein ligase, TRIAD3A, was found to suppress TLR9 immune responses by ubiqutinating the receptor, and thus marking it for degradation, in experiments done in cell lines overexpressing TRIAD3A (371). Interestingly, imiqimod (a non-biological ligand of TLR7) and R848 (a non-biological ligand of TLR7 and TLR8) were found to abrogate the interferon-α production initiated by specific types of CpGs in peripheral blood mononuclear cells isolated from healthy humans, by downregulating TLR9 mRNA production (372).

LSECs depend on MR-mediated recruitment of lysosomal enzymes for normal degradation capacity

In paper II we present evidence that the MR, which is regarded as an important PRR in macrophages and dendritic cells, is also needed for proper catabolic function in the LSECs by the recruitment of lysosomal enzymes (87).

Animal model: To study the role of the MR in recruitment of lysosomal enzymes to LSECs, we used an $MR^{-/-}$ mouse model (kindly provided by professor M. Nussensweig, Rockefeller University). The gene for MR was knocked-down by introducing a reporter gene with a STOP codon at exon 1 producing a much smaller mRNA that is not translated into protein (219). The $MR^{-/-}$ mice (C57BL/6 background) have an apparently normal phenotype and are fertile. However, they have enhanced blood levels of several lysosomal enzymes and some connective tissue turnover by-products (i.e. PICP, PIIICP) (219), indicating that this receptor is an essential regulator of serum glycoprotein homeostasis (219).

Cathepsin-D, an aspartic protease (373) was used as a model ligand to study blood clearance, tissue distribution, LSEC endocytosis and production of lysosomal enzymes in cells from $MR^{-/-}$ and wild-type control mice. Lysosomal enzymes belong to the a group of endogenous macromolecules that express

mannose in terminal positions of their sugar side-chains. Cathepsin-D is synthesized in the rough endoplasmic reticulum as a pre-pro-enzyme, which is further processed into the mature two-chain form of cathepsin-D as it moves through the Golgi apparatus (374). In cell types other than LSECs, the transport of cathepsin-D from the Golgi to the lysosomes usually follows the classical mannose 6-phosphate receptor-mediated (M6PR)-route (374). Glycosylation patterns are used as signals to transport enzymes into the correct cellular compartment and the intracellular traffic of lysosomal enzymes is well studied (reviewed in (375)). Cathepsin-D is not only found in lysosomes but can also be secreted from cells and can be recovered in tissue stroma and plasma. In fact, plasma cathepsin-D is used as a prognostic marker in breast cancer as it has been shown that cancer cells that secrete this protein have a higher metastatic potential (374). It has also been shown that PCs secrete proforms of cathepsin-D (376).

To study LSEC degradation of internalized material in $MR^{-/-}$ mice versus normal mice we used the SR ligand FSA that was taken up at equal rates in LSECs from both groups. This ligand has been reported previously to be rapidly cleared from blood almost exclusively by the LSECs (129, 142, 377) and in the present study we found no difference in rates of blood clearance of FSA in $MR^{-/-}$ and wild-type mice, suggesting that SR endocytosis is normal in $MR^{-/-}$ deficient mice. Furthermore, FSA is easy to work with in the laboratory: it is stable, easy to label with radioactive iodine and fluorochromes, and is effectively degraded in the endo/lysosomal pathway after uptake (123, 129, 142, 179, 193). The degradation products are released into the blood or culture medium and can be measured as the non-precipitable fraction after mixing with trichloroacetic acid (86, 129).

The main findings of this work can be summarized as follows: Cathepsin-D clearance was significantly slower in the $MR^{-/-}$mice, and the enzyme was not recognized by LSECs isolated from these mice, indicating that the LSEC MR is essential for clearance of mannosylated lysosomal enzymes, as also indicated by the enhanced plasma levels of various lysosomal enzymes in this mouse model, reported by others (219). In accordance with this finding the activity of 4 mannosylated lysosomal enzymes (cathepsin-D, α-mannosidase,

β-hexosaminidase, aryl sulphatase) but not the non-mannosylated acid phosphatase was markedly and significantly reduced in LSECs isolated from $MR^{-/-}$ mice. A direct effect of the inability of the $MR^{-/-}$ LSECs to obtain lysosomal enzymes from their surroundings was seen as a 50% reduction in the catabolism (degradation) of FSA compared to wild-type controls: this was seen both in vivo after intravenous injection and in cultured LSECs. Interestingly the specific activities of the analyzed lysosomal enzymes, including that of cathepsin-D, were significantly higher in wild-type LSECs than in the corresponding PCs. This has also been reported in rat (73). However, the relative expression of pro-cathepsin-D versus mature cathepsin-D in LSECs and PCs (analyzed by immunoblotting) showed a lower expression of pro-cathepsin-D in LSECs than in PCs. This finding strengthens our hypothesis that the high lysosomal activity of LSECs reflects uptake from the cell surroundings via MR rather than the novo synthesis by the LSEC. Lysosomal enzymes are stable proteins, which may retain their activity for several days after uptake in cells (378), and the MR-mediated recruitment of lysosomal enzymes to the cells represents an M6PR independent mechanism for transport of lysosomal enzymes to degradation compartments in the endocytic pathway.

M6PR independent recruitment of lysosomal enzymes to cells has also been suggested in the kidney proximal convoluted tubules (379): Mice lacking the megalin receptor in these cells were defective in cathepsin-B activity, whereas tubuli epithelial cells in normal mice were able to take up cathepsin-B from the circulation (interestingly, only 10% of the injected dose reached the kidney). In addition cathepsin-B knockout mice were able to regain normal activity of the enzyme after one single injection of cathepsin-B (379).

Paper II shows that the LSEC is not only a sink for elimination of unwanted waste products are eliminated: the cells are also a very effective and energy saving recycling station.

The effect of aging on LSEC scavenger function

In paper III (380) we were not only interested *per se* in the clearing functions of the cells, we also wondered if and how this function is affected at old age.

In this study we used a hybrid rat model (F344/BN F1 males) to study endocytosis of FSA in LSECs isolated from young and old rats. Compared to inbred strains (like F344 rats or C57BL/6 mice) hybrid animals have few diseases associated with high age, and are recommended for aging research by the guidelines of the National Institute on Aging (381). Thus the results obtained better reflects the effect of aging alone and not of diseases associated with aging.

Our reason for choosing a rat model was also the higher number of LSECs needed for endocytosis capacity studies and parallel morphology studies (approximately 60-100 million cells/ rat liver compared to mice 5-10 million cells/ mouse liver).

Comparing endocytosis in cells isolated from two age groups generated several methodological challenges: for example LSECs gradually loose their endocytic capacity in vitro (382, 383). Therefore cells used for this type of experiment had to be freshly isolated, and all experiments had to be started at exactly the same time point after plating: 2h were chosen since the cells were well spread and highly viable at this time. All methods and ligand handling procedures had to be strictly standardized. Cell culture purity had to be similar for young and old animals and was tested by SEM; an average purity of > 97% was obtained in each group. Cell numbers were also assessed by SEM of parallel cultures to those used for endocytosis assays as this was found to be a more reliable procedure than cell protein for comparing uptake per cell. Control experiments in vitro showed that collagenase (same concentration and time as used in vivo during the dissociation of liver cells) had no effect on the ability of LSEC to endocytose ^{125}I-FSA.

The main finding of the paper was a marked age-related reduction in the LSEC capacity for FSA uptake when the ligand was added in increasing concentrations to the cultures, whereas endocytosis of FSA was similar in the two age groups at low doses (i.e. “trace amounts”; 0.1μg/ml). To my knowledge this is the first time that an endocytosis capacity study of LSECs has been carried out using optimal culture and experimental conditions. Similar studies carried out in the past i.e. the endocytic capacity of KCs and

LSECs for heparin (among other substances) was compared in (384) and the conclusion was that LSECs endocytosed these ligands several times more effectively than KCs. These studies were performed in the presence of serum and with cells cultured for longer time (127, 384, 385), which are factors that may affect endocytosis negatively, as reported by Hansen et al. (193). They found that endocytosis of FSA in rat LSECs in vitro was inhibited 10-80% by serum (human, rat, bovine and fetal) in a dose dependent manner (193). The present study was therefore carried out under serum free conditions.

In spite of the significantly (~30%) reduced maximum endocytic capacity of LSECs isolated from old rats, the cells from the old animals still had a high endocytic activity compared to other endothelial cells. For example, after a 4h incubation with acetylated LDL (25 μg/ml) cultures of bovine aortic endothelial cells and capillary endothelial cells from bovine adrenal cortex, were reported to take up 1-2 μg ligand per mg cell protein (386). Human umbilical vein endothelial cells incubated with acetylated-LDL (50 μg/ml) or oxidized-LDL (100 μg/ml) for 6h showed uptake of 2.5 μg ligand protein per mg cell protein (387). Since we calculated uptake of FSA per cell and not per mg cell protein these results cannot be directly compared with our results presented in paper III. However, based on the reported mass (46 μg/million cells) of rat LSEC (73), uptake of FSA per mg cell protein during 2h incubation with 32μg/ml FSA (ligand concentration at the curve flattening point) was approximately 40 μg/mg and 60 μg/mg, in the young and old rat LSECs, respectively.

In accordance with previous findings in other species and other rat strains, the old F344/BN F1 rat livers showed increased thickening of the sinusoidal endothelium, reduced LSEC fenestration, as well as accumulation of large lipid droplets in SCs. However, no accumulation of basal lamina deposits or collagen was seen in the space of Disse from young to old age, suggesting that functional and structural changes in the endothelium precede the age-related accumulation of extracellular matrix in the space of Disse reporterd observed in other studies (22, 326, 328, 329). We also made a morphological assessment of the isolated LSECs. Surprisingly we did not find a statistical significant difference in the number of the fenestrae, fenestrae diameter or

LSEC porosity (% area of fenestrae/area cell) between the age groups, other than a slightly less fenestrated phenotype in the old rat LSEC cultures (not statistically significant). This difference between the in vivo and in vitro observations may be explained in several ways: One possibility is that the isolation procedure selects the most active cells (given that fenestration is correlates positively with high cell viability), resulting in less differences between LSECs from young and old rats in vitro. O´Reilly et al (388) reported a small, but statistically age-associated difference in the diameter of fenestrae from cultured LSECs from F344 rats (in this case the cells were observed 18 hours after isolation; compared to after 2 hours in our study). They suggested a selection of the best cells as a possible mechanism, but also mentioned the possibility that "hyperfenestration" might be an artifact of the culture due to an actin disruption or that other liver cells are needed for proper regulation of fenestration. In our study, the average plating efficiency was approximately equal in young and old rats, so if we have selected the best cells in our study, we may rather have underestimated than overestimated the difference in endocytic capacity between the cells from the two age groups.

When the LSEC endocytosis capacity data and porosity data per animal were compared, no relationship was found, neither between nor within the age groups. This is not unexpected when taking a brief look at the vertebrate kingdom: e.g. in Atlantic salmon (*Salmo salar*) the LSECs are fenestrated (69) but does not have the scavenger function of the mammalian LSECs (104, 105). Instead the scavenger endothelial cells of salmonid species are located in the kidney (104, 105).

Immunohistochemistry of liver sections and immunoblotting of LSEC cultures indicated that the expression of the major SRs for FSA uptake, stabilin-1 (11) and stabilin-2 (93), was not affected by aging. The endocytosis studies of ^{125}I-FSA also indicated that the internalized ligand was as effectively degraded in the old rat cells as in the cells from the young group, even at high ligand concentrations, suggesting that the lysosomal degradation capacity was not significantly affected by animal age. The high catabolic activity in the LSEC also at high age is in accordance with a previous study in rat comparing lysosomal enzyme activity in LSECs from old and young animals showing that

the activity of acid phosphatase, aryl-sulfatase B, β-galactosidase and cathepsin-D increased at old age (344).

We proposed the hypothesis in paper III that the observed changed endocytic capacity might be due to the age-related increase in the thickness of the sinusoidal endothelium that is observed in many species in vivo (reviewed in (71))(380). Increased endothelial cell thickness may lead to a slow down ("traffic jam") in the intracellular vesicle traffic. However, this hypothesis needs to be tested. A search for the exact explanation(s) for the age-associated decrease in endocytic capacity was unfortunately too big a challenge to be completed under this PhD study; it would require both genomic and proteomic screening and validation of data. Different receptors, as well as the different components of the endocytic machinery may as well be differently affected by aging.

An important consequence of a decreased endocytic capacity of LSECs at old age might be seen first of all when the organism is challenged; e.g. in situations with massive trauma or massive tumor lysis as in chemotherapy treatment when there is a sudden increase in circulating debris due to massive cell death. Other challenges are chronically elevated blood levels of harmful modified substances (e.g. AGEs and oxidized LDLs) such as diabetes or cardiovascular disease.

CONCLUSIONS

- The presence of a functional TLR9 in LSECs emphasizes the importance of these cells in the innate defense mechanisms of the liver

- SR-mediated endocytosis is necessary to deliver the agonist (unmethylated CpG) to the TLR9 in LSECs

- LSECs depend on MR-mediated recruitment of lysosomal enzymes from their surroundings to maintain their high degradation capacity

- The LSEC capacity for endocytosis of a model SR ligand (FSA) was significantly reduced at old age
- No correlation was found between LSEC endocytic capacity and cell fenestration, however, both properties can be affected by aging

FINAL REMARKS

The high endocytic activity of LSECs of unwanted molecules of foreign and self-origin is of major importance for the correct maintenance of the liver and for the body equilibrium of various macromolecules. In addition to a mere scavenging role of the LSECs the present study show that LSEC endocytosis is involved in regulation of liver immune responses by delivering ligands to TLR9, and is also necessary to maintain the very effective lysosomal degradation of endocytosed material that signify the physiological function of LSECs. However this function appears to be negatively affected by aging. The liver has a large reserve capacity, but under certain conditions such as in massive trauma or acute tumor lysis, which may lead to a rapidly increased burden of circulating waste/potentially dangerous material, a reduced endocytic capacity of the LSECs may have direct and serious effects on the health of the elderly.

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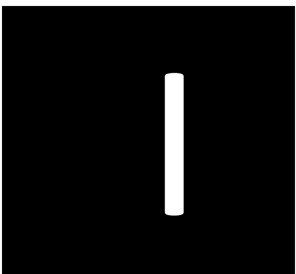

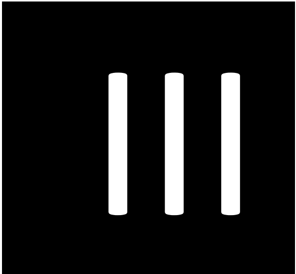

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