The scavenger endothelial cell – a new player in homeostasis and immunity

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Abstract

To maintain homeostasis the animal body is equipped with a powerful system to remove circulating waste. This review presents evidence that the scavenger endothelial cell (SEC) is responsible for the clearance of blood-borne waste macromolecules in vertebrates. SECs express pattern-recognition endocytosis receptors (mannose and scavenger receptors), and in mammals, the endocytic Fc gamma-receptor IIb2. This cell type has an endocytic machinery capable of super-efficient uptake and degradation of physiological and foreign waste material, including all major classes of biological macromolecules. In terrestrial vertebrates the majority of SECs line the wall of the liver sinusoid. In phylogenetically older vertebrates SECs reside instead in heart, kidney or gills. SECs thus, by virtue of their efficient non-phagocytic elimination of physiological and microbial substances, play a critical role in the innate immunity of vertebrates. In major invertebrate phyla, including insects, the same function is carried out by nephrocytes. The concept of a dual cell principle of waste clearance is introduced to emphasize that professional phagocytes (macrophages in vertebrates; haemocytes in invertebrates) eliminate larger particles (> 0.5 µm), by phagocytosis, whereas soluble macromolecules and smaller particles are eliminated efficiently and preferentially by clathrin-mediated endocytosis in non-phagocytic SECs in vertebrates or nephrocytes in invertebrates. Including these cells as important players in immunology and physiology provides an additional basis for understanding of host defense and tissue homeostasis.

Key words

Liver sinusoidal endothelial cell, endocytosis, blood clearance, scavenger cells, nephrocyte
**Abbreviations**

AGE, advanced glycation end product

DC, dendritic cell

FcγR, IgG Fc receptor

LDL, low density lipoprotein

LPS, lipopolysaccharide

LSEC, liver sinusoidal endothelial cell

MHC, major histocompatibility complex

MPS, mononuclear phagocyte system

PAMP, pathogen-associated molecular pattern

DAMP, danger-associated molecular pattern

PRR, pattern recognition receptor

RES, reticuloendothelial system

SEC, scavenger endothelial cell

SR, scavenger receptor

TLR, toll like receptor
Major cellular waste clearance systems

Every second the animal body is challenged with waste material, both self-made and foreign. Most macromolecules in the body are highly dynamic structures, with natural turnover rates spanning from minutes to months. This natural turnover includes some limited extracellular degradation, but in fact complete degradation to single building blocks (amino acids, monosaccharides, fatty acids, and nucleotides) will primarily take place after uptake and intralysosomal processing in specialized scavenger cells. In addition to natural turnover mechanisms, chemical and mechanical wear and tear processes constantly turn macromolecules into harmful waste substances such as advanced glycation end products and oxidized lipoproteins that are recognized and eliminated by the same scavenger cells. Moreover, foreign material (primarily microbes and microbial products) that constantly invade the body are removed by these scavenger cells.

Cells responsible for the removal of waste macromolecules have long been the subject of research in immunology, metabolism and physiology. About 150 years ago the tradition of injecting “vital stains”, i.e. colloidal dyes, into living organisms to identify anatomical sites of waste accumulation began. After several decades of such studies, Aschoff in 1924 summed up the state of the art and launched the concept of “the reticuloendothelial system” (RES), which included both macrophages and cells lining the sinusoids of liver, lymph nodes, spleen, bone marrow, and adrenal and pituitary glands (3). The role of the macrophage in uptake and degradation of waste substances was extensively studied over the years that followed after Aschoff’s publication, leading to a major focus on the role of macrophages in this process. The last 3-4 decades led to a significant development in our understanding of the waste generating and disposal processes in the body, and we now know that extracellular macromolecules as well as entire cells are turned over by uptake and lysosomal degradation in specialized scavenger cells, namely macrophages and scavenger endothelial cells (SECs) in vertebrates (136). Clearly, a deeper knowledge of the SEC, which is much less studied...
than the macrophage, will provide insight into disease formation, and blood clearance functions, i.e. the beneficial elimination of blood borne virus and other pathogens as well as unwanted uptake of biopharmaceuticals. This review focuses on the least studied of these two cell types, namely the SEC, and the role of these cells in tissue homeostasis and host defence. In the last part of the review we argue that the nephrocyte, an essential pinocytic cell of many invertebrate phyla, carries out a scavenger function with striking analogy to the vertebrate SEC.

**Scavenger endothelial cells (SECs)**

In mammals, birds, reptiles and amphibians, the body’s largest SEC reservoir is located in the liver where they make up the entire hepatic sinusoidal endothelium (136). Interestingly, phylogenetically older vertebrates carry their SECs in organs other than the liver. Thus, bony fish have their SECs in either heart (endocardium) or kidney (venous sinusoids), whereas in cartilagenous fish, and the jawless vertebrates hagfish and lamprey, SECs are located in special blood vessels of the gills (136). The vast majority of the literature on SEC scavenger function and receptor expression deals with the mammalian liver sinusoidal endothelial cell (LSEC). Accordingly the following outline of the special features of SECs refers mainly to the mammalian LSEC.

**The liver sinusoidal endothelial cell (LSEC)**

The numerous sinusoids of liver are lined by an endocytically highly active endothelium, which efficiently eliminates large amounts of circulating macromolecules of foreign and endogenous origin (142). The LSECs were first distinguished from the resident liver macrophages or Kupffer cells in rat, on the basis of ultrastructure, by Wisse in 1970 (171). These endothelial cells lack a basal lamina, and are characterized by numerous fenestrae (diameter approx 150 nm) without diaphragms, allowing passage of molecules between the sinusoidal lumen and the underlying hepatocytes (Fig.1)
The fenestrations act as a permeable and selective ultrafiltration system which is important for the hepatic uptake of many substrates, and fenestrae size affects the balance of different lipoprotein moieties in plasma, modulating cholesterol metabolism and thus susceptibility to atherosclerosis (34, 172).

In his pioneering work, Wisse also noted numerous endocytic vesicles that suggested active endocytosis of blood plasma proteins (171). A physiological role of the scavenger activity of these cells was revealed for the first time when their very active endocytosis of the connective tissue macromolecule hyaluronan was discovered in 1982 (144). Later studies revealed that LSECs are the major elimination site for an array of physiological and pathophysiological waste macromolecules, and carry high affinity endocytosis receptors that efficiently mediate the uptake of a great number of waste substances (Table 1; Fig.2). In addition these cells avidly pinocytose small/colloidal particles such as colloidal silver and latex beads < 200 nm (122), but are not normally phagocytic (67).

Endocytosis receptors on LSEC

Scavenger receptors

The term "scavenger receptor" (SR) was originally coined to describe a macrophage receptor that mediates endocytosis of a broad range of polyanionic molecules (39). However, this term has now become the common name for an expanding number of identified SRs with various ligand specificities/affinities expressed on a number of different tissues and cells, including the LSEC. Currently, there are eight known structurally unrelated SR sub-classes (SR-A to SR-H) that recognise common ligands (104).

The LSEC expresses SR-A (aka macrophage SR) (52), SR-B (SR-B1 and CD36) (86), and SR-H (stabilin-1/FEEL-1 and stabilin-2/FEEL-2/HARE) (1, 42, 94, 120, 177). SR-D (CD68), SR-E (LOX-
1), SR-F (SREC) and SR-G (SR-PSOX) are not normally expressed on LSECs (24). The main work-horse SR on this cell type appears to be SR-H/stabilin-2 (possibly together with stabilin-1; Table 1) based on the following: i) SR-A knockout mice clear SR ligands equally well as wild type mice (79, 159, 165), and cultured LSECs from the same knockout mice endocytose and degrade SR ligands equally well as wild types (41); ii) an antibody to CD36 that inhibits CD36 mediated uptake of SR ligands in other cells has no effect on the uptake of SR ligands by LSECs (107); iii) an antibody to stabilin-2 can inhibit the LSEC mediated uptake of hyaluronan by more than 80% and other SR ligands by over 50% (94).

Stabilin-1 and -2 are 41% homologous and mediate the uptake and degradation in LSECs of oxidized low-density lipoproteins (LDL) (76), extracellular matrix macromolecules, and protein turnover by-products (94) that could otherwise accumulate in vascular tissues and interfere with homeostasis. Advanced glycation end-products (AGEs) and SPARC (secreted protein acidic and rich in cysteine, also known as osteonectin and BM-40) have also been identified as ligands for the LSEC stabilins (42, 66).

While the two stabilins have a largely similar ligand profile (Table 1), there are some important differences: stabilin-1 binds SPARC but not hyaluronan, and stabilin-2 binds hyaluronan but not SPARC. Another difference is their organ and intracellular distribution. High expression of stabilin-2 appears to be restricted to fenestrated endothelium e.g. in liver sinusoids (31), bone-marrow sinusoids (126), lymph nodes (89), and choriocapillaris (75). These endothelia also show high endocytic activity. Comparative studies in lower vertebrates show stabilin-2 expression in cod endocardial endothelial cells (Fig.3) which represents the major SEC population of this species (154, 155). Stabilin-1, while being expressed on the same endothelia as stabilin-2 in mammals, is also found on alternatively activated macrophages (120). Recent data suggests that stabilin-1 is expressed
in foam cells (11) and stabilin-2 in endothelial cells abutting atherosclerotic plaques (72).

Interestingly, during embryonic development, stabilin-2 is expressed in all liver vascular endothelia, while becoming restricted to the liver sinusoids at embryonic day 19.5 in mouse (175). In mature LSECs, stabilin-2 can be found throughout the cell, on the cell surface and associated with various structures associated with endocytosis, while stabilin-1 appears to have a predominantly intracellular distribution. However, stabilin-1 in LSECs, like stabilin-2, is associated with clathrin and adaptor protein-2 (42), mediates uptake of oxidized LDL and formaldehyde-treated serum albumin (76), and rapidly cycles between the cell surface and endosomes (125). A recent study reporting that stabilin-1/2 double knockout mice had mild liver fibrosis, and also severe kidney pathology suggests that the stabilins are vital for the removal of compounds toxic for the kidneys (130).

In summary, stabilin-1 and -2 appear to be the main SRs for circulating colloidal and soluble macromolecular waste in the mammalian LSEC (Table 1), but their scavenging function can be replaced somewhat in stabilin-1/2 double knockout mice.

**Mannose receptor**

Another important LSEC endocytosis receptor is the mannose receptor (CD206), a 175-180 kDa type I integral membrane protein belonging to the C-type lectin family (96). The receptor mediates uptake of a wide range of endogenous glycoproteins and microbial glycans and has a proposed role both in immunity (157) and glycoprotein homeostasis (73, 150).

The mannose receptor is expressed in several mammalian cell types, including LSECs (27, 83, 87, 89), sinusoidal endothelial cells of lymph nodes (89), subpopulations of immature dendritic cells (DCs) (96), and tissue macrophages (157). However, its expression in Kupffer cells is debatable. It is absent in human Kupffer cells (89), and its expression in rat and mouse Kupffer cells is low.
compared to LSECs (78, 83). This is supported by the consistent observation that soluble mannose receptor ligands are taken up in the LSECs, rather than in Kupffer cells and other macrophages, following their intravenous injection (142).

Mannose receptor deficient mice show elevated levels of lysosomal enzymes in plasma, and increased fetuin B, and C-terminal procollagen propeptides in sera, compared to control mice (73). These molecules are upregulated in inflammation and wound healing, and are cleared from the bloodstream mainly in LSECs (25, 147). In fact, LSECs were shown to depend on the mannose receptor for recruitment of lysosomal enzymes to maintain normal degradation capacity for endocytosed material (25). Other physiological ligands for this receptor in LSECs include tissue type plasminogen activator (143), neutrophil granulocyte-derived myeloperoxidase (137), salivary amylase (111), and denatured collagen fragments (87) (Table 1). These molecules are released to the body fluids during normal and pathophysiological tissue turnover, and may be harmful to the organism if allowed to accumulate.

The extraordinarily broad ligand specificity of the mannose receptor is explained by its three different ligand binding regions: i) an outer cysteine-rich amino-terminal domain, which recognizes specific sulfated sugars (32); ii) a fibronectin type II repeat which binds collagens (108), 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 (29, 161). There is little or no competitive inhibition of ligands for the different domains, which explains why it was believed for more than 20 years that LSECs carried a distinct collagen α-chain receptor (142, 145). The LSEC collagen α-chain receptor was found to be identical to the mannose receptor (87). This receptor clearly plays a vital role in homeostasis in that the normal remodelling of bone by osteoclasts releases more than half a
gram of partially degraded collagen into the blood stream per day in humans, and this material is efficiently cleared by LSEC (87).

The mannose receptor expression in liver is influenced by inflammatory stimuli and cytokines. Interleukin-1 (IL-1) upregulates receptor expression, and endocytosis via this receptor in LSECs (2), whereas IL-10 diminishes the LSEC mannose receptor activity (64).

**Fc gamma-receptor IIb2**

Although hepatic Fc-gamma-receptor- (FcγR)-mediated clearance of circulating IgG immune complexes was previously assumed to be mediated by Kupffer cells only (33), we now know that the LSEC also contributes importantly to the uptake of small soluble immune complexes (46, 57, 81, 140). Using immune complexes consisting of dinitrophenylated (DNP) albumin in complex with anti-DNP IgG, it was found following intravenous injection in rats that the proportions removed by Kupffer cells and LSECs depended on the number of antibodies in complex with DNP-albumin. The larger the complex the more was directed to Kupffer cells (140). A similar size dependency is also observed when comparing uptake of SR-ligands in Kupffer cells, and LSECs (56, 76, 146).

Mousavi et al. (102) demonstrated that FcγRIIb2 is the only FcγR in rat LSECs. The expression of FcγRIIb2 in rat and human LSEC is unique and can be used as a biomarker for these cells (88). In mouse, 72% of the total body pool of FcγRIIb2 is expressed in the liver, and 90% of this is associated with LSECs (36).

**Other endocytic receptors**

The SRs, mannose receptor, and FcγRIIb2 constitute the main waste clearing receptors on LSEC (Table 1). In addition, several other receptors are expressed in these cells that may be involved in
scavenging activity. Recently, Øie et al reported the presence of a functional LRP-1 (low-density lipoprotein receptor-related protein-1) in LSECs, mediating endocytosis of trypsin-activated alpha 2-macroglobulin and receptor associated protein (RAP) in these cells (114). In liver, this receptor is mainly expressed in hepatocytes, and only around 10% of the liver LRP-1 activity is expressed in the LSECs. This finding suggests another role for LSECs in liver lipid homeostasis, besides the structural lipid filtration function of their cell fenestrae.

Other receptors include L-SIGN (liver/lymph node-specific ICAM-3 grabbing non-integrin; DC-SIGNR), LSECtin (liver and lymph node sinusoidal endothelial cell C-type lectin; DC-SIGN), and LYVE-1 (lymphatic vessel endothelial hyaluronan receptor-1) (69, 119, 124). L-SIGN binds virus, including hepatitis C virus (38) and human immunodeficiency virus (119), whereas LSECtin, which is predominantly expressed on LSECs, is potentially involved in the regulation of immune responses in liver through interaction with L-SIGN in response to hepatitis C virus (77). However, the role of these receptors in LSEC endocytosis is unknown.

LYVE-1 is a hyaluronan binding protein, and is expressed in lymphatic vascular endothelium and sinusoidal endothelia of lymph nodes, liver and spleen (5, 103, 124), and in subsets of tissue macrophages co-expressing stabilin-1 (129). Initially, the role assigned for this receptor was hyaluronan clearance from lymph (124), but the receptor is also suggested to be involved in lymphangiogenesis, wound healing and tumor formation (129). Its expression in normal liver is restricted to LSECs (103, 113). Of note, stabilin-2 (previously known as the hyaluronan receptor) is the major endocytic receptor for hyaluronan in LSEC (94, 149) and the relative contribution (if any) of LYVE-1 in this process is unknown.
Endocytosis mechanisms and metabolism

Endocytosis mechanisms

The main type of receptor-mediated endocytosis in LSECs is clathrin-dependent. The cells are well equipped with molecules involved in clathrin-dependent endocytosis, and contain approximately twice as many clathrin-coated pits per membrane unit than hepatocytes and Kupffer cells (62). LSECs also express caveolin (86), and at least one receptor that mediates endocytosis (FcγRIIb2) has been observed in lipid rafts (102), but uptake (“podocytosis”) via caveolae has not been demonstrated, and fluid phase endocytosis is negligible as entrance gate for waste macromolecules (62).

The internalization rate for mannosylated albumin bound to the mannose receptor is only 10-15 sec (84), which is ten-fold faster than internalization via the asialoglycoprotein-receptor in hepatocytes (84). Both stabilins (42), FcγRIIb2 (102) and the mannose receptor (85) are recycled from the early endosomes to the cell surface, so these receptors therefore mediate uptake of ligands at a relatively constant rate over time. Even the ligands may to some extent be recycled. This has been demonstrated particularly well for endocytosis via the FcγRIIb2 (102), but may also occur for mannose receptor ligands (85), due to incomplete dissociation of ligand from the receptor in the early endosome.

Following intravenous injection (into rodents) of ligands that typically distribute to LSEC, such as collagen α-chains, formaldehyde-treated albumin, and AGE-albumin, degradation products usually appear in the circulation already within 10-12 min (25, 87, 146). The transport of ligand along the LSEC endo/lysosomal route proceeds sequentially via Early Endosome Antigen 1-positive endosomes (48), lgp120 (rat lysosomal membrane glycoprotein 120) positive late endosomes (61),
and finally to lysosomes where degradation is completed (48, 61). Degradation of collagen α-chains begins already in the early endosomes (48).

The pathways followed by immune complexes internalized by the FcγRIIb2 in LSECs deviate from those followed by ligands of the mannose receptor and SRs. The internalized immune complexes are largely returned to the cell surface (80, 102) leading to a slow net internalization of cell-surface bound ligand (half time of internalization approximately 15 min as compared to < 1 min by mannose receptors and SRs). A consequence of the extensive recycling of immune complexes is that a relatively small proportion (about 10%) of internalized ligand is directed to later parts of the endocytic pathway. Whereas activated FcγRs are downregulated following ligand binding in other immune cells (8, 163) the FcγRIIb2 is not degraded as a result of endocytosis of immune complexes in LSECs (102). The functional significance of the extensive recycling of immune complexes bound to its receptor in LSECs is not known.

**LSEC metabolism**

The high capacity uptake and degradation activities of the LSECs place these cells as an important metabolic actor in liver. The cells are equipped with very high levels of lysosomal enzymes (25, 65) to maintain the high catabolic activity needed to degrade all the waste macromolecules that constantly enter the blood. Using biosynthetically 14C- or 3H-labeled hyaluronan, or a chondroitin sulfate proteoglycan labeled biosynthetically in the sugar moiety with 14C, 3H or [35S]sulfate, or chemically with 125I in the tyrosine groups of the core protein it was found that the endocytosed macromolecules were degraded intralysosomally in LSECs by acid hydrolases to basic building blocks (amino acids, monosaccharides and sulfate), that were then transported across the lysosomal membrane for further processing of the monosaccharides to lactate and acetate, that were in turn transported out of the cells (30, 141). It is worth noting that lactate is a major product released by
LSECs independently of degradation of endocytosed macromolecules in rat (91) and pig (109, 110). The high production of lactate by LSECs signifies that their metabolism is largely anaerobic, which is supported by the facts that the cells i) carry few mitochondria (7), and ii) operate in the O₂ poor environment of the hepatic sinusoid (91).

**LSEC immune functions**

*Role in innate immunity*

LSECs contain pattern recognition receptors (PRRs), the two most important being the mannose receptor and SRs. These receptors recognize a number of structures carried by microbes and their products, or so called pathogen-associated molecular patterns (PAMPs), as well as potentially harmful endogenous ligands, such as matrix fragments, DNA and other molecules leaking from injured or dying cells (100). Interestingly the PAMPs recognized and scavenged by the LSEC fit the definition of DAMPs (danger associated molecular patterns) (93).

LSECs also express toll-like receptors (TLRs) which are major regulators of innate immune responses against viruses, bacteria, fungi and protozoa (59). So far TLR9 and TLR4 have been identified in LSECs (90, 162), and indirect evidence points to LSEC expression of TLR2 (50). A recent study reported that murine LSECs produced TNF-α when treated with ligands for TLR1-4, -6, -8 and -9, IL-6 by agonists for TLR3 and -4, and interferon-β following treatment with TLR3 ligands (174). Thus future studies are likely to reveal the existence of several types of TLRs in these cells. TLR9 recognizes microbial DNA, in particular immune stimulating unmethylated CpG motifs. Since TLR9 is localized in the endo/lysosomal compartment, CpG must first bind to an endocytosis receptor (probably stabilin 1 and/or 2) expressed on the surface of LSEC, which transports it to the compartment where TLR9 is located (90). Upon binding to its ligand, TLR9 in LSECs initiates cytosolic MyD88-mediated signalling leading to translocation of NfκB, and eventually to production
and release of IL-1 and IL-6. TLR4 binds LPS in various cell types, including the LSEC (162). Although TLR4 is expressed on the cell surface, additional molecules are required for successful binding to LPS; LPS binds to LPS-binding protein (LBP) in blood, and this LPS-LBP complex is subsequently recognized by CD14, a molecule expressed not only in phagocytes, but also in LSECs (131). LPS recognition is followed by increased physical proximity between CD14 and TLR4, suggesting that CD14 and TLR4 may interact in LPS signalling. MD-2 is another protein that has an important role in the LPS response by macrophages, DCs and B cells, and probably also in LSECs (173).

The presence of FcγRIIb2, a type of receptor normally associated with cells of the immune system places LSEC in a unique position among the endothelial cells of the body. In fact, LSECs and placental endothelial cells are the only endothelia that have so far been reported to express this receptor (82, 102).

**Role in uptake of blood borne virus**

Most virus and phage particles represent small particles, or colloids, less than 200 nm in diameter, that are taken up by pinocytosis rather than phagocytosis (128). It is therefore likely that several blood-borne viruses are cleared by the highly endocytically active LSEC. The uptake of virus has traditionally been studied in connection with disease forming virus, and the important research field of how virus infects cells has been unaware of the naturally high scavenger capacity of the LSECs. A recent study showed that 90% of adenovirus 1 injected into mice ended up in the LSEC (37). One may therefore speculate that most viruses that reach the blood are eliminated “silently” by uptake and degradation in the LSEC, without causing any infection.
Role in adaptive immunity

The LSECs has been implicated as an inducer of immune tolerance in the liver. Knolle et al. reported that LSECs express MHC II and the co-stimulatory molecules CD40, CD80 and CD86 (63) and perform antigen processing and presentation with similar efficacy as those of DCs. However, in contrast to conventional antigen-presenting cells, LSECs fail to induce differentiation of naive CD4+ T cells toward a Th1 phenotype. This feature of LSECs, along with the production of negative immune modulatory cytokines by LSEC-primed T cells upon restimulation, is suggested to contribute to the unique hepatic immune tolerance (16). In this model, the LSEC represents the sessile, organ-resident antigen-presenting cell inducing local tolerance in the liver toward soluble antigens such as food antigens or self-proteins. This view of LSECs as functional antigen presenting cells was challenged by Katz et al. (58). They reported that, unlike DCs, LSECs have low or absent expression of MHC II, and CD86, and are poor stimulators of allogeneic T cells, despite high capacity for antigen uptake in vitro and in vivo. They concluded that LSECs alone are insufficient to activate naive T cells. Of note, a study by Onoe et al. (116), in contrast to the results of Katz et al. (58) but in agreement with Knolle et al. (63), concluded that primary LSECs from mice do indeed express MHC II and CD86. The conflicts associated with the expression of MHC II in LSECs are further underlined by earlier studies that failed to demonstrate this molecule on rat or human LSECs (80, 131). Obviously, further studies are needed to elucidate the role of LSECs in adaptive immunity.

Role of the LSEC in age-related conditions

Distinct age-related changes in otherwise healthy livers are described in mouse, rat, baboon (Papio hamadryas), and human (15, 54, 70, 71, 97, 169) and in two premature aging syndromes transgenically-induced in mice (40, 92). These changes include increased endothelial thickness, and loss of cell porosity, changes in endothelial cell markers, and sporadic depositions of basal lamina and collagens in the subendothelial space (the space of Disse). This may have important implications
for lipid transfer between blood and hepatocytes, which normally occurs through the liver sieve (71). The reduced LSEC porosity leads to impaired blood clearance of lipids after meals, because circulating triglyceride rich chylomicron remnants produced from dietary fat are hindered from entering the space of Disse (34, 49). Defenestration may therefore exacerbate postprandial hypertriglyceridaemia, which is a common condition in old age and a major risk factor for atherosclerosis (71). Interestingly, age-related defenestration can be prevented by caloric restriction (55) and the sirtuin-activator, resveratrol (68), and reversed by the serotonergic drug, 2,5-dimethoxy-4-iodoamphetamine (35).

Although a signature function of LSECs are their enormous endocytic capacity, only a few reports have addressed this issue in relation to aging. These studies have all used modified (heat-aggregated or chemically treated) albumin as model substances. Typically such modifications lead to neutralization of positively charged amino-groups, increasing the net negative charge of the protein and thus their affinity for SRs (22, 166). Results are not fully consistent (12), but most point to significantly reduced LSEC endocytic capacity at old age (14, 47, 54, 138).

In addition to the negative effect of hepatic defenestration on blood lipid contents, a decreased LSEC endocytic capacity in old individuals will further increase the risk for deposition of potentially dangerous waste macromolecules in extrahepatic tissues which may lead to systemic complications. The risk may be especially important in situations with a sudden increase in circulatory waste loads, such as in massive trauma or disseminated intravascular coagulation, as well as in long-term conditions such as obesity and diabetes where there is an increased burden of modified proteins and lipoproteins that are normally efficiently removed by LSEC.
Invertebrate analogues to the vertebrate SEC

True endothelial cells are only found in vertebrates (13), but insect haemocytes and nephrocytes have similar scavenger functions to vertebrate macrophages and SECs, sharing the task of waste clearance and defence against foreign intruders (20, 136). Haemocytes represent the primary phagocyte, and are located in the circulating haemolymph (74). Nephrocytes (synonym “athrocytes”; (18)) on the other hand, are highly pinocytic cells tethered to the oesophagus (“garland cells”) or to the heart (“pericardial nephrocytes”) (Fig.4), and are bathed in haemolymph (18, 170).

The scavenger function of insect nephrocytes has been explored for more than 100 years (18). Soluble vital dyes such as ammonia carmine and trypan blue were found to be rapidly and preferentially taken up by insect pericardial and garland nephrocytes (118, 121). Morphologically, these nephrocytes are podocytes, with a filtration slit diaphragm, and deeply infolded plasma membranes forming labyrinthic channels lined with numerous coated pits and vesicles (19, 170) (Fig.4). However, instead of being passed to a nephric tubule, filtered material is endocytosed from the side of these channels (20). Like the vertebrate SEC, the insect nephrocyte is not phagocytic (18, 20).

Clathrin-mediated endocytosis mechanisms have been extensively studied in the fruit fly Drosophila melanogaster. However, little is known about endocytosis receptors on nephrocytes. Recently Soukup et al. (156) reported the expression of the Lipophorin receptor-1 receptor, a member of the low density lipoprotein family, in Drosophila garland and pericardial nephrocytes. They also demonstrated for the first time that the serpin-family inhibitor, Necrotic, which controls a proteolytic cascade which activates the innate immune response to fungal and gram⁺-bacterial infections, was cleared from haemolymph via this receptor, and then delivered to lysosomes for degradation,
suggesting an important regulatory role of the nephrocytes in humoral immune responses in *Drosophila*.

In addition, nephrocytes avidly sequester soluble tracer molecules such as horseradish peroxidase, ferritin, juvenile hormone esterase, maleylated bovine serum albumin (an SR ligand), and acid dyes (9, 18-20), suggesting the expression of one or more SRs. We found that larval *Calliphora* (blowfly) nephrocytes endocytose and rapidly degrade formaldehyde-treated albumin. They also endocytose hyaluronan, and AGE-albumin, but do not sequester mannan, suggesting that they do not express a mannose receptor (Smedsrød group; unpublished observations). Fig. 4 shows the uptake of trypan blue and formaldehyde-treated albumin in *Calliphora* larval nephrocytes, and the well-developed endo/lysosomal network of these cells. The lack of a distinct mannose receptor (which is found in vertebrates) may reflect the fact that invertebrate glycoproteins often contain high-mannose groups (105).

Nephrocytes that strongly resemble insect nephrocytes are found in ancestors of insects such as Onychophorans (132), myriapods (127), and spiders (176). Decapod branchial nephrocytes, which are located at the stem of the gill, also show functional equivalence to the nephrocytes of insects, and are implicated in the removal from the haemolymph of macromolecules produced during tissue maintenance, growth and molting (158), as well as vital dyes (112), and virus particles (95).

Pinocytic nephrocytes are also present in bivalve molluscs (101), and snails (152).

Based on their pinocytic scavenging function, we argue that invertebrate nephrocytes are functionally similar to the vertebrate SEC. Interestingly, nephrocytes in insects have been suggested to be evolutionarily related to both vertebrate glomerular podocytes (170), and endothelial cells (20). Nephrocyte pinocytic systems incorporate ultrafiltration filter-slits which resemble those of
vertebrate glomerulus (19). Orthologues of the major constituents of the vertebrate kidney podocyte slit diaphragm, including nephrin and podocin, are expressed in the *Drosophila* nephrocyte and form a complex of interacting proteins that closely mirror the vertebrate slit diaphragm complex, suggesting that the two cell types are evolutionarily related (170). Thus some functional elements of invertebrate nephrocytes evidently evolved into components of the vertebrate kidney glomerulus, whilst others appear in SECs. Arguments have also been put forward for Rudhira as a molecular link between pericardial nephrocytes in *Drosophila* and vertebrate SECs (20). Rudhira is a cytoplasmic WD40 domain protein suggested to regulate colloid uptake in *Drosophila* pericardial nephrocytes (20), where it is expressed in high levels (while not found in other tissues) (21), while its murine ortholog is expressed in endothelial precursors during vasculogenesis and angiogenesis (139). The scavenger systems of invertebrates may thus represent waypoints in the molecular evolution of scavenger processes in vertebrates.

**Conclusions: Proposal of a common principle of cellular waste handling**

In his account on the waste clearing cells of animals Aschoff launched the name RES to denote the cells that were most actively taking part in the uptake of intravenously administered vital stains (3). He observed that macrophages and sinusoidal cells of liver and certain other organs took up massive amounts of vital stains. Over the years that followed after Aschoff’s work, it became common to think that the RES consisted of macrophages only. This was partly due to the misconception that the cells of the RES were professional phagocytes only and used phagocytosis to clear vital stains from the circulation. In line with this view van Furth proposed in 1972 that the term RES be replaced with the mononuclear phagocyte system (MPS) (167).

A major aim of this review is to present evidence that the old and ill-defined concept of RES includes more than the MPS. Using modern technology and contemporary knowledge based on a
reflective understanding of historical findings Kawai et al. (1998) showed that the major site of uptake of vital stain in liver was LSECs rather than macrophages (60). What seems clear today is that the major scavenger cell systems of vertebrates and invertebrates are based on a dual cell principle of waste clearance (Fig. 5). In vertebrates the SEC represents the professional pinocyte, clearing the blood of a wide range of soluble macromolecules, and small particles (< 200 nm) by clathrin-mediated endocytosis, whilst the macrophage represents the professional phagocyte, eliminating larger particles (136). A corresponding dual scavenger cell system is present in invertebrates, with the nephrocyte and haemocyte being geared to clathrin-mediated endocytosis and phagocytosis, respectively.

While the cells of the MPS have been studied in great detail since Metchnikoff’s discovery of the macrophage about 130 years ago (99), our knowledge of the SEC is the result of investigations over the last 40 years. The shift of paradigm from RES ≡ MPS to RES ≡ MPS + SEC is a major achievement, allowing novel understanding of how the blood is cleared of own and foreign waste material. We now know that the cellular arm of the innate immune system consists of both the MPS and the SEC. In spite of this fact, text books in pathology and immunology still teach students that RES ≡ MPS, thus giving the false impression that the macrophage alone is responsible for the blood clearance of both soluble macromolecules and most types of particles. To understand how viruses and an array of pathogens and biopharmaceuticals are eliminated from the blood, we need a better understanding about the blood clearance function of SEC.

SECs of vertebrates share some striking common traits: they i) are extremely active in clathrin-mediated endocytosis; ii) are rarely, if ever, observed to perform phagocytosis (uptake of particles > 0.5 µm (42)) under normal conditions; iii) have a well-developed intracellular apparatus to process internalized substances; iv) have super high specific activity of lysosomal enzymes; v) carry out an
overall anaerobic metabolism as reflected by the fact that the major end products of degraded ligands are lactate and acetate (135, 141); vi) are located at sites where epithelial cells display a very high metabolic activity (136); vii) express SRs and mannose receptors (136).

It is worthy of note that the invertebrate nephrocyte shares several of these traits with the vertebrate SEC: i) extremely active clathrin-mediated endocytosis; ii) inability to perform phagocytosis; iii) a well-developed intracellular apparatus to process internalized substances; vi) selective uptake of ligands that in vertebrate SECs are endocytosed via the SR.

6. Perspectives and significance

The dual cell principle of waste clearance in the animal kingdom raises some obvious questions and issues:

i) Any trait that has been conserved up through evolution must be regarded as successful and important. Why has it been important to maintain a dual cell principle of waste clearance?

ii) What is the ontogenetic origin of the vertebrate SEC? It has been suggested that the LSEC in mammals is of bone marrow origin (44).

iii) What is the “phylogenetic reason” for “moving” the population of SECs from the gill vessels in the three phylogenetically oldest vertebrate classes, via heart or kidney in the bony fishes, and finally to the liver of the four land-based vertebrate classes?

iv) Why do SECs in general reside in an oxygen poor environment? Is oxygen incompatible with such waste recycling?

v) What is the exact physical nature of the ligand binding properties of SEC scavenger receptors? These need to be studied in more detail to determine to what extent hydrophobicity and hydrophilicity along with electric charge (168) determine the ability of these receptors to recognize material that should be removed from the blood to maintain homeostasis.
vi) Clearly, a number of the substances that are categorized by immunologists as carrying “danger signals” (133) are recognized and cleared by the “new” scavenger cells SECs and nephrocytes. Why not therefore view the vertebrate SEC and invertebrate analogue nephrocyte as an integral component of the innate immune systems. Studying these fundamental questions will open new avenues to a more complete understanding of physiology, immunology and medicine.

**Acknowledgments**

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Figure legends

**Figure 1: Morphology of scavenger endothelial cells in mammalian liver**

A) Scanning electron micrograph (EM) of a mouse liver sinusoid. The liver sinusoidal endothelial cells (LSECs), which are specialized scavenger endothelial cells, are highly fenestrated. Arrows point to LSEC fenestrae arranged in sieve plates. SD, space of Disse (the subendothelial space); HC, hepatocyte.

B) Transmission EM of rat liver sinusoid. Arrows point to clathrin coated pits, and arrow heads to fenestrae in LSECs. SD, space of Disse; HC, hepatocyte.

C) Scanning EM of rat LSEC culture on day 0. The cells are all highly fenestrated.

D) High magnification of insert in C) showing details of cell fenestration (arrows).

**Figure 2: Endocytosis in liver sinusoidal endothelial cells (LSECs)**

A) Fluorescence micrographs of a rat liver section following double intravenous injection of 0.5 mg TRITC-labelled formaldehyde-treated albumin (red), a substance endocytosed by LSECs (6) and 5x10^8 FITC-labelled latex beads (2 µm diameter; green), which are taken up in Kupffer cells (KCs). (Reproduced from (26) with permission from the American Journal of Physiology).

B) Endocytosis of denatured collagen in mouse LSECs. Primary LSEC cultures (day 0) were incubated at 37°C for 2h with 10µg/ml DTAF-labelled denatured collagen, a ligand for the LSEC mannose receptor (87). Ligand uptake is seen in green discrete vesicles throughout the cell cytoplasm. TRITC, tetramethyl rhodamine isothiocyanate; FITC, fluorescein isothiocyanate; DTAF, 5-isomer of fluorescein dichlorotriazine.

**Figure 3: Scavenger function and tissue location of SECs in a lower vertebrate**

LSECs in fish and other lower vertebrates do not have a scavenger function, but depend instead
depend on SECs in kidney, heart or even gills for removal of blood borne colloids and waste macromolecules (136). Cod SECs are shown as an example below:

A) Scanning EM of the endocardium of cod heart atrium. The muscular trabeculae are lined with high endothelial cells (arrows), which are interconnected at the base of the cells. Cod SECs exhibit high endothelial morphology, similar to SECs in gills of hagfish.

B) Fluorescence micrograph of a section of cod atrium 1h after intravenous injection of FITC-hyaluronan, a ligand for stabilin-2 in mammals (94, 120). The fluorescence can be seen in discrete vesicles throughout the cytoplasm of the endocardial endothelial cells (arrows). e, Endothelium; m, myocardium; hl, heart lumen. (Reproduced from (153) with permission from Springer Verlag).

C) Uptake of FITC-LPS in cod endocardial SEC culture. The cells were incubated with FITC-LPS for 1 h at 12°C. The fluorescence is confined to endocytic vesicles. (Reproduced from (134) with permission from the Journal of Experimental Biology). Abbreviations: FITC, fluorescein isothiocyanate; LPS, lipopolysaccharide from Vibrio salmonicida.

D) Stabilin-2 expression in cod endocardial SEC. Western blot showing that purified lysates from cod atrial endocardial endothelial cells (24000 cells), pig liver sinusoidal endothelial cells (LSECs)(20000 cells), and rat LSECs (5000 cells) all contain stabilin-2 epitopes that react with an antibody to whole rat stabilin-2 (94, 120).

Figure 4: Endocytosis in insect nephrocytes (3rd instar Calliphora larva)

A) Calliphora larva dissected 24h after haemocoel injection with 0.4% Trypan Blue in physiological saline for insects (17, 19). Only the pericardial nephrocytes (n) and peri-oesophageal nephrocytes (garland cells; g) sequester this blue vital dye. The unstained heart (ht) with blue stained lateral nephrocytes (n) run horizontally parallel to a tracheal trunk (tr), and the garland cells (g) surround the oesophagus (lower right). Malphigian tubules (m, chalky white or yellow), and fat body cells (f) remain unstained.
B) Scanning electron micrograph (EM) of heart and nephrocytes in the posterior abdominal region of the larva. The heart (ht) and giant pericardial nephrocytes (N) are suspended from the abdominal wall by elastic connective tissue strands and are stretched by alary muscles (al). Smaller nephrocytes (n) occupy the pericardial space and extend anteriorly along the heart. All nephrocytes are bathed in haemolymph and are strategically situated in areas of maximal blood flow.

C) Low magnification transmission EM showing nephrocyte (n) lying alongside the contracted heart muscle (ht). A thin basal membrane (bm) encloses the nephrocytes, whilst a much thicker basal membrane encloses the muscular tissue of the heart. The cortical region (Cr) of the nephrocytes contains numerous labyrinthine channels (highlighted (*) in D).

D) Transmission EM of the cortical region of a nephrocyte. Note the podocyte configuration, with deeply infolded plasma membranes forming channels studded with numerous coated pits (arrows) and coated vesicles. These labyrinthine channels (*) are separated from haemolymph by a thin basal membrane (bm) and filter slits (arrow heads), which together serve as a haemolymph ultrafilter.

E) Immune transmission EM of the cortical region of a nephrocyte, showing FITC-labelled formaldehyde-treated albumin uptake in endosomes 1 h after injection into the haemocoel. The localisation of FITC-formaldehyde-treated albumin is visualized by 10 nm gold particles (black dots; arrows). The endocytosed ligand was detected with a mouse anti-FITC-antibody, rabbit anti-mouse antibody, and protein A-conjugated 10nm gold particles. (*), labyrinthine channels; FITC, fluorescein isothiocyanate.

**Figure 5: Representation of the dual cell principle of waste clearance**

*Panel a:* Large particles (> 0.5 µm) are taken up by phagocytosis (23), whereas small particles (colloids) and soluble waste* are taken up by pinocytosis.

*Panel b:* The major scavenger cells that are responsible for the clearance of blood borne waste are of two very different types: professional phagocytes (the macrophage in vertebrates and the haemocyte
in invertebrates) and professional pinocytes (the scavenger endothelial cell (SEC) in vertebrates and the nephrocyte in invertebrates).

*The term waste is defined here as material whose presence in the blood is incompatible with homeostasis.
Ligands cleared from the blood circulation mainly by the mammalian liver sinusoidal endothelial cells (LSECs) and their corresponding endocytosis receptors.

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<tr>
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<th>Reference</th>
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SR: Scavenger receptor; type of SR not yet established.

AGE, advanced glycation end-product; LDL, low density lipoprotein.

Stabilin-1 synonym: FEEL-1 (fasciclin endothelial growth factor (EGF)-like, laminin-type EGF-like and link domain-containing scavenger receptor 1) (1).


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End notes

1 The term “waste” is defined here as material whose presence in the blood is incompatible with homeostasis.
The dual cell principle of waste clearance in the animal kingdom

Cellular uptake of insoluble and soluble macromolecules is divided into:

- **Phagocytosis** + **Pinocytosis**

and

Clearance of circulating waste is performed by two very different cell types:

- Professional phagocytes
  - Vertebrates: *The macrophage*  
  - Invertebrates: *The haemocyte*
- Professional pinocytes
  - Vertebrates: *The scavenger endothelial cell*  
  - Invertebrates: *The nephrocyte*
Table 1:

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