Stabilin-1 mediates phosphatidylserine-dependent clearance of cell corpses in alternatively activated macrophages

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Summary

Stabilin-1 is specifically expressed in alternatively activated macrophages. These macrophages participate in anti-inflammatory and healing processes, and display a high phagocytic capacity. In this study, we provide evidence that stabilin-1 is a membrane receptor that performs a crucial function in the clearance of cell corpses. Stabilin-1 is expressed on the cell surface of alternatively activated macrophages and is recruited to the sites of recognition and engulfment of apoptotic bodies, as well as to early phagosomes. Blocking stabilin-1 in macrophages results in defective engulfment of aged red blood cells. Ectopic expression of stabilin-1 induces the binding and engulfment of aged cells in mouse fibroblast L cells. The binding and phagocytosis are dependent on phosphatidylserine (PS), which is well known as an engulfing ligand. Furthermore, using PS-coated beads, we demonstrate that PS directly interacts with stabilin-1 and is sufficient for stabilin-1-mediated phagocytosis. EGF-like domain repeat in stabilin-1 is responsible for PS recognition and binding. Thus, our results demonstrate that stabilin-1, found on alternatively activated macrophages, is a phagocytic receptor mediating the clearance of apoptotic cells in a PS-dependent manner. Therefore, this protein might play an important role in the maintenance of tissue homeostasis and prevention of autoimmunity.

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Key words: Stabilin-1, Phagocytosis, Aged RBC, Apoptotic cells, Macrophages

Introduction

Macrophages are responsible for the maintenance of tissue homeostasis and for orchestrating defence responses and healing processes in adult tissues (Hume, 2006). One of the major functions of macrophages is the clearance of exogenous non-self as well as unwanted-self components present in the extracellular environment. The selectivity of ligand recognition is a result of a highly specific pattern of scavenger receptor expression by resident tissue macrophages and by newly recruited monocytes undergoing differentiation to macrophages (Gordon, 2002). A distinction between apoptotic cells (unwanted-self) and microbial pathogens (non-self) must be made because the clearance of apoptotic cells by phagocytes occurs in the absence of inflammatory responses (Savill et al., 2002). Various subpopulations of alternatively activated (M2) macrophages express molecular repertoires leading to tolerance and the resolution of inflammation, and display a high phagocytic capacity (Gratchev et al., 2005). Using our in vitro model for alternatively activated macrophages we demonstrated that combination of interleukin-4 (IL-4) and dexamethasone stimulates the phagocytic capacity of macrophages and induces expression of scavenger receptors including CD163, MARCO, and stabilin-1 (Gratchev et al., 2005; Kzhyshkowska et al., 2006c; Politz et al., 2002). In vivo stabilin-1 is expressed by cells specialized in the clearance of unwanted-self and in the maintenance of tissue homeostasis, including different subsets of tissue macrophages as well as non-continuous endothelial cells in lymph nodes, liver, spleen and bone marrow (Goerd et al., 1993; Goerd and Orfanos, 1999; Goerd et al., 1999; Martens et al., 2006; Politz et al., 2002).

Stabilin-1 (also called FEEL-1 and CLEVER-1) and stabilin-2 (also called FEEL-2 and HARE) are type I transmembrane receptors with multiple functions (Kzhyshkowska et al., 2006a). They were initially identified as high molecular weight proteins produced by sinusoidal endothelial cells and have been reported to function as scavenger receptors. These proteins affect the endocytosis of acetylated low-density lipoprotein (acLDL) and advanced glycation end products (AGE) and have binding activities with Gram-negative and Gram-positive bacteria (Adachi and Tsujimoto, 2002; Kzhyshkowska et al., 2005; Tamura et al., 2003). They have also been reported to contribute to the endothelial adhesion of lymphocytes (Irjala et al., 2003; Jung et al., 2007; Salmi et al., 2004). Although stabilin-1 shares common features with stabilin-2, its function appears to be significantly different from that of stabilin-2. For example, while stabilin-2 efficiently mediates the uptake of hyaluronic acid (HA) and AGE-modified proteins, stabilin-1 does not bind HA and binds AGE-modified proteins with a lower affinity. A recent study reported that stabilin-1 mediates the internalization...
and clearance of SPARC (secreted protein acidic and rich in cysteine), a multifunctional regulator of tissue remodeling (Kzhyshkowska et al., 2006c). These data lead to the proposal that stabilin-1 in alternatively activated macrophages can actively regulate unwanted self content in the extracellular matrices and spaces. In addition to functioning as a scavenger receptor, stabilin-1 in macrophages is involved in intracellular sorting and lysosomal delivery of the novel stabilin-1-interacting chitinase-like protein (SI-CLP) (Kzhyshkowska et al., 2006b). Furthermore, in alternatively activated macrophages stabilin-1 internalizes hormone placental lactogen and targets part of it for degradation in lysosomes and part to a novel trans-Golgi-network-mediated transcytosis (Kzhyshkowska et al., 2008).

The efficient removal of cell corpses is crucial to the protection of normal healthy cells from the harmful contents and debris of dying cells, thereby reducing the amount of tissue damage that occurs as a result of inappropriate inflammation or autoimmune responses (Ravichandran and Lorenz, 2007; Savill and Fadok, 2000). Phagocytes mediate the clearance of apoptotic cells through the recognition of phosphatidylserine (PS) on the cell surface (Erwig and Henson, 2008; Wu et al., 2006). Recently, Tim4 and BA11 were suggested as novel members of a PS-receptor (Miyanishi et al., 2007; Park et al., 2007). More recently, we demonstrated that stabilin-2 is a PS-receptor that mediates the clearance of cell corpses (Park et al., 2008a). Although endocytic ligands of stabilin-1 are different from those of stabilin-2, stabilin-1 is specifically expressed in alternatively activated macrophages that actively participate in anti-inflammatory and healing processes. These macrophages also display a high phagocytic capacity, suggesting that stabilin-1 could also participate in the clearance of apoptotic cells. In this study, we present evidence that stabilin-1 can function as a receptor involved in the clearance of cell corpses via PS recognition.

Results

Stabilin-1 colocalizes with apoptotic cells in phagosomes

Expression of stabilin-1 by macrophages requires stimulation with IL-4 and dexamethasone. This combination of factors results in the generation of alternatively activated macrophages in vitro. We attempted to determine whether stimulation with IL-4 and dexamethasone can also increase the surface expression of stabilin-1 in human monococyte-derived macrophages (HMDMs) using flow cytometry with an anti-stabilin-1 antibody (N-13). The levels of stabilin-1 on the surface of HMDMs are significantly increased in macrophages stimulated with IL-4 and dexamethasone (IL-4/Dex) (supplementary material Fig. S1). Thus the data obtained using a goat N-13 antibody correspond to the data obtained earlier using the MS-1 antibody (Kzhyshkowska et al., 2004). Therefore, we confirmed that stabilin-1 is exposed on the surface of alternatively activated macrophages. We next investigated the localization of stabilin-1 in macrophages during the engulfment of apoptotic cells. IL-4/Dex-stimulated human macrophages derived from the peripheral blood mononuclear cells (PBMCs) of six healthy donors were used for this assay. Apoptotic Jurkat cells were used as target cells. Apoptotic cells were efficiently internalized by stabilin-1-positive macrophages. Stabilin-1 shows typical partial localization in early endosome antigen 1 (EEA1)-positive endosomes prior to addition of apoptotic cells (Fig. 1A). After 3 hours of continuous phagocytosis, stabilin-1 was accumulated in the early phagosomes, which were positive for EEA1 (Fig. 1, B-D). In order to examine the involvement of stabilin-1 on the early stage of recognition and engulfment of apoptotic bodies, we used apoptotic Jurkat cells labeled with the red dye DiD. Confocal microscopy revealed that stabilin-1 is intensively relocated to the sites of uptake of apoptotic bodies at the early (1 hour) time point of phagocytosis (Fig. 2; supplementary material Fig. S2). Z-stack images confirmed that stabilin-1 is localized around the internalized apoptotic Jurkat cells after 3 hours of phagocytosis (supplementary material Fig. S3). These data suggest that stabilin-1 might be involved in the clearance of apoptotic cells, specifically during the stage of apoptotic body-recognition and during the formation of early phagosomes.

Stabilin-1 is involved in the clearance of aged red blood cells in macrophages

To investigate the involvement of stabilin-1 in the clearance of cell corpses by macrophages, we attempted to determine whether an anti-stabilin-1 antibody that recognizes stabilin-1 on the cell surface inhibits the engulfment of aged red blood cells (RBCs). We selected aged RBCs as a prey because they do not bind to phagocytes without

![Fig. 1. Concentration of stabilin-1 in phagosomes in human macrophages stimulated with IL-4 in combination with dexamethasone. (A) When no apoptotic cells were added, stabilin-1 was partially localized in the EEA1-positive endosomes (Kzhyshkowska et al., 2004). (B-D) Incubation with apoptotic Jurkat cells for 3 hours induced the accumulation of stabilin-1 in early phagosomes, which are positive for EEA1. Stabilin-1 is shown in red, and the early/sorting endosomal marker EEA1 is shown in green. Yellow indicates colocalization of EEA1 and stabilin-1 (merge of green and red). White arrows indicate colocalization of stabilin-1 and EEA1 (merge of green and red) in phagosomes. Scale bars: 11.35 μm (A), 6.71 μm (B), 9.21 μm (C), and 9.91 μm (D).](317x374 to 562x719)
Stabilin-1 as a phagocytic receptor

apoptotic signals, and they allow for the distinction between binding and engulfment via hypotonic lysis (Gigli and Nelson, 1968; Park et al., 2008a). Confocal microscopy analysis revealed that stabilin-1 is intensively relocalized, presumably to the sites of aged RBC recognition, after 1 hour of aged RBC phagocytosis, and is redistributed to the periphery of phagosomes after 3 hours of aged RBC phagocytosis (supplementary material Fig. S4). Active recruitment of stabilin-1 to the sites of recognition and phagosomes during phagocytosis of both apoptotic Jurkat cells and aged RBC was specific, because stabilin-1 was not involved in the phagocytosis of fluorescently labeled microspheres (supplementary material Fig. S5). Pretreatment with an anti-stabilin-1 antibody (N-13) significantly reduced the percentage of HMDMs engulfing aged RBCs (from 32.1±7.1 to 20.1±2.5), whereas an isotype-matched antibody had no effect (from 32.1±7.1 to 32.5±4.4) (Fig. 3A,B). In unstimulated HMDMs, treatment with anti-stabilin-1 antibody resulted in minimal inhibition of aged RBC engulfment (supplementary material Fig. S6).

In an effort to further confirm the involvement of stabilin-1 in removal of cell corpses, we designed three short hairpin RNAs (shRNA) that targeted stabilin-1 and cloned them into the pSuper vector. The suppression of stabilin-1 expression was evaluated in CHO-K1 cells by co-transfecting the shRNA-expressing vector along with a stabilin-1-expressing vector. Expression of stabilin-1 was strongly suppressed by pSuper/shRNA-1-2, and to a lesser extent by pSuper/shRNA-1-3, but only marginally by shRNA-1 (Fig. 4A). In order to effectively suppress the expression of stabilin-1 in macrophages, lentiviruses encoding for stabilin-1 shRNA or its scrambled shRNA, along with green fluorescent protein were prepared (Fig. 4B). Flow cytometry analysis confirmed that stabilin-1 shRNA and control shRNA have similar transduction efficiencies (Fig. 4C, right panel). Stabilin-1 shRNA, but not control shRNA, significantly inhibited the expression of the stabilin-1 protein in HMDMs (Fig. 4C, left panel). In addition, analysis of immunofluorescence staining also revealed that stabilin-1 shRNA effectively downregulates the expression of stabilin-1 in macrophages (Fig. 4D). The knockdown of stabilin-1 in macrophages caused a decrease in the uptake of aged RBCs (38.7±11.8) as compared to irrelevant knockdown (57.5±7.7) (Fig. 4E). Collectively, our results demonstrate that stabilin-1 plays a role in the clearance of aged cells in stabilin-1-expressing macrophages.

Stabilin-1 mediated the clearance of aged RBCs in a PS-specific manner

To further examine whether stabilin-1 is involved in the phagocytosis of aged cells, we generated three cell lines that constitutively express stabilin-1 (Fig. 5A) and examined their phagocytic activity. As expected, stabilin-1-expressing L cells (L/Stab-1) were shown to bind and engulf aged RBCs to a significant degree, whereas L cells transfected with the empty vector (L/Mock) showed little binding to and absolutely no phagocytosis of aged RBCs (Fig. 5B,C). Phagocytosis assays were conducted, and the percentages of macrophages that harbored aged RBCs were determined. The results are expressed as the means ± s.d. from three independent experiments. ANOVA: *P<0.05.
cells in the presence of PS liposomes, or phosphatidylcholine (PC) liposomes (negative control). The PS liposomes inhibition the engulfment of aged RBCs by L/Stab-1 cells in a dose-dependent manner, whereas the PC liposomes exerted no such effects (Fig. 6A). The inhibition of aged RBC engulfment was restricted to the PS liposome-treatments; other anionic and neutral phospholipids exerted no detectable effects (Fig. 6B).

PS directly interacts with stabilin-1 and is sufficient for stabilin-1-mediated phagocytosis
Stabilin-1 is a type I membrane protein consisting of a signal sequence and extracellular, transmembrane and cytoplasmic regions (Fig. 7A) (reviewed by Kzhyshkowska et al., 2006a). To examine whether stabilin-1 directly interacts with PS, we expressed the recombinant protein corresponding to the extracellular region of stabilin-1 as an Fc-fusion protein (Stab1-Fc) (Fig. 7A). We also generated PS-coated beads to mimic apoptotic cells and rule out any possible binding via other molecules on the apoptotic cell surface. Coating with PS on the bead surface was quantified by the binding of FITC-labeled annexin V. PS-coated beads acquired the ability to bind annexin V (Fig. 7B). When beads coated with phospholipids were incubated with Stab1-Fc, PS-coated beads, but not PC-coated beads, bound significantly to Stab1-Fc proteins (Fig. 7C). These results suggest that stabilin-1 can directly recognize PS exposed on apoptotic cells as an engulfment signal.

We then conducted cell-based phagocytosis assays using beads coated with phospholipids (PC or PS) in order to directly assess the role of stabilin-1 as a PS-receptor involved in the binding and engulfment of apoptotic cells. L cells stably expressing stabilin-1 (L/Stab-1) preferentially bound to PS-coated beads relative to PC-coated beads, whereas L cells transfected with the empty vector (L/Mock) showed only basal levels of binding to either PC- or PS-coated beads (Fig. 7D). PS-coated beads, but not PC-coated beads, were effectively engulfed by L/Stab-1 cells (Fig. 7E). None of the beads were engulfed by L/Mock cells. These results indicate that PS alone is sufficient to mediate the tethering or engulfment of apoptotic cells by stabilin-1, suggesting that stabilin-1 is a candidate PS-receptor for cell-corpse clearance.

EGF-like domain repeat is involved in PS recognition and binding
In order to further evaluate the PS-binding domain within the extracellular region of stabilin-1, we next generated recombinant proteins of the sixth FAS1 domain (FAS1-6) and the third EGF-like domain repeat (EGFrp-3) and then examined their effects on the activity of binding to apoptotic Jurkat cells. EGFrp-3 protein bound to apoptotic cells in a dose-dependent manner, whereas FAS1-6 protein did not (Fig. 8A). We also examined whether EGFrp-3 protein directly binds to PS using PC- and PS-coated beads. In agreement with the results using apoptotic cells, EGFrp-3 protein significantly bound to PS-coated beads but not PC-coated beads. These results indicate that EGFrp in stabilin-1 is responsible for PS recognition and binding.
Discussion

Stabilin-1 as a multifunctional receptor that mediates endocytosis of extracellular soluble factors such as acLDL and SPARC, as well as intracellular sorting of cargo proteins such as lysosomal protein SI-CLP (Kzhyshkowska et al., 2006b; Kzhyshkowska et al., 2006c; Tamura et al., 2003). However, the characterization of stabilin-1 as a membrane receptor has been controversial for some time because previous histological data has indicated its predominant localization in intracellular vesicles in both macrophages and sinusoidal endothelial cells (Kzhyshkowska et al., 2004). The expression of stabilin-1 can be induced by treatment of peripheral blood-derived monocytes with a combination of IL-4 and dexamethasone. In this study, we have confirmed that surface expression of stabilin-1 was also upregulated by combination of IL-4 and dexamethasone. This type of monocyte stimulation represents a well-established, in vitro model of alternative macrophage activation (M2) (Goerdt et al., 1993; Gordon, 2003; Kodelja et al., 1997; Politz et al., 2002). Alternatively activated macrophages actively participate in anti-inflammatory and healing processes and display high phagocytic activity (Gratchev et al., 2005). A recent study showed that IL-10-producing macrophages (M2) preferentially clear early apoptotic cells (Xu et al., 2006). In addition, we have demonstrated that stabilin-2, which is highly homologous to stabilin-1, is a PS receptor that mediates the engulfment of apoptotic cells (Park et al., 2008a). On the basis of these findings, we hypothesized that stabilin-1 in macrophages could also be involved in the clearance of apoptotic and aged cells.

Four lines of evidence bolster the claim that stabilin-1 mediates the clearance of apoptotic cells in alternatively activated macrophages. First, stabilin-1 is recruited to the sites of recognition and engulfment of apoptotic bodies and colocalizes with phagocytosed apoptotic bodies in early phagosomes. Second, the blockade of stabilin-1 with shRNA or blocking antibody resulted in reduced uptake of aged RBCs in HMDMs. Third, stabilin-1-expressing cells were shown to mediate the binding and engulfment of aged RBCs in a PS-specific manner, and this engulfment activity was inhibited significantly by stabilin-1-specific shRNA. Fourth, a substantial portion of the PS-coated beads, but not the PC-coated...
beads, were bound and engulfed by stabilin-1-expressing cells. EGFrp in stabilin-1 directly binds to PS-coated beads, thereby suggesting that stabilin-1 might function as a receptor that potentially binds directly to PS via its EGFrrps. Taken together, our results indicate that stabilin-1 performs an important function as a membrane receptor for clearance of cell corpses in macrophages.

Stabilin-1 is involved in two traffic pathways in human macrophages. The first is early endocytosis and recycling, and the second is trafficking between the endosomal system and the biosynthetic Golgi compartment (Kzhyshkowska et al., 2006a). In addition to acting as a scavenger receptor for acLDL and SPARC, we demonstrated that stabilin-1 mediates the clearance of apoptotic and aged cells and is recruited to the sites of apoptotic cell recognition and engulfment in macrophages. The engulfment of apoptotic cells by phagocytes leads to actin polymerization and to the extension of membrane protrusions to form a closed phagosome. Membrane delivery is considered to play an important role in pseudopodia extension during phagocytosis (Huynh et al., 2007). Considering the fact that stabilin-1 is a rapidly recycled receptor localized in intracellular vesicle, membrane targeting of stabilin-1-containing vesicles might act as a source for ‘membrane delivery’. Stabilin-1 is also involved in intracellular sorting and in secretion of a novel chitinase-like protein, SI-CLP, and hormone placental lactogen, indicating that stabilin-1 is involved in the delivery of both newly synthesized and endocytosed proteins to distinct secretory pathways (Kzhyshkowska et al., 2008; Kzhyshkowska et al., 2006b).
al., 2006b). There is growing evidence that active immunosuppressive and anti-inflammatory responses can be induced by phagocytes that bind and ingest apoptotic cells. Therefore, it is possible that the clearance of apoptotic cells by stabulin-1 might change the secretory repertoire of a macrophage via the induction or inhibition of its function as a sorting receptor. Stabulin-1 and stabulin-2 are multifunctional receptors with a similar domain structure. Nevertheless, the function of stabulin-1 appears to be significantly different from stabulin-2, and the only ligand shared by stabulin-1 and stabulin-2 is acLDL. We previously showed that stabulin-2-mediated phagocytosis is partially inhibited by acLDL (data not shown), suggesting that an apoptotic cell is another common ligand of stabulin-1 and stabulin-2. Although stabulin-1 and stabulin-2 share similar expression in sinusoidal endothelial cells of spleen, liver and lymph node, and in our alternatively activated HMDM model, they show a different expression pattern in macrophages. Firstly, stabulin-1-positive macrophages are found in many tissues including placenta, skin, colon and lymph node (Kzhyshtokska et al., 2006a; Martens et al., 2006), whereas the expression of stabulin-2 is restricted to a few macrophages including alveolar macrophages (data not shown) and HMDMs (Park et al., 2008a). Secondly, while stabulin-2 is abundant on the cell surface in HMDMs regardless of activation conditions, surface expression of stabulin-1 in macrophages requires the stimulation by IL-4 and dexamethasone, suggesting that stabulin-1 acts as a receptor for apoptotic-cell clearance in alternatively activated macrophages. Furthermore, we found that expression of stabulin-1 and stabulin-2 in macrophages is regulated by different transcriptional factors (unpublished data). Thus, it is possible that these receptors are acting in different macrophage populations in different physiological or pathological conditions.

Our results showed that the extracellular region of stabulin-1 directly interacts with PS-coated beads but not PC-coated beads. We also observed that the coating of beads with PS induced their uptake by stabulin-1, suggesting that PS alone is a sufficient signal for phagocytosis mediated by stabulin-1. Recently, we demonstrated that the EGFr and stabulin-2 recognizes PS during cell-corpse clearance and that at least four EGFr-like domains containing an atypical EGFr-like domain and calcium are required for PS recognition (Park et al., 2008c). In this study, we found that stabulin-1 also mediates PS recognition via its EGFr-like domains. Furthermore, our recent study showed that GULP/CED-6 (PTB domain-containing engulfment adaptor protein 1/cell death protein 6) is involved in stabulin-2-mediated phagocytosis, and that its phosphotyrosine-binding (PTB) domain is able to specifically interact with one specific NPXY motif in the stabulin-2 cytoplasmic tail (Park et al., 2008b). GULP/CED-6 contains the PTB domain, which is known to interact with the NPXY/F motif (Caldwood et al., 2003; Su et al., 2002). Considering that stabulin-1 contains a NPXY motif in its cytoplasmic tail, stabulin-1-mediated phagocytosis might also operate via intracellular signal transduction pathways similar to those for stabulin-2.

The blockade of stabulin-1 receptor with shRNA or blocking antibody resulted in a partial reduction of aged-cell engulfment. This could be due to significant redundancy in cell-corpse recognition by using several receptors of HMDM at the same time. Current studies showed that the transmembrane receptors Tim4 (Miyaniishi et al., 2007), BAII (Park et al., 2007) and stabulin-2 might serve as PS-receptors that are involved in cell-corpse clearance. Although we have showed that stabulin-2 is also involved in aged-cell engulfment in our alternatively activated HMDM model, there is still no evidence to indicate whether Tim4 and BAII are involved in apoptotic cell phagocytosis in alternatively activated macrophages. Whether stabulin-1 and stabulin-2 are functionally linked or act independently is also unclear at present. However, the results of this study provide evidence that stabulin-1 is a receptor for the clearance of aged and apoptotic cells, particularly in alternatively activated macrophages. Our results should help elucidate the molecular mechanism by which apoptotic cell clearance occurs in the human body, and by which PS is recognized during many physiological and pathological conditions.

Materials and Methods

Reagents

Normal goat IgG and rabbit IgG were obtained from Chemicon. For immunofluorescent analysis, a rabbit polyclonal anti-stabulin-1 antibody (RSI) was used as previously described (Kzhyshtokska et al., 2008). For fluorescence-activated cell sorting (FACS) analysis and function blocking, a goat polyclonal anti-stabulin-1 antibody (N-13) was purchased from Santa Cruz Biotechnology. The anti-EEA1 mouse monoclonal antibody was from BD Biosciences. A monoclonal antibody recognizing the FLAG epitope (clone M2) was obtained from Sigma. Monoclonal anti-stabulin-2 antibody (SG3) was used as previously described (Park et al., 2008a). Alexa-Fluor-488-conjugated anti-goat IgG antibody and Alexa-Fluor-568-conjugated anti-rabbit IgG were obtained from Molecular Probes. Anti-human IgG and horseradish peroxidase (HRP)-conjugated anti-His antibody were purchased from Santa Cruz Biotechnology. Polybrene was acquired from Sigma. NBD-PC (1-oleoyl-2-[6-[N-(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-sn-glycero-3-phosphocholine), NBD-PS (1-oleoyl-2-[6-[N-(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-sn-glycero-3-phospho-L-serine) and all phospholipids were purchased from Avanti-Polar Lipids (Alabaster, AL).

Cell culture

Human macrophages were obtained from normal donors, isolated using monocyte isolation kit II systems (Miltenyi Biotec, Bergisch Gladbach, Germany) and cultured in X-vivo 10 (BioWhittaker, Walkersville, MD) containing 10% human serum. The cells were stimulated with the combination of IL-4 at 10 ng/ml (R&D Systems) and dexamethasone at 100 nM (Sigma). The differentiated macrophages (HMDMs) were utilized after 7-10 days of culturing. Alternatively, stabulin-1-positive macrophages were produced in the absence of serum as previously described (Kzhyshtokska et al., 2008). HEK293 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) (high glucose) containing 10% fetal bovine serum (FBS). Jurkat T cells were maintained in Roswell Park Memorial Institute (RPMI)-1640 culture medium containing 10% FBS.

Stable transfection

Mouse fibroblasts L cells (ATCC CCL-1), kindly donated by Masatoshi Takeichi (Kyoto University, Japan), were grown in DMEM supplemented with 10% heat-inactivated FBS and the appropriate antibiotics. The complete stabulin-1 cDNA was amplified from the KG-1 cell line and cloned into pcDNA3 (+) (Invitrogen) with the FLAG epitope sequence. L cells were transfected in OptiMEM I medium using Lipofectamine (Invitrogen). For stable transfection, L cells were selected in G418 (400 μg/ml). Individual G418-resistant colonies were isolated after 10-12 days of culture. The final clones were designated L/Stab-1-#. Negative control clones were produced in the absence of serum as previously described (Kzhyshtokska et al., 2008). HEK293 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) (high glucose) containing 10% fetal bovine serum (FBS). Jurkat T cells were maintained in Roswell Park Memorial Institute (RPMI)-1640 culture medium containing 10% FBS.

Fluorescence-activated cell sorting (FACS) analysis

For the analysis of surface staining, the cells were incubated with 1 μg/ml of anti-stabulin-1 antibody (N-13) on ice for 30 minutes with gentle agitation every 5 minutes. The cells were washed twice, resuspended in phosphate-buffered saline (PBS), and incubated with 4 μg/ml of Anti-Fluor-488-conjugated anti-goat secondary antibody for 45 minutes on ice. After three washes with PBS, the cells were resuspended in PBS, and then analyzed via flow cytometry on a FACScalibur cytometer (BD Biosciences).

Binding and phagocytosis of aged red blood cells

Aged RBCs were prepared by incubating cells in PBS (20% hematocrit) at 37°C for 4 days as previously described (Oka et al., 1998; Park et al., 2008a). Exposure of PS on the surface of aged RBCs was detected using annexin-V–fluorescein isothiocyanate (FITC) and an Annexin V–Apoptosis detection kit (Santa Cruz Biotechnology). Next, aged RBCs were added to the transfected L cells or HMDMs and then incubated for 2-3 hours at 37°C. After washing away the unbound RBCs, the unbound RBCs were lysed by adding deionized H2O for 10 seconds followed by immediate replacement with DMEM, as previously described (Ogden et al., 2001). The cells were then fixed with methanol and stained using a Diff Quick staining kit (IMEB, 3371).
San Marcos, CA). Binding and phagocytosis of aged RBCs were quantified via light microscopy prior to and following hypotonic lysis, respectively. The percentages of binding and phagocytosis of aged RBCs were determined on the basis of the percentages of phagocytes that were positive for binding and engulfment, respectively, as described previously (Park et al., 2008b; Park et al., 2008c). At least 100 cells were scored per well, and all experiments were repeated at least three times. In certain experiments, the phagocytosis assay was performed in the presence of an anti-stabilin-1 antibody or anti-stabilin-2 antibody (S3G3). Isotype-matched antibody was used as control. Aged RBCs were labeled with FITC prior to their addition to HMDMs. In experiments using phospholipid liposomes, phospholipid liposomes were pre-incubated with L/STab-1 cells for 1 hour at 37°C prior to the addition of aged RBC.

Phagocytosis of apoptotic cells
Apoptosis in Jurkat cells was induced by exposure to UV irradiation at 254 nm 300 J/cm² in a UV-Gel-Crosslinker/Stratalinker and further maintained for 6 hours or 16 hours. Apoptosis was assessed by morphological changes under light microscopy. After 6 hours, over 80% of cells were apoptotic. After 16 hours, all cells were apoptotic. Both 6-hour and 16-hour apoptotic cells were used in phagocytosis assay with similar results. In order to visualize apoptotic cells using a confocal microscope, Jurkat cells were labeled with 0.2 μM propidium iodide. Apoptotic cells were added into culture medium at a ratio of 1:50 (10,000 apoptotic cells/100,000 macrophages) and allowed to take place for 1 or 3 hours. Then, fixation and immunofluorescence analysis were performed as previously described (Kzhyshkowska et al., 2008). Confocal microscopy was performed with a Leica TCS SP2 laser scanning spectral confocal microscope, equipped with a 63 × 1.32 objective. Excitation was with an argon laser emitting at 488 nm, and Alexa Fluor 647 derivative at 568 nm, and a helium-neon laser emitting at 633 nm. Data were acquired and analyzed using Leica Confocal software. All two-color images were acquired using a sequential scan mode.

Lentivirus-mediated shRNA production
Three 20-nt shRNA molecules derived from three different parts of the human stabilin-1 gene (GenBank accession no. NM_015136) were designed. Candidate oligonucleotides were synthesized and cloned into the pSuper basic vector (OligoEngine, Seattle, WA). Effective shRNA vectors were selected by co-transfection with a vector expressing stabilin-1 in CHO-K1 cells. pSuper/shStab-1 significantly downregulated the expression of the stabilin-1 protein. The expression cassette of pSuper/shStab-1-2 was subcloned into the pRNAi/shRNA vector (GenScript, Piscataway, NJ). Scrambled shRNA (shCont) was used as a control for stabilin-1 shRNA (shStab-1).

All constructs were confirmed via sequence analysis. Lentiviral transduction was conducted in accordance with the manufacturer’s instructions (GenScript). Briefly, the pRNAT-shStab-1 vector was co-transfected with the packaging vector (Invitrogen) into 293FT cells (Invitrogen). The supernatant was collected after 72 hours and filtered through a 0.22 μm pore size filter. The lentivirus-containing supernatants were then used to infect HMDMs. Two days after transduction, HMDMs were seeded at 2 x 10⁶ cells/ml into six-well plates. Lentiviral particles and HIV-1 env were used to infect the cells using 8 μg/ml of polybrene (Sigma). The differentiated macrophages were then incubated with recombinant HIV-1 Env to allow infection. On day 3 post-infection, cells were treated with 1 μM cAMP and cultured in serum-free media containing 20% FBS. The supernatants were collected for 48 hours and analyzed for the production of shRNA. The levels of shRNA expression were determined by RT-qPCR using primers specific for each shRNA sequence.

Preparation of recombinant protein in mammalian cells and binding assays
To generate an expression vector for Stab1-Fc fusion protein, the cDNA encoding for the immunoglobulin Fc region was amplified by PCR, cloned into plasmid D3.1(+) (Invitrogen), and designated as pDNA-Fc. Next, the cDNA corresponding to the extracellular region of stabilin-1 (amino acids 1-2476) was amplified from a full-length stabilin-1 cDNA by PCR, cloned into pDNA-Fc vector, and designated as pDNA-Stab1-FC. HEK293T cells were then transfected with the pDNA-Stab1-FC vector for 6 hours using Lipofectamine (Invitrogen), in accordance with the manufacturer’s instructions. At 24 hours after transfection, the culture medium was exchanged with serum-free DMEM. The supernatant was collected after 48 hours and incubated with PS- or PC-coated beads at 4°C for 4 hours in the presence of 5% CO². After washing, the beads were washed three times with PBS (pH 7.4). The precipitated proteins were dissolved by boiling in SDS-sample buffer and subjected to SDS-PAGE on a 6% gel. The bound Stab1-Fc proteins were detected via immunoblotting with anti-human IgG (Santa Cruz Biotechnology).

Binding and phagocytosis of PS-coated beads
Phospholipid-coated beads were labeled with PC-NBD and added to L/STab-1 cells. After 2 hours of incubation, the non-ingested beads were removed by extensive washing. In order to further identify the engulfed cells, fluorescence derived from bound beads (remnant beads) was quenched using trypan blue as previously described (Park et al., 2008a). The binding and uptake of phospholipid-coated beads was quantified by light microscopy prior to and following trypan blue staining, respectively. The binding index was determined by calculating the number of bound beads per cell. The percentage of phagocytosis was determined by the percentage of phagocytes positive for engulfment.

Preparation of bacterial recombinant protein and binding assays
To produce His-tagged recombinant proteins corresponding to the third EGF-like domain repeat (EGFIII-3) and the sixth FAS1 domain (FAS1-6) of stabilin-1, fragments of stabilin-1 cDNA encoding amino acids 1317-1582 and 1762-1867 were generated by PCR, cloned into the BamHI and XhoI sites of PET-28a (Novagen/Merck, Madison, WI). These His-tagged recombinant proteins were then expressed in BL-21 cells, harvested and purified using Ni-NTA resin (Qiagen, Valencia, CA) according to the manufacturer’s instructions. A binding assay was performed as described previously (Jung et al., 2007) with slightly modifications. Apoptotic Jurkat cells were suspended in medium at a density of 1 x 10⁶ cells/ml and 1 ml of the cell suspension was incubated with His-tagged stabilin-2 derivatives in serum-free medium containing 0.1% BSA and 5 mM CaCl₂ for 5 hours at 4°C. The cells were then washed three times with PBS (pH 7.4), before lysis at 4°C in ice-cold buffer A (10 mM Tris-C1 pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.5% SDS, 0.02% sodium azide, 1 mM EDTA, 1 mM PMSF). Equal amounts of protein were then separated by SDS-PAGE on 8% gel. The amounts of His-tagged proteins associated with the apoptotic cells were determined by immunoblotting with anti-His antibody (Santa Cruz Biotechnology).

In some experiments, recombinant proteins were incubated with PS- or PC-coated beads at 4°C for 5 hours. The beads were then washed three times with PBS (pH 7.4). The precipitated proteins were detected via immunoblotting with anti-His antibody (Santa Cruz Biotechnology).

Statistical analysis
The statistical significance was assessed using ANOVA or the Student t-test. A P value of <0.05 was considered to be statistically significant.

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References


Supplementary figure 1
Supplementary figure 4