CAVEOLAE-DEPENDENT ENDOCYTOSIS IS REQUIRED FOR CLASS A MACROPHAGE SCAVENGER RECEPTOR-MEDIATED APOPTOSIS IN MACROPHAGES

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Running head: Endocytic pathways of SR-A

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Class A macrophage scavenger receptor (SR-A) is a transmembrane receptor that can bind many different ligands including modified lipoproteins that are relevant to the development of vascular diseases. However, the precise endocytic pathways of SR-A-mediated ligands internalization are not fully characterized. In this study, we show that SR-A/ligand complex can be endocytosed by both clathrin- and caveolae-dependent pathways. Internalizations of SR-A/lipoprotein (such as acLDL) complexes primarily go through the clathrin-dependent endocytosis. By contrast, macrophage apoptosis triggered by SR-A-fucoidan internalization requires caveolae-dependent endocytosis. The caveolae-dependent process activates p38 kinase and JNK signaling; whereas the clathrin-mediated endocytosis elicits ERK signaling. Our results suggest that different SR-A endocytic pathways have distinct functional consequences due to the activation of different signaling cascades in macrophages.

Two main pathways have been identified for receptor-mediated endocytosis: the clathrin-dependent and the caveolae/lipid raft-dependent endocytic pathways (1,2). Clathrin-dependent endocytosis is the most well-characterized mechanism for mediating the internalization of membrane receptors into cells. It is also important for intracellular trafficking at the trans-golgi network and endosomes (3). Caveolae/raft-dependent endocytosis is involved in multiple biological processes, including mediating virus entry into host cells, internalizing GPI-anchored proteins and regulating certain signaling cascades. Caveolae are cholesterol and sphingolipid-rich plasma membrane invaginations of a diameter of 60–80 nm, of which caveolin-1 is the main protein component required for caveolae biogenesis (4). Trafficking through these two endocytic pathways affects the amplitude and the on–off status of many signaling pathways. Thus, these endocytic processes contribute to the regulations of cell migration, cell cycle, cell polarity, apoptosis, and gene transcription (1,4,5).

Class A macrophage scavenger receptor (SR-A) is a transmembrane receptor expressed mainly in macrophages (6,7). SR-A can bind with an unusually broad range of polyanionic ligands, which includes modified lipoproteins,
lipopolysaccharide (LPS) of Gram-negative bacteria, and advanced glycation end products. Its broad specificity in binding with the ligands supports the multiple functions of SR-A in macrophage growth, adhesion to the substratum, cell-cell interactions, phagocytosis, and host defense (8-10). However, the molecular mechanisms enabling SR-A to exert multiple functions are still not well understood. It is reported that acetylated low density lipoprotein (acLDL), a specific ligand of SR-A (17), is internalized via coated-pit mediated endocytosis (14,18,19). Macropinocytosis may also contribute to the uptake of acLDL at a low level (19). On the other hand, the VirB-dependent bacterial internalization induces localization of SR-A into the detergent-resistant membrane lipid rafts (20), which are sterol- and sphingolipid-enriched, and caveolins-containing domains that compartmentalize cellular processes. We and others previously identified a few unique signal motives in the cytoplasmic domain of SR-A required for its internalization (11-14). We also showed that the internalization of SR-A and its ligand complex into cell is regulated by interaction of SR-A to its coupler (15,16). In this study, we want to address the question of whether different functions of SR-A are carried out by different endocytosis pathways. We show that SR-A can be internalized by both clathrin- and caveolae-dependent pathways. The clathrin-dependent SR-A endocytosis activates extracellular signal-regulated kinase (ERK) signaling. The caveolae-dependent pathway is required for p38 kinase and c-Jun NH2-terminal kinase (JNK) signaling, as well as caspase activation. Our results indicate for the first time that SR-A-mediated macrophage apoptosis may be selectively regulated by the caveolae-dependent endocytosis.

**Experimental Procedures**

*Cell culture-* RAW264.7 cells (American Type Culture Collection, ATCC) were cultured in Roswell Park Memorial Institute medium 1640 (RPMI-1640, Gibco) containing 10% (v/v) fetal calf serum (FCS, Gibco), supplemented with 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. To examine the effect of chlorpromazine or nystatin, cells were first incubated with 5 μM chlorpromazine (CP, Sigma) or 25 μg/ml nystatin (Sigma) in growth medium for 30 min at 37°C (21,22), and then harvested for measurements of SR-A activity or apoptosis. To examine the effect of different kinase inhibitors, cells were first incubated with either 10 μM SB203580 (Sigma), or 5 μM SP600125 (Sigma), or 100 μM PD98059 (Sigma) in growth medium for 1 h at 37°C, and then harvested for measurements of SR-A activity or apoptosis.

*SiRNA transfection-* Transfections were performed by using Lipofectamine 2000 (Invitrogen). Target sequence in mouse clathrin heavy chain gene is: GGCUUCUAAUAUCACGAAtt; UUCGUGAUAAUUAGAGCCac. The target sequence for caveolin-1 gene is: CCAUCUACGUCCAUACCUCUt; AAGGUAGGACGUAGAUGGag. A scrambled SiRNA was used as a negative control. All oligonucleotides were obtained from Ambion RNA. RAW264.7 cells were seeded 1 day prior to transfection. They were 30% confluent when they were transfected with 30 nM positive or scrambled oligonucleotides in Lipofectamine 2000 and Opti-MEM (Invitrogen) without serum for 72 h. The reduction in clathrin heavy chain and caveolin-1 protein was estimated by western blot analysis using antibodies against clathrin heavy chain (Sigma), caveolin-1 (Sigma) and β-actin (Santa Cruz).
**Immunoprecipitation and Western Blotting**

Cells were washed twice with phosphate-buffered saline (PBS) and lysed in lysis buffer (50 mM Tris-HCl, pH 7.6, 0.15 M NaCl, 1% Triton X-100, 5 mM EDTA, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). For immunoprecipitation, SR-A was immunoprecipitated with a mouse monoclonal antibody (E20) (Santa Cruz) and normal goat IgG (Santa Cruz). Immunoprecipitates were collected on Pansorbin, washed three times in lysis buffer, and eluted into Laemmli sample buffer. Cell lysates or immunoprecipitates were separated by SDS-PAGE with 12% acrylamide gels. Proteins were transferred to PVDF membrane and blocked for 30 min in blocking buffer (Tris-buffered saline, pH 7.6, 0.05% Tween, and 3% BSA). After incubation with primary antibody diluted in blocking buffer for 60 min and washing, the blot was incubated for 30 min with appropriate secondary anti-IgG-horseradish peroxidase conjugate. The membrane was washed three times for 10 min each and developed with Supersignal chemiluminescent substrate (Pierce). The primary antibodies against p-p38, p38, p-JNK, JNK, p-ERK, and ERK were obtained from Cell Signaling Technology. Quantification was performed by measurement of the intensity of the signals with the use of ImageJ software.

**Measurements of Dil-acLDL uptake by SR-A**

Human LDL (d=1.019–1.063 g/ml) was isolated from EDTA-treated plasma by ultracentrifugation. LDL was labeled with 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI, Molecular Probe) to specific activities of 20–40 ng/mg LDL. Acetylation of LDL was performed as described previously (14). The protein concentrations of LDL were measured using the method of BCA protein assay. AcLDL, an artificially modified LDL, serves as a specific ligand of SR-A, it is widely used for in vitro determination of SR-A activities. SR-A uptake of acLDL was evaluated by flow cytometry with Dil-acLDL (14,23).

**Immunofluorescence Analysis**

RAW264.7 cells were grown on coverslips for 24 h at 37°C. After fixation with 3% paraformaldehyde in PBS for 15 min at room temperature, cells were permeabilized with 0.1% Nonidet P-40, PBS for 5 min, and blocked with 3% BSA, 0.01% Tween 20, PBS (PBST-BSA) for 30 min. The primary antibody against SR-A (2F8, BMA), clathrin (Sigma), or caveolin-1 (Sigma) in PBST-BSA was incubated with cells for 60 min and the fluorochrome-conjugated secondary antibody (FITC goat anti-rat IgG, Cy3 goat anti-rabbit IgG, FITC rabbit anti-rat IgG, or Cy3 goat anti-mouse IgG) in PBST-BSA was added for 60 min. Morphologic observation was performed with a Zeiss LSM 710 META confocal microscope. Pictures were obtained using sequential scanning, and the exposure settings and gain of laser were kept the same for each condition.

**Peritoneal Macrophages (PM)**

PM were harvested 4 days after thioglycollate injection of the peritoneal cavity. Cells were washed with chilled PBS (pH 7.4), and macrophages were resuspended in RPMI 1640 containing 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin and plated on 60-mm round Petri dishes. After a 2 h incubation at 37 °C, 5% CO2 non-adherent cells were removed, and the remaining adherent cells were cultured in RPMI 1640 containing 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin.

**Apoptosis Assay**

After treatment with RNAi, RAW264.7 cells were washed, resuspended in the staining buffer, and examined with the Annexin V-FITC & propidium iodide (PI) Apoptosis kit (Biouniquer) according to the manufacturer’s instructions. Stained cells were detected by FACS (FACScalibur; BD Biosciences). The Annexin...
V-positive and PI-negative cells were regarded as apoptotic cells.

Statistical analysis- Results are reported as the mean ± S.D. Statistical significance between the groups was assessed by one-way analysis of variance (ANOVA). The level of significance was chosen as $P < 0.05$.

RESULTS

SR-A-mediated ligand endocytosis happens via both clathrin- and caveolae-dependent pathways in RAW264.7 macrophages. It was known that certain SR-A-mediated endocytosis goes through the clathrin-coated vesicles (19). Our previous results showed a direct interaction between SR-A and clathrin (14), which was further confirmed by a coimmunoprecipitation assay in this study (Figure 1A). In order to investigate whether SR-A can also be endocytosed by the caveolae pathway, we performed a coimmunoprecipitation assay to examine the interactions between SR-A and caveolin-1. Both endogenous caveolin-1 and SR-A could be precipitated by an antibody against SR-A in RAW264.7 cells (Figure 1B). Double immunofluorescence experiments confirmed that the partial co-localization of SR-A with caveolin-1 (Figure 1C) and clathrin (Figure 1D) in cells. The co-localization of SR-A with caveolin-1 was weaker than that of SR-A with clathrin. Similar co-localization status of SR-A with caveolin-1 (Figure 1E) and clathrin (Figure 1F) were detected when SR-A ligand fucoidan was added into RAW264.7 cells.

To further characterize the relevance of these two endocytic pathways to SR-A functions, we used RNA interference to knock down clathrin heavy chain or caveolin-1 (Figure 2A). When clathrin was downregulated by 65%, the uptake of Dil-acLDL (5 μg/ml), a specific SR-A agonist, was decreased by 61%. However, downregulation of caveolin-1 by 60% caused only a slight reduction in Dil-acLDL uptake (decrease by 25%) (Figure 2Ba). Similar results were obtained by the use of a higher concentration (50 μg/ml) or a lower concentration (0.5 μg/ml) Dil-acLDL. Knocking-down clathrin decreased the uptake of 50 μg/ml Dil-acLDL by 55%, whereas knocking-down caveolin-1 led to only 26% reduction (Figure 2Bb). Similar results were obtained when low concentration Dil-acLDL was used (Figure 2Bc). These results suggest that SR-A-mediated endocytosis can go through both clathrin- and caveolae-dependent pathways. Clathrin-coated vesicles are the main carriers for SR-A-mediated uptake of Dil-acLDL.

Caveolae-dependent endocytosis may contribute to the SR-A-mediated apoptosis in macrophages. We next examined the biological significance of the caveolae-mediated SR-A-ligand endocytosis. It was shown previously that accumulation of excess free cholesterol leads to macrophage apoptosis, and this apoptotic process requires both endoplasmic reticulum (ER) stress-triggered unfolded protein response pathway and the engagement of SR-A (ligand binding to SR-A). As such, the apoptosis inducing effect of excess cholesterol can be mimicked by thapsigargin (Tg, a drug causing ER stress) plus fucoidan (a ligand of SR-A) (25,26). How and why SR-A engagement is needed for the apoptosis remains unclear. We first established this apoptosis model in our lab. Briefly, SR-A-/- +/+ and SR-A+/- macrophages were treated with fucoidan, Tg, or fucoidan plus Tg. Consistent with previous study, we confirmed that fucoidan or Tg alone could not induce macrophage apoptosis in either SR-A+/+ or SR-A-/- macrophages. By contrast, treatment with fucoidan plus Tg resulted in a significant increase in the number of apoptotic SR-A+/+ macrophages,
but not SR-A/−/− cells (Figure 3A). Apoptotic cells were detected using Annexin V and PI staining. Annexin V-positive and PI-negative cells are considered as apoptotic cells.

Using RAW264.7 cells, we found that fucoidan plus Tg treatment induced high level of cleaved caspase-3 (Figure 3B). Interestingly, this activated caspase response was dramatically suppressed by the knockdown of caveolin-1 but not by clathrin-RNAi (Figure 3B). Using FACS analyses of Annexin V and PI staining (Figure 3C) or PI alone (Figure 3D), we found that the fucoidan/Tg-induced cell death was significantly rescued by caveolin-1-RNAi (58~78% rescue), but was only mildly rescued by clathrin-RNAi (11~16% rescue). Thus, caveolae-mediated SR-A-ligand endocytosis may be a primary pathway responsible for the SR-A-engaged apoptosis in macrophages.

**SR-A-fucoidan complex activates MAPKs in macrophages and the activation depends on endocytosis.** To investigate cell signaling linked to the endocytosed SR-A, we examined the activation of mitogen-activated protein kinases (MAPKs) in acLDL- or fucoidan-treated RAW264.7 cells. As shown in Figure 4A, phosphorylation of p38 kinase, JNK, and ERK MAPKs in cells were all simultaneously increased by treatment with either acLDL or fucoidan at 37°C. When macrophages were incubated with these two SR-A ligands at 4°C, at which temperature SR-A can only bind with its ligands but can not be internalized into the cell (23), different responses were detected for acLDL and fucoidan (Figure 4C). AcLDL caused a simultaneous activation of p38 kinase, JNK and ERK even when applied at 4°C. By contrast, fucoidan did not activate any of these three kinases at 4°C. These results suggest that the fucoidan-induced MAPKs signaling is dependent on SR-A-fucoidan complex internalization into macrophage, which is different from that of SR-A-acLDL complex. Thus, we used fucoidan in the following endocytosis-related experiments.

**Caveolae dependent SR-A endocytosis activates p38 kinase and JNK, whereas clathrin mediated SR-A endocytosis activates ERK in macrophages.** To further determine which of the MAPKs are activated by caveolae mediated endocytosis of SR-A, we used RNAi and specific inhibitors of this pathway. Nystatin is a sterol-binding agent that disassembles caveolae and cholesterol in the membrane. It does not affect the clathrin-dependent internalization (4,21,27,28). Importantly, nystatin inhibited the fucoidan-activated phosphorylation of p38 kinase and JNK but had no effect on ERK phosphorylation (Figure 5A). Knockdown of endogenous caveolin-1 led to the same result as nystatin treatment (Figure 5B). These results strongly supported that SR-A-fucoidan induced activation of p38 kinase and JNK requires caveolae-mediated endocytic pathway.

Similar strategy was also used to determine the MAPKs activated by the SR-A-clathrin pathway. Chlorpromazine (CP) causes redistribution of the assembly protein complex-2 (AP-2) from the plasma membrane to endosomes. It thus inhibits clathrin-dependent endocytosis without affecting the caveolae-dependent internalization processes (29,30). Application of CP to the fucoidan-treated cells abolished ERK phosphorylation but did not affect the activation of p38 kinase and JNK (Figure 6A). These results were further confirmed by knockdown of clathrin heavy chain in these RAW264.7 macrophages (Figure 6B). These results suggest that clathrin-related SR-A-fucoidan endocytosis activate ERK rather than p38 kinase and JNK signaling.

**p38 kinase and JNK activation contribute to SR-A-engaged apoptosis in macrophages.** Finally,
we investigated whether p38 kinase and JNK activation is part of the signaling mechanisms involved in the SR-A-dependent macrophage apoptosis. Selective inhibitors for p38 kinase (SB203580), JNK kinase (SP600125), and ERK kinase (PD98059) were used in the study. We first showed that these inhibitors can efficiently attenuate the activation of their respective kinase targets in RAW264.7 macrophages (Figure 7A). Next, we examined the effects of these inhibitors on fucoidan plus Tg induced increase in cleaved caspase-3. Addition of either p38 kinase inhibitor SB203580 or JNK inhibitor SP600125 diminished the level of cleaved caspase-3 to the background level (decrease by 56% or 53% respectively). By contrast, the ERK inhibitor PD98059 had a mild and insignificant effect on caspase activation (decrease by 17%) (Figure 7B). These data suggest that p38 kinase and JNK activity are required for the fucoidan/Tg- induced macrophage apoptosis. Taken together with the results that caveolae but not clathrin mediated endocytosis is the primary pathway for the SR-A-fucoidan-induced p38 kinase and JNK activation, our study provides a cellular pathway selectively required for SR-A-dependent apoptosis in ER stressed macrophages.

**DISCUSSION**

The uptake of modified lipoproteins by SR-A is thought to be central to foam cell formation, from which atherosclerotic lesion is generated and develops. However, the conflicting outcomes from multiple studies on the impact of SR-A in mouse models of atherosclerosis suggest the existence of pathogenesis mechanisms beyond lipid uptake (7,9,31). Foam cell is believed to represent one of the major activation events stimulating the proinflammatory phenotype of lesional macrophages. SR-A has been found to initiate signaling cascades regulating not only lipid metabolism, but also macrophage activation and inflammatory programs that may influence the development and stability of the atherosclerotic plaque. More importantly, SR-A has roles in the induction of apoptosis that may differentially impact early versus later more complex lesions (25,32). The molecular background underlying these multiple functions of SR-A is its unique ability to bind with broad array of ligands. As a member of the group of pattern recognition receptors that mediate the innate immune host response, SR-A can bind and “scavenge” modified forms of LDL, apoptotic cells, anionic phospholipids, β-amyloid peptide, and advanced glycation end-products as well as pathogens and pathogen-associated molecules (8,10). Here, we demonstrated that SR-A can endocytose various ligands via different pathways. Multiple endocytic pathways are utilized by many other receptors. For example, bone morphogenic protein receptor (BMPR), epidermal growth factor receptor (EGFR), and platelet-derived growth factor receptor (PDGFR) have been shown to exert distinct functions by choosing different endocytic pathways (21,33). We found that the distinct endocytic pathways of SR-A are independent on the concentration of ligand. Presence of ligand fucoidan did not influence SR-A choosing its encocytic routes. This is different from EGFR, which enters into cell via different routes based on the dose of ligand (24,34).

Endocytosis is originally regarded as a mechanism to terminate signaling through receptor internalization and subsequent lysosomal degradation. But it is widely accepted now as a fundamental organizer of the cell signaling events (2,35,36). As we showed different signaling responses to SR-A ligands at 4°C and 37°C, signaling persists throughout the endocytic route of SR-A. Thus, endocytic signaling is not merely
a ‘passive’ extension of binding to the ligands by SR-A at the plasma membrane, but is a tool to achieve signal diversification and specificity (37-39). This may constitute the molecular mechanisms for the multiple functions of SR-A.

Macrophage apoptosis occurs at all stages of atherosclerosis. In early lesions it is mildly dangerous because apoptotic cells are efficiently cleared by neighboring macrophage. In advanced atherosclerotic lesions macrophage cell death leads to necrotic core formation and plaque destabilization (32,40,41). SR-A plays an important role in both the induction of macrophage apoptosis and the clearance of these dying cells. Under conditions of hypercholesterolemia macrophages in vessel wall are overloaded by modified lipoproteins, leading to a toxic accumulation of free cholesterol in the cell that result in endoplasmic reticular stress. Subsequently, engagement of SR-A pathways by modified lipoproteins or fucoidan triggers apoptotic cell death, indicating that SR-A signaling contributes to macrophage death and necrotic core formation (25,32). Caveolin-1 is believed to play a role in macrophage apoptosis (42). We showed that the SR-A-engaged apoptosis is primarily mediated by the caveolae-dependent endocytosis in macrophages. Furthermore, Frank et al. demonstrated that loss of caveolin-1 gene expression is protective against the development of aortic atheromas, with up to an 70% reduction in atherosclerotic lesion area (43). It is possible that blockade of caveolae-mediated endocytic pathway may diminish macrophage apoptosis and, thus, inhibit atherosclerosis lesion development.

In the present study we further showed that the fucoidan/Tg-induced macrophage apoptosis is dependent on activation of p38 kinase and JNK. The pro-apoptosis properties of p38 kinase and JNK have also been reported in some other apoptosis models (44-46). In addition, JNK2 is shown to be required for SR-A-mediated foam cell formation and atherogenesis (47). Known as a signalosome, caveolae concentrate many signaling molecules, including H-Ras, Src family, and endothelial nitric oxide synthase (eNOS), within specialized plasma membrane domains (48). Regulation of LPS-induced cytokine production by caveolin-1 involves the p38 kinase pathway (49). Thus, caveolin-1 and JNK2 may become potential targets to prevent SR-A-dependent apoptosis in ER stressed macrophages.

In conclusion, our data demonstrated that SR-A/ligand internalization occurs through two endocytosis routes: clathrin- and caveolae-dependent pathways. Dosage of the ligands seems not to influence choice of the route by SR-A. Uptake of modified LDL by SR-A primarily go through clathrin route. The SR-A-induced apoptosis requires endocytosis through the caveolae route, which is linked to p38 kinase and JNK activation (Figure 8). SR-A may exert multiple functions by choosing distinct endocytic routes and the linked signaling in macrophages.

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FOOTNOTES

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The abbreviations used are: SR-A, Class A macrophage scavenger receptor; LPS, lipopolysaccharide; acLDL, acetylated low density lipoprotein; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH2-terminal kinase; CP, chlorpromazine; PBS, phosphate-buffered saline; Dil 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; PM, Peritoneal Macrophages; PI propidium iodide; ER, endoplasmic reticulum; Tg, thapsigargin; MAPK, mitogen-activated protein kinase; BMPR, bone morphogenic protein receptor; EGFR, epidermal growth factor receptor; PDGFR, platelet-derived growth factor receptor; eNOS, endothelial nitric oxide synthase

FIGURE LEGENDS

Fig. 1. Association of SR-A with caveolin-1 or clathrin in RAW264.7 macrophages. A. coimmunoprecipitation of endogenous SR-A and clathrin. RAW264.7 cell lysates were incubated with an anti-SR-A specific or nonimmune goat IgG. The immunoprecipitated complexes were analyzed by western blot for SR-A (SR-A) or clathrin heavy chain (cltc) as indicated. The presented blot is one of three independent blots. B. coimmunoprecipitation of endogenous SR-A and caveolin-1. RAW264.7 cell lysates were incubated with an anti-SR-A specific or nonimmune goat IgG. The immunoprecipitated complexes were analyzed by western blot for SR-A (SR-A) or caveolin-1 (cav1) as indicated. The presented blot is one of three independent blots. C. colocalization of endogenous SR-A and the caveolin-1 in RAW264.7 cells. Immunofluorescence experiments were performed by using antibody against SR-A (a, SR-A) and antibody against caveolin-1 (b, cav1) as indicated. (c) Merged images show overlapping subcellular distribution that appears yellow. D. colocalization of endogenous SR-A and clathrin in RAW264.7 cells. Immunofluorescence experiments were performed by using antibody against SR-A (a, SR-A) and antibody against clathrin heavy chain (b, cltc) as indicated. (c) Merged images show overlapping subcellular distribution that appears yellow. E. colocalization of endogenous SR-A and the caveolin-1 in RAW264.7 cells after incubated with fucoidan. RAW264.7 cells were incubated with 50 μg/ml fucoidan for 10 min. Immunofluorescence experiments were performed by using antibody against SR-A (a, SR-A) and antibody against caveolin-1 (b, cav1) as indicated. (c) Merged images show overlapping subcellular distribution that appears yellow. F. colocalization of endogenous SR-A and the clathrin in RAW264.7 cells after incubated with fucoidan. RAW264.7 cells were incubated with 50 μg/ml fucoidan for 10 min. Immunofluorescence experiments were...
performed by using antibody against SR-A (a, SR-A) and antibody against clathrin heavy chain (b, cltc) as indicated. (c) Merged images show overlapping subcellular distribution that appears yellow.

**Fig. 2.** Effects of endocytic pathways on lipid internalization. *A.* RAW264.7 cells were transfected with 30 nM irrelevant SiRNA (SiRNA-(-)), clathrin heavy chain-targeting SiRNA (SiRNA-cltc), or caveolin-1-targeting SiRNA (SiRNA-cav1) for 72 h. Cell lysates were subjected to western blot analysis with the indicated antibodies. Untreated cells were used as a control. The presented blot is one of three independent blots. *B.* RAW264.7 cells were transfected with 30 nM irrelevant SiRNA (SiRNA-(-)), clathrin heavy chain-targeting SiRNA (SiRNA-cltc), or caveolin-1-targeting SiRNA (SiRNA-cav1) for 72 h. Untreated cells were used as control. After incubated with 5 μg/ml DiI-acLDL (a), 50 μg/ml DiI-acLDL (b), or 0.5 μg/ml DiI-acLDL (c) for 2 h at 37°C, the cellular uptake of DiI-acLDL was quantified by a flow cytometry. Unlabeled acLDL in excess amounts (300 μg/ml) was added together with the fluorescent lipoproteins for competition assays (acLDL). Results were expressed as mean ± S.D. of triplicate samples. *, P < 0.05.

**Fig. 3.** Effects of endocytic pathways on macrophage apoptosis. *A.* SR-A<sup>−/−</sup> and SR-A<sup>+/+</sup> macrophages were incubated with 50 μg/ml fucoidan, 0.5 μM Tg, or 50 μg/ml fucoidan plus 0.5 μM Tg for 12 h. Non-treated cells were used as a control. Cells were stained with annexin V and PI and analyzed by FACS. The apoptotic cells (the annexinV-positive and PI-negative cells) were indicated as the percentage of gated cells. Results were expressed as mean ± S.D. of triplicate samples. *, P < 0.05. *B.* RAW264.7 cells were transfected with 30 nM irrelevant SiRNA (SiRNA-(-)), clathrin heavy chain-targeting SiRNA (SiRNA-cltc), or caveolin-1-targeting SiRNA (SiRNA-cav1) for 72 h. Untreated cells were used as control. Cells were incubated with 50 μg/ml fucoidan plus 0.5 μM Tg for 12 h. Cell lysates were subjected to western blot with indicated antibodies. The presented blot is one of three independent blots. *C.* cells were stained with annexin V and PI and analyzed by FACS. The apoptotic cells (the annexin V-positive and PI-negative cells) were indicated as the percentage of gated cells. Results were expressed as mean ± S.D. of triplicate samples. *, P < 0.05. *D.* cells were stained with PI and analyzed by FACS. The apoptosis cells (PI-positive cells) were indicated as the percentage of gated cells. Results were expressed as mean ± S.D. of triplicate samples. *, P < 0.05.

**Fig. 4.** Changes in MAPKs activities induced by SR-A. RAW264.7 cells were incubated with 5 μg/ml acLDL or 50 μg/ml fucoidan at 37°C (A and B) or 4°C (C and D) for 0, 30, or 60 min. Cell lysates were subjected to western blot with the indicated antibodies. The presented blot (A and C) is one of three independent blots and quantitative data (B and D) are presented (fold over controls). Results were expressed as mean ± S.D. of triplicate samples.

**Fig. 5.** Downregulation of caveolae on SR-A signal pathway. *A.* RAW264.7 cells were treated with 25 μg/ml nystatin for 30 min and then stimulated with 50 μg/ml fucoidan at 37°C for 0, 30, or 60 min. Cell lysates were subjected to western blot analysis. The presented blot is one of three independent blots. *B.* RAW264.7 cells were transfected with 30 nM irrelevant SiRNA (SiRNA-(-)) or caveolin-1-targeting SiRNA (SiRNA-cav1) for 72 h. Non-treated cells were as control. After incubated with 50 μg/ml fucoidan...
at 37°C for 0, 30, or 60 min, cell lysates were subjected to western blot analysis. The presented blot is one of three independent blots.

**Fig. 6.** Downregulation of clathrin on SR-A signal pathway. A. RAW264.7 cells were treated with 5 μM CP for 30 min and then stimulated with 50 μg/ml fucoidan at 37°C for 0, 30, or 60 min, Cell lysates were subjected to western blot analysis. The presented blot is one of three independent blots. B. RAW264.7 cells were transfected with 30 nM irreverent SiRNA (SiRNA -(-)), or clathrin heavy chain-targeting SiRNA (SiRNA-cltc) for 72 h. Non-treated cells were as control. After incubated with 50 μg/ml fucoidan at 37°C for 0, 30, or 60 min, cell lysated were subjected to western analysis. The presented blot is one of three independent blots.

**Fig. 7.** Inhibition of MAPKs on cell apoptosis. A. RAW264.7 cells were treated without (control) or with a MAPK inhibitor (10 μM SB203580, 5 μM SP600125, or 100 μM PD98059) for 1 h prior to incubation with 50 μg/ml fucoidan for 30 min. Non-treated cells were as control. Cell lysates were subjected to western blot with indicated antibodies. The presented blot is one of three independent blots. B. RAW264.7 cells were treated without (control) or with a MAPK inhibitor (SB203580, SP600125, or PD98059) for 1 h prior to incubation with 50 μg/ml fucoidan and 0.5 μM Tg for 12 h. Non-treated cells were as control. Cell lysates were subjected to western blot with indicated antibodies. The presented blot is one of three independent blots. Results were expressed as mean ± S.D. of triplicate samples.

**Fig. 8.** Hypothetic model for the SR-A-fucoidan complex endocytosis in macrophage.
Endocytic pathways of SR-A

Figure 1
Figure 2

A

control  SiRNA-(-)  SiRNA-cav1

actin

cav1

control  SiRNA-(-)  SiRNA-cltc

cltc

actin

B

a  5 μg/ml

Relative intensity of red fluorescence

acLDL  control  SiRNA-(-)  SiRNA-cav1

b  50 μg/ml

Relative intensity of red fluorescence

acLDL  control  SiRNA-(-)  SiRNA-cltc  SiRNA-cav1

C

0.5 μg/ml

Relative intensity of red fluorescence

acLDL  control  SiRNA-(-)  SiRNA-cltc  SiRNA-cav1
Figure 3

A

B

C

D

Endocytic pathways of SR-A
Figure 4

A

37°C acLDL fucoidan
p-p38
p38
p-JNK JNK
p-ERK ERK

B

37°C
Relative p-p38 level
0 30 60
acLDL fucoidan
0 30 60
Relative p-JNK level
0 30 60
acLDL fucoidan
0 30 60
Relative p-ERK level
0 30 60
acLDL fucoidan

C

4°C acLDL fucoidan
p-p38
p38
p-JNK JNK
p-ERK ERK

D

4°C
Relative p-p38 level
0 30 60
acLDL fucoidan
0 30 60
Relative p-JNK level
0 30 60
acLDL fucoidan
0 30 60
Relative p-ERK level
0 30 60
acLDL fucoidan

Endocytic pathways of SR-A

Figure 6
Figure 7
Figure 8
Caveolae-dependent endocytosis is required for class A macrophage scavenger receptor-mediated apoptosis in macrophages
Xu-Dong Zhu, Yan Zhuang, Jing-Jing Ben, Ling-Ling Qian, Han-Peng Huang, Hui Bai, Jia-Hao Sha, Zhi-Gang He and Qi Chen

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