Major Vault Protein Regulates Class A Scavenger Receptor-mediated Tumor Necrosis Factor-α Synthesis and Apoptosis in Macrophages*⑤

Jingjing Ben[‡§1], Yan Zhang[‡1], Rongmei Zhou[‡], Haiyang Zhang[¶], Xudong Zhu[‡], Xiaoyu Li[‡], Hanwen Zhang[‡], Nan Li[‡], Xiaodan Zhou[‡], Hui Bai[‡], Qing Yang[‡], Donghai Li[¶], Yong Xu[‡§], and Qi Chen[‡§2]

From the ‡Atherosclerosis Research Center, Key Laboratory of Cardiovascular Disease and Molecular Intervention and §State Key Laboratory of Reproductive Medicine, Nanjing Medical University, Nanjing 210029, China and the ¶Jiangsu Engineering Research Center for microRNA Biology and Biotechnology, State Key Laboratory of Pharmaceutical Biotechnology, Nanjing University, Nanjing 210029, China

Background: Class A scavenger receptor (SR-A) influences the synthesis of pro-inflammatory mediators and apoptosis in macrophages.
Results: Major vault protein (MVP) interacts with SR-A and modulates SR-A-caveolin-p38/JNK-mediated TNF-α production and apoptosis in macrophages.
Conclusion: MVP may fine-tune SR-A activity in macrophages contributing to atherogenesis.
Significance: Targeting MVP-SR-A complex in macrophages may yield viable solutions for the intervention of atherosclerosis.

Atherosclerosis is considered a disease of chronic inflammation largely initiated and perpetuated by macrophage-dependent synthesis and release of pro-inflammatory mediators. Class A scavenger receptor (SR-A) expressed on macrophages plays a key role in this process. However, how SR-A-mediated pro-inflammatory response is modulated in macrophages remains ill defined. Here through immunoprecipitation coupled with mass spectrometry, we reported major vault protein (MVP) as a novel binding partner for SR-A. The interaction between SR-A and MVP was confirmed by immunofluorescence staining and chemical cross-linking assay. Treatment of macrophages with fucoidan, a SR-A ligand, led to a marked increase in TNF-α production, which was attenuated by MVP depletion. Further analysis revealed that SR-A stimulated TNF-α synthesis in macrophages via the caveolin- instead of clathrin-mediated endocytic pathway linked to p38 and JNK, but not ERK, signaling pathways. Importantly, fucoidan invoked an enrichment of MVP in lipid raft, a caveolin-reliant membrane structure, and enhanced the interaction among SR-A, caveolin, and MVP. Finally, we demonstrated that MVP elimination ameliorated SR-A-mediated apoptosis in macrophages. As such, MVP may fine-tune SR-A activity in macrophages which contributes to the development of atherosclerosis.

Atherosclerosis is a chronic inflammatory disease characterized by the development of arterial plaques with both early infiltration and persistent presence of macrophages. Macrophages derived from recruited monocytes participate in a maladaptive, nonresolving inflammatory response that expands the subendothelial layer by the accumulation of cells, lipids, and matrix. Activated macrophages not only transform into foam cells via uptake of modified low density lipoproteins (LDL), but also aberrantly produce a sea of inflammatory cytokines that play pivotal roles in atherogenesis (1–4).

Class A scavenger receptor (SR-A)[3] is a multifunctional macrophage receptor that is up-regulated during monocyte differentiation into macrophages, and its role in atherosclerosis has been extensively investigated. SR-A expression is largely confined to macrophages and dendritic cells but can be induced in the endothelium and smooth muscle cells in atherosclerotic plaques. A range of endogenous and exogenous substances, including modified LDL, fucoidan, advanced glycation end products, and apoptotic cells, have been reported as the ligands for SR-A. By binding with them, SR-A can influence a wide variety of macrophage functionalities including foam cell formation, phagocytosis of bacteria, cell adhesion, and apoptosis (5, 6). Of particular interest, several studies have demonstrated that SR-A regulates cytokine production in response to various kinds of inflammatory stimuli, positioning SR-A as an integral part of the macrophage-dependent pro-inflammatory machinery (7–12).

Attempts have been made to dissect the network that impacts the ability of SR-A to modulate the inflammatory response in macrophages. For instance, Hsu *et al.* (7) and Coller *et al.* (8) have independently reported that protein-tyrosine kinase and protein kinase C are involved in SR-A-dependent

[3] The abbreviations used are: SR-A, class A scavenger receptor; ER, endoplasmic reticulum; MPM, mouse peritoneal macrophage; MVP, major vault protein; PI, propidium iodide; EDC, l-ethyl-3-(3-dimethylaminopropyl)carbodiimide; NHS, N-hydroxysulfosuccinimide.

secretion of pro-inflammatory cytokines. It has also been documented that HSP90 and HSP70, among others, interact with the cytoplasmic domain of SR-A, likely affecting the signaling cascades downstream of SR-A that results in accelerated synthesis and release of pro-inflammatory cytokines (13). Our previous investigation has led to the discovery of the regulatory property of glucose-regulated protein 78 on SR-A-mediated internalization of acetylated LDL in macrophages (14). Further investigation along this line would potentially reveal more mechanistic insight into the intricate nature of SR-A-dependent pathobiological processes in macrophages.

In the present study, we have identified major vault protein (MVP) as a novel binding partner for SR-A exploiting proteomic tools. MVP promotes SR-A-mediated TNF-α synthesis and apoptosis in macrophages. Therefore, targeting the MVP-SR-A complex in macrophages may yield viable solutions for the intervention of atherosclerosis.

EXPERIMENTAL PROCEDURES

Cell Culture—Mouse peritoneal macrophages (MPMs) were harvested from the peritoneal cavity, washed with PBS, resuspended in Roswell Park Memorial Institute 1640 medium (RPMI 1640 medium; Hyclone) containing 10% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin and plated on dishes. After a 2-h incubation at 37 °C, nonadherent cells were removed, and the remaining adherent cells were cultured. Human peripheral blood mononuclear cells were isolated from healthy donors by a density gradient centrifugation. Monocytes were further purified by attachment assay. Macrophages were obtained by culturing adherent monocytes in tissue culture flask containing 1 ng/ml macrophage colony-stimulating factor (M-CSF; Sigma) for 5 days in X-Vivo 15 medium (Bio-Whittaker) supplemented with 10% human serum. RAW264.7 and THP-1 cells (American Type Culture Collection, ATCC) were cultured in RPMI 1640 medium containing 10% FCS, supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin. Phorbol 12-myristate 13-acetate (100 nM; Sigma,) was added to THP-1 cells for 3 days to induce a macrophage phenotype of differentiation.

Immunoprecipitation and Western Blotting—Cells were washed twice with PBS and treated in lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, protease inhibitors; Beyotime China). For immunoprecipitation, mouse SR-A was immunoprecipitated with a monoclonal antibody (2F8; BMA), and human SR-A and MVP were immunoprecipitated with anti-SR-A (Santa Cruz Biotechnology) and anti-MVP antibodies (Abcam). Cell lysates were incubated with antibodies at 4 °C overnight, and immune complexes were recovered by incubation at 4 °C for 2 h with protein G PLUS-agarose (Santa Cruz Biotechnology). Immunoprecipitates were collected, washed three times in lysis buffer at 4 °C, and eluted into Laemmli sample buffer by boiling.

Cell lysates or immunoprecipitates were separated by SDS-PAGE. Proteins were transferred to a PVDF membrane and blocked for 30 min in blocking buffer (Tris-buffered saline, pH 7.6, 0.05% Tween 20, and 3% BSA). After incubation with primary antibody diluted in blocking buffer overnight at 4 °C and washing, the blot was incubated for 60 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 SR-A, MVP, clathrin, caveolin (Sigma), *p*-p38, p38, *p*-JNK, JNK, *p*-ERK, ERK, caspase 3 (Cell Signaling Technology), and β-actin (Santa Cruz Biotechnology) were used. Quantification was performed by measurement of the intensity of the signals with the use of ImageJ software.

Indirect Immunofluorescence—MPMs 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 15 min, and blocked with 3% BSA, 0.01% Tween 20, PBS (PBST-BSA) for 30 min. The primary antibody against SR-A (2F8, rat; BMA), MVP (goat; Santa Cruz Biotechnology), caveolin (rabbit; Sigma), Syntaxin6 (rabbit; Abcam) or Erp 72 (rabbit; Abcam) in PBST-BSA was incubated with cells overnight at 4 °C, and the fluorochrome-conjugated secondary antibodies (FITC-donkey anti-rat, Cy3-donkey anti-goat, Alexa Fluor 647-donkey anti-rabbit; Invitrogen) in PBST-BSA was added for 30 min. Morphologic observation was performed with a confocal fluorescence microscope (LSM710; Carl Zeiss).

Chemical Cross-linking Reactions—Cells were dissociated by trypsin-EDTA solution and centrifuged at 1000 rpm for 5 min, and then cells were washed by PBS and centrifuged as described above. Subsequently, the precipitation was resuspended with 100 μl of PBS gently, and the concentration of total protein was determined by BCA assays. The suspension of cells was diluted to 2 mg/ml with PBS, and a final volume of 500 μl was needed for the following chemical cross-linking assays. For cross-linking reaction with EDC/sulfo-NHS, 10 μl of a freshly prepared aqueous cross-linker solution was added into 500 μl of cell suspension, with final concentrations of EDC and sulfo-NHS of 10 and 5 mM, respectively. The reaction mixtures were gently incubated at room temperature, and the reactions were quenched by the addition of 10 μl of aqueous dithiothreitol (DTT); the final concentration of DTT was 20 mM. Finally, cells were centrifuged at 3000 $\times$ *g* and 4 °C for 10 min, and the precipitation was used for the following Western blotting assays (15).

siRNA Transfection—Transfections were performed by using Lipofectamine 2000 (Invitrogen). The target sequence for the MVP was as follows: sequence 1, CAUAAGAACUCAG-CACGUAtt and UACGUGCUGAGUUCUUAUGga; sequence 2, CCAUCGAAACUGCAGAUCAtt and UGAUCUGCAGU-UUCGAUGGtg. The target sequence for mouse clathrin heavy chain gene was as follows: GGCUUCUAAAUAUCACGAAtt and UUCGUGAUAAUUUAGAAGCCac. The target sequence for the caveolin 1 gene was CCAUCUACGUCCAUACCUUtt and AAGGUAUGGACGUAGAUGGag. 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) for 72 h.

ELISA—RAW264.7 cells were incubated with fucoidan (50 μg/ml) for 18 h after siRNA transfection or after treatment with inhibitors for p38 (SB203580), JNK (SP600125), or ERK

(PD98059). Then supernatant of cells was collected and stored frozen until assayed. The TNF-α was measured by sandwich ELISA using the Quantikine TNF-α ELISA kit (R&D Systems) according to the manufacturer's instruction.

Isolation of Lipid Raft—Cells (1×10^8) from flasks were washed and resuspended in 5 ml of ice-cold PBS, centrifuged, and resuspended in 2 ml of MES buffer (25 mM MES, pH 6.5, and 150 mM NaCl) containing 1% Triton X-100, 1 mM phenyl-methylsulfonyl fluoride (PMSF), and 1 μg/ml leupeptin and homogenized by pipetting up and down 10 times through a 1000-μl blue tip. The extract was adjusted to 40% sucrose by adding 2 ml of 80% sucrose, and a total volume of 4 ml was transferred to an ultracentrifuge tube and overlaid with 1 ml of 35, 30, 25, 20, 15, and 5% sucrose in MES buffer as above but minus the Triton X-100. All of the materials were kept at 0 °C. The gradient was centrifuged at 39,000 rpm for 18 h at 2 °C in a Beckman SW50.1 rotor. Ten 1-ml fractions were removed from the top of the tubes. These fractions were diluted 1:2 in MES buffer containing Triton X-100, and the complexes were collected by centrifugation for 15 min in a microcentrifuge at 12,000 rpm. Pellets were solubilized in Laemmli sample buffer, boiled, and analyzed by Western blotting (16, 17).

Apoptosis Assay—After treatment with siRNA, RAW264.7 cells were washed, resuspended in the staining buffer, and examined with the annexin V-FITC and 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.E. Statistical significance between the groups was assessed by one-way analysis of variance. The level of significance was chosen as $p < 0.05$.

RESULTS

Identification and Verification of Major Vault Protein as a Novel Binding Partner for SR-A—To screen for protein couplers with SR-A that may potentially impact SR-A activity, we purified SR-A from either wild type (WT) or SR-A-null (KO) MPMs by immunoprecipitation. The immune complex was separated by SDS-PAGE and analyzed by silver staining (Fig. 1A). By comparing the patterns of the WT group and the KO group, we then analyzed the identities of discrete bands in the WT group by mass spectrometry. Among of the newly discovered SR-A binding partners was MVP, which achieved a score of 65 and 16% sequence coverage by the MASCOT search engine (Fig. 1B).

To further prove the existence of an SR-A-MVP complex in macrophages, the following experiments were conducted. We first probed the MVP-SR-A interaction by co-immunoprecipitation followed by Western blotting. Indeed, MVP was brought down by the anti-SR-A antibody using lysates derived from MPMs (Fig. 2A) and human peripheral blood monocytes/macrophages (supplemental Fig. S1). Next, we employed immunofluorescence staining to demonstrate the co-localization of SR-A and MVP. As shown in Fig. 2C, there was a clear overlap (*yellow*) of MVP (*red*) and SR-A (*green*) on the membranes and cytoplasm of MPMs. Also, MVP and SR-A could be
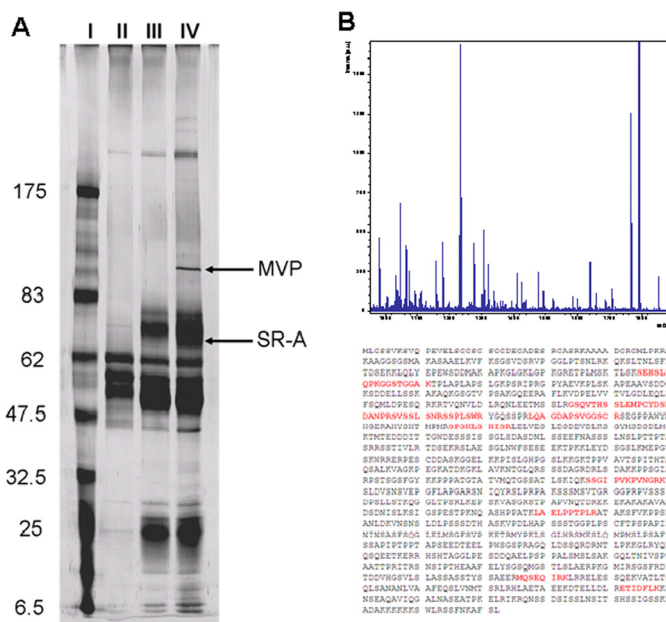

FIGURE 1. **MVP is a novel binding partner for SR-A.** *A*, SR-A was immunoprecipitated from MPMs, and the protein complexes were analyzed by SDS-PAGE and silver staining. *Lane I*, protein marker. *Lane II*, MPMs lysates incubated with protein G PLUS-agarose. *Lane III*, SR-A KO MPM lysates incubated with protein G PLUS-agarose and anti-SR-A antibody. *Lane IV*, WT MPM lysates incubated with protein G PLUS-agarose and anti-SR-A antibody. *B*, the predominant 110-kDa protein bound to the SR-A was identified as MVP by a mass spectrometry analysis.

co-localized on Golgi (Syntaxin6-positive staining) and ER (Erp 72-positive staining) (supplemental Fig. S2). Finally, we examined whether SR-A and MVP are in direct contact with each other using chemical cross-linking assays. Only after the addition a cross-linker (EDC/sulfo-NHS) did the SR-A-MVP complex started to appear (Fig. 2B), suggesting that MVP interacts with SR-A directly.

MVP Knockdown Inhibits SR-A-mediated TNF-α Production—Having established and confirmed the interaction between SR-A and MVP, we sought to determine whether and how MVP might affect SR-A function. Fucoidan, a polyanionic polysaccharide, has been used as a ligand for SR-A to stimulate the production of cytokines in macrophages (7, 18, 19). WT and SR-A KO MPMs were treated with fucoidan, and cytokine expression was examined by quantitative RT-PCR and ELISA. Interestingly, the induction of TNF-α, but not that of IL-6, by fucoidan was significantly dampened in SR-A KO MPMs compared with WT macrophages (Fig. 3A and supplemental Fig. S3). Accordingly, we focused on TNF-α expression hereafter.

To investigate the role of MVP in SR-A-mediated TNF-α expression in macrophages, we used siRNA to knock down MVP. Two different pairs of siRNAs reduced the protein levels of MVP by >60% (Fig. 3B). Accompanying the decrease in MVP expression was the attenuation of TNF-α release from macrophages (Fig. 3C). Therefore, MVP is required for SR-A-mediated TNF-α production in macrophages.

MVP Is Required for SR-A-Caveolin-p38/JNK Pathway-mediated TNF-α Production—We have previously shown that endocytosis of SR-A is through both the caveolae-p38/JNK pathway and the clathrin-ERK pathway (18). To determine how these two pathways might differentially contribute to TNF-α
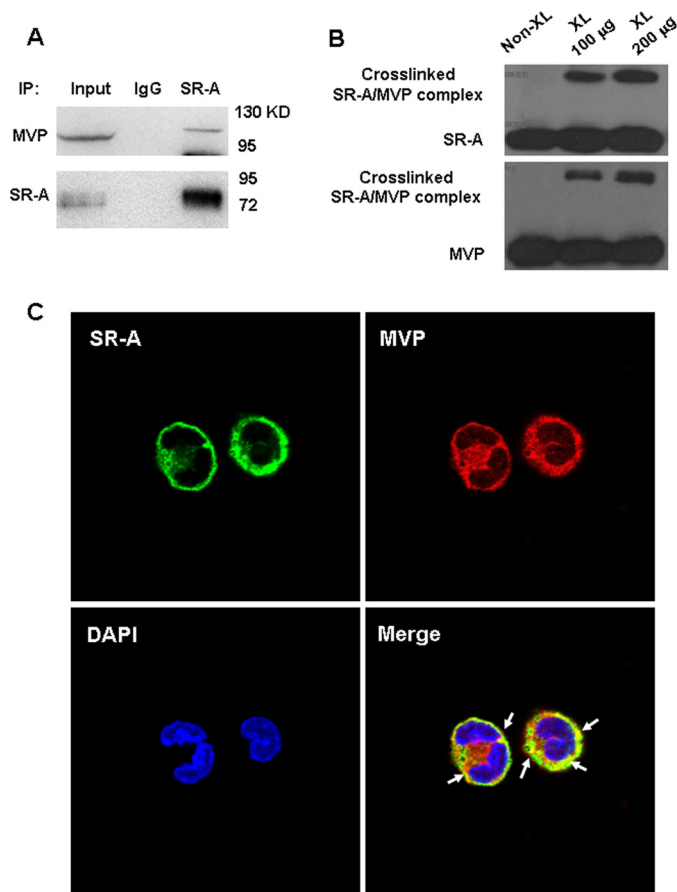
FIGURE 2. **Co-localization and interaction of endogenous SR-A and MVP in macrophages.** *A*, the MPM lysates were immunoprecipitated (*IP*) with antibody against SR-A or IgG. The immunoprecipitates were analyzed by Western blotting using the antibodies against SR-A and MVP. *B*, a chemical cross-linking (*XL*) reaction was carried out to establish the direct interaction between MVP and SR-A in RAW264.7 cells. After the chemical cross-linking reaction, the reaction mixtures were analyzed by Western blotting with the indicated antibodies. The *top* and *bottom panels* were probed with anti-SR-A or anti-MVP antibody, respectively. The *top bands* on both *panels* are MVP-SR-A complexes. *C*, indirect immunofluorescence experiments were performed using antibodies against SR-A and MVP in MPMs. Nuclei were stained with DAPI. *Merged images* showed overlapping distribution which appears *yellow*.

production in macrophages, endogenous caveolin (Fig. 4*Aa*) or clathrin (Fig. 4*Ab*) was individually silenced by siRNA. The production of TNF-α was blunted by caveolin knockdown but remained unaltered by clathrin knockdown (Fig. 4*B*). These data point to a SR-A-caveolin route in mediating TNF-α synthesis in response to fucoidan treatment in macrophages.

Based on the discovery of requirement of MVP in SR-A-mediated TNF-α production, we next examined the involvement of MVP in the activation of MAP kinases initiated by SR-A-caveolae endocytosis. As shown in Fig. 4*C*, treatment with fucoidan led to a rapid activation of all three MAP kinases in macrophages. MVP elimination crippled the activation of p38 and JNK while leaving ERK activation intact, affirming the notion that MVP is coupled to the SR-A-caveolae pathway rather than the SR-A-clathrin pathway. Lending additional support to this model is our observation that treatment with selective inhibitor for p38 (SB203580) or JNK (SP600125), but not ERK (PD98059), ameliorated TNF-α release from fucoidan-treated macrophages (Fig. 4*D*). Collectively, these

data suggest that MVP be required for the SR-A-caveolae-p38/JNK pathway to promote TNF-α synthesis in macrophages.

MVP Is Recruited to Lipid Raft in Response to Fucoidan Treatment—To verify whether MVP co-localizes with SR-A in the caveolae on cell membranes, we employed immunofluorescence staining to demonstrate the co-localization of SR-A, MVP, and caveolin. As shown in Fig. 5*A*, there was a clear overlap (*white*) of MVP (*red*), SR-A (*green*), and caveolin (*purple*) on MPMs. Also, we fractionated lipid rafts using RAW264.7 cell lysates by ultracentrifugation on a 5–40% sucrose gradient. Western blot analysis of the gradient fractions with an anticaveolin antibody showed that lipid rafts excluding other components were distributed in 25–30% sucrose fractions. Interestingly, we found that both MVP and SR-A could be detected in lipid rafts. Furthermore, treatment of cells with fucoidan caused more enrichment of MVP but not SR-A in lipid rafts (Fig. 5*B*).

Consistent with the above results, we further confirmed the interaction between MVP and caveolin, a marker of lipid rafts, by immunoprecipitation assay. Western blot analysis showed that fucoidan stimulation enhanced the complex formation between MVP and caveolin (Fig. 5*C*). Simultaneously, the interaction between MVP and SR-A was also augmented by fucoidan (Fig. 5*D*). Together, these data suggest that MVP be topologically attached to the SR-A-caveolae compartment in macrophages during the TNF-α synthesis induced by fucoidan.

MVP Knockdown Inhibits SR-A-Caveolae-dependent Apoptosis—It has been reported that SR-A indulged macrophage apoptosis is mediated by caveolae-p38/JNK pathway (18, 19). To address whether MVP could exert a regulative effect on SR-A-mediated apoptosis, we knocked down the expression of MVP using siRNA and measured cell apoptosis by FACS analysis and Western blotting. The staining of annexin V, an indicator of early apoptosis, was significantly up-regulated in macrophages by co-treatment with fucoidan and thapsigargin. This was averted by MVP silencing (Fig. 6*A*). Similarly, MVP ablation also blocked the increase in caspase 3 cleavage (Fig. 6*B*). Together, these results indicate that MVP may also dictate SR-A-indulged apoptosis, in addition to influencing SR-A-mediated TNF-α synthesis in macrophages.

DISCUSSION

Macrophages play a key role in atherosclerosis at all stages of disease development primarily by creating and maintaining a pro-inflammatory niche within the vasculature (20). The pattern recognition receptor SR-A is indispensible in the induction of innate immune and inflammatory responses (7–11). However, it remains to be sorted out how SR-A, sensing intrinsic and extrinsic cues, steers these responses and how the activity of SR-A itself is regulated. In the present study, we have characterized the interaction between SR-A and a newly identified binding partner, MVP. Our data as summarized here illustrate a modulating mechanism of MVP on SR-A-caveolae-p38/JNK pathway-mediated TNF-α production and apoptosis in the macrophage (Fig. 7). Treatment with SR-A ligand fucoidan can promote recruitment of MVP to lipid rafts to form an SR-A-MVP complex. This in turn activates p38 and JNK signaling pathways, causing TNF-α synthesis and cell apoptosis. The
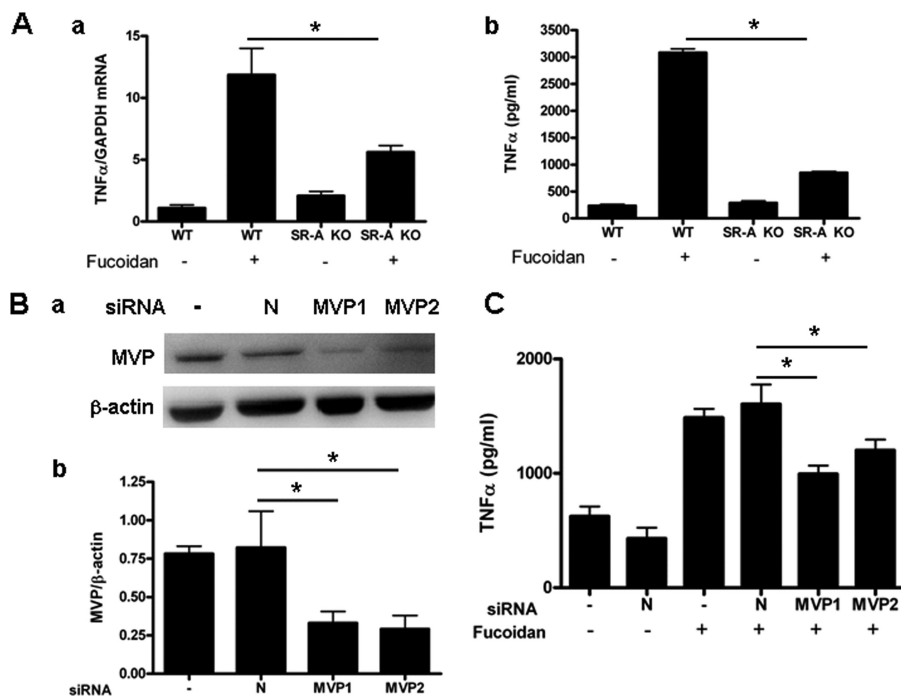
FIGURE 3. **MVP knockdown inhibits fucoidan/SR-A-mediated TNF-α production.** *A*, WT and SR-A KO MPMs were treated with fucoidan for 18 h. The cells and supernatants were harvested. TNF-α expression level was detected by quantitative RT-PCR (*a*) and ELISA (*b*). *B*, MVP was knocked down by siRNA in RAW264.7 cells. *a*, RAW264.7 cells were transfected with 30 nM irrelevant siRNA (siRNA-N), MVP-targeting siRNA-1 (siRNA-MVP1), or MVP-targeting siRNA-2 (siRNA-MVP2) for 72 h, respectively. Cell lysates were subjected to Western blot analysis with the indicated antibodies. Untreated cells were used as a control. *b*, MVP levels were determined upon normalization for β-actin. *C*, MVP knockdown inhibits fucoidan-SR-A-mediated TNF-α production. RAW264.7 cells were transfected with siRNA-N, siRNA-MVP1, or siRNA-MVP2 for 72 h, respectively. They were then incubated with fuciodan for 18 h. The supernatant was harvested and detected for TNF-α production by ELISA.
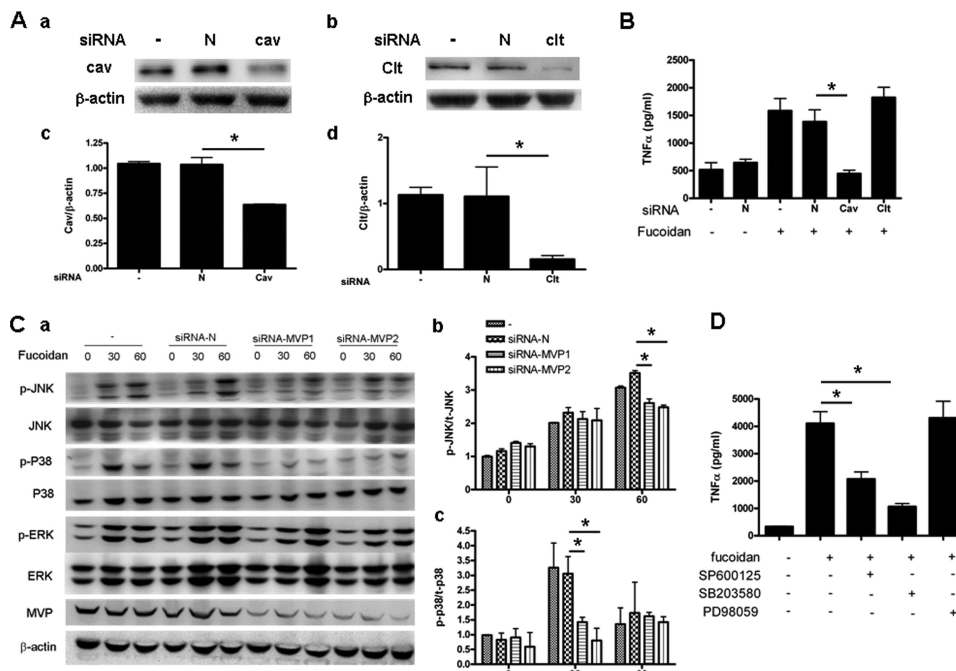

FIGURE 4. **Caveolae-p38/JNK pathway is required for modulation of MVP on SR-A-mediated TNF-α production.** *A*, RAW264.7 cells were transfected with 30 nM siRNA-N, caveolin-1-targeting siRNA (siRNA-cav), or clathrin heavy chain-targeting siRNA (siRNA-clt) for 72 h, respectively. Cell lysates were subjected to Western blotting with the indicated antibodies. Untreated cells were used as a control. The presented blot (*a* and *b*) is one of three independent blots, and quantitative data (*c* and *d*) are presented. *B*, knockdown of caveolin inhibits SR-A-mediated TNF-α production. RAW264.7 cells were transfected with siRNA-N, siRNA-clt, or siRNA-cav for 72 h, respectively, and incubated with fucoidan for 18 h. The supernatant was harvested and detected for TNF-α production by ELISA. *C*, MVP knockdown inhibits caveolae and p38/JNK-dependent SR-A endocytosis pathway. *a*, RAW264.7 cells were transfected with siRNA-N, siRNA-MVP1, or siRNA-MVP2 for 72 h, respectively. The cells were then incubated with fucoidan for 0, 30, or 60 min, respectively. Cell lysates were subjected to Western blotting with the indicated antibodies. *b*, *p*-JNK levels were determined upon normalization for JNK. *c*, *p*-p38 levels were determined upon normalization for p38. *D*, the inhibitors for JNK and p38 block SR-A-mediated TNF-α production. RAW264.7 cells were incubated with inhibitor for p38 (SB203580), JNK (SP600125), or ERK (PD98059), respectively, and then treated with fucoidan. The supernatant was collected and detected for TNF-α production by ELISA. Results are expressed as mean $\pm$ S.E. (*error bars*) of triplicate samples. *, $p < 0.05$.
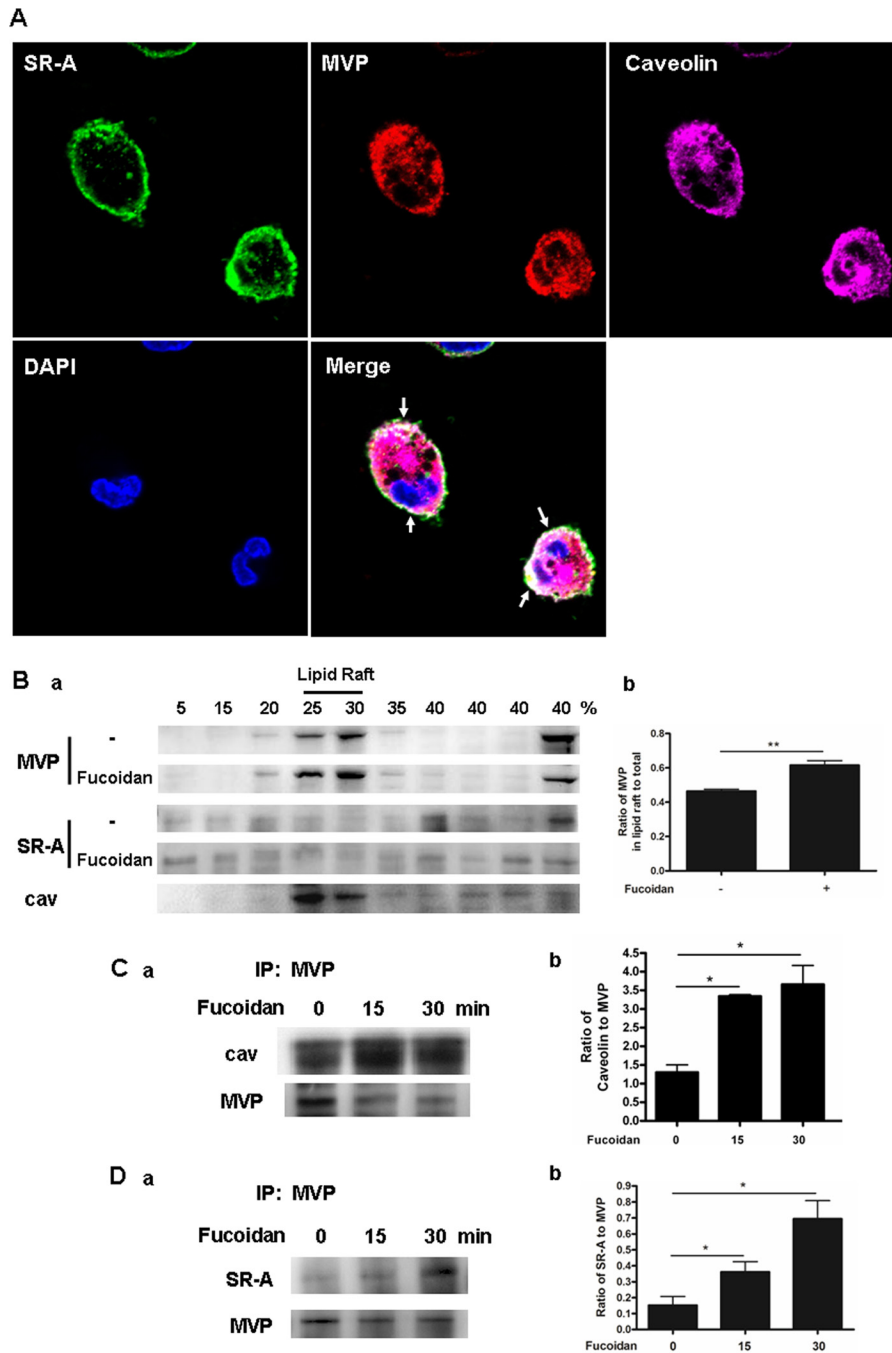
FIGURE 5. **MVP is recruited to lipid raft in response to fucoidan treatment.** *A*, indirect immunofluorescence experiments were performed using antibodies against SR-A, MVP, and caveolin in MPMs. Nuclei were stained with DAPI. *Merged images* show overlapping distribution which appears *white*. *B*, MVP is recruited to lipid raft in response to fucoidan treatment. *a*, RAW264.7 cells were incubated with fucoidan for 30 min. The whole cell lysates were used for sucrose gradients ultracentrifugation as described under "Experimental Procedures." The fractions collected were subjected to Western blotting with anti-MVP, anti-SR-A, or anti-caveolin antibodies. *b*, MVP levels were determined as described in *a*. MVP levels in 25–30% fractions upon normalization for total MVP are shown. Results are expressed as mean ± S.E. (*error bars*) of triplicate samples. *, $p < 0.05$. *C*, fucoidan treatment stimulates interaction of MVP with caveolin in THP-1 cells. *a*, phorbol 12-myristate 13-acetate-treated THP-1 cells were incubated with fucoidan for 0, 15, or 30 min, respectively, and the cell lysates were immunoprecipitated (*IP*) with the antibody against MVP. The immunoprecipitates were analyzed by Western blotting using the indicate antibodies. *b*, caveolin levels are shown upon normalization for total MVP immunoprecipitated by the antibody against MVP. *D*, fucoidan treatment stimulates interaction of MVP with SR-A in THP-1 cells. *a*, phorbol 12-myristate 13-acetate-treated THP-1 cells were incubated with fucoidan for 0, 15, or 30 min, respectively, and the cell lysates were immunoprecipitated by the antibody against MVP. The immunoprecipitates were analyzed by Western blotting using the indicated antibodies. *b*, SR-A levels are shown upon normalization for total MVP immunoprecipitated by the antibody against MVP. Results are expressed as mean ± S.E. of triplicate samples. *, $p < 0.05$.

functional relevance of SR-A-MVP in the cytoplasm including ER and Golgi warrants further investigation.

Initially discovered as a lung resistance-related protein, MVP is the predominant component of a large, cytoplasmic ribonu- cleoprotein particle, and the vault complex. MVP expression and/or activity is known to fluctuate according to environmen- tal signals, essentially positioning MVP as a stress protein (21– 24). It has been proposed to carry out important pathobiologi-
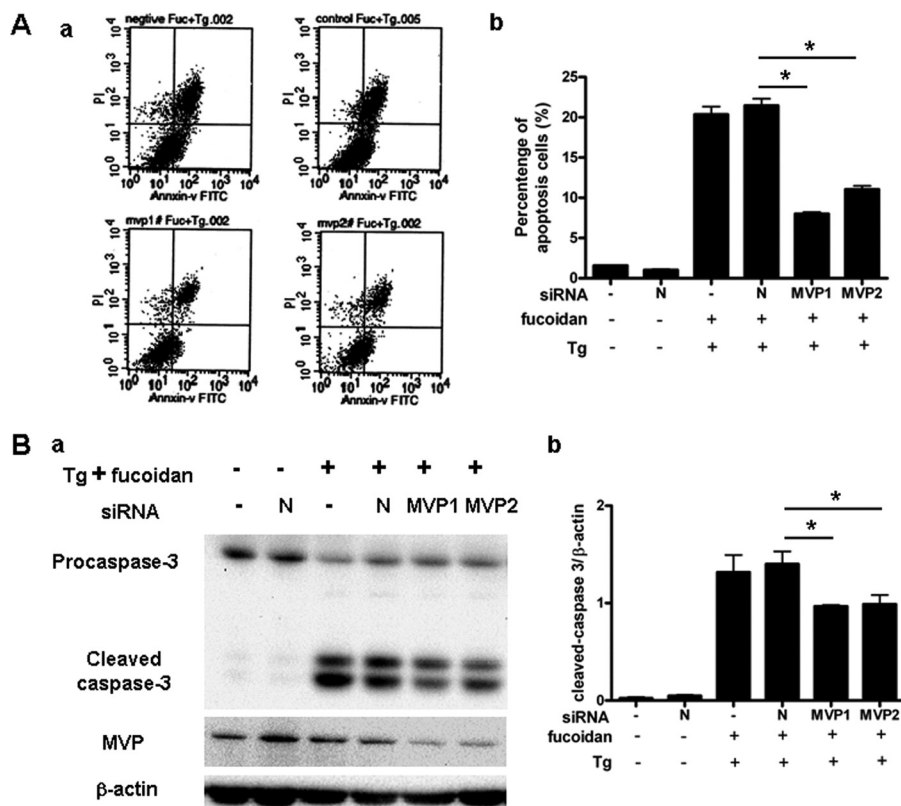
FIGURE 6. **MVP knockdown inhibits SR-A-mediated macrophage apoptosis.** *A*, RAW264.7 cells were transfected with siRNA-N, siRNA-MVP1, or siRNA-MVP2 for 72 h, respectively. Untreated cells were used as a control. Cells were incubated with fucoidan plus 0.5 μM thapsigargin (Tg) for 12 h. Cells were stained with annexin V and PI and analyzed by FACS. The apoptotic cells (the annexin V-positive and PI-negative cells) are indicated as the percentage of gated cells. Results were expressed as mean $\pm$ S.E. (*error bars*) of triplicate samples. *, $p < 0.05$. *B*, RAW264.7 cells were transfected with siRNA-N, siRNA-MVP-1, or siRNA-MVP2, respectively. Untreated cells were used as a control. Cells were incubated with fucoidan plus 0.5 μM thapsigargin for 12 h. Cell lysates were subjected to Western blotting with the indicated antibodies. Results are expressed as mean $\pm$ S.E. of triplicate samples. *, $p < 0.05$.
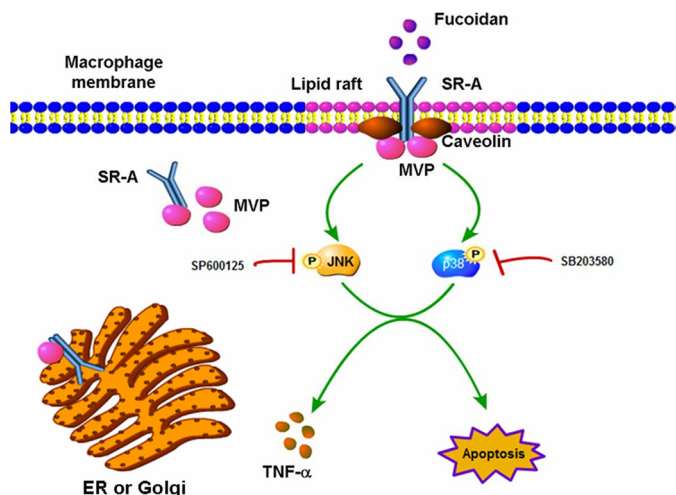

FIGURE 7. **Hypothetical modulation pattern of MVP on fucoidan-SR-A complex-induced TNF-α synthesis and apoptosis in macrophages.** MVP co-localizes with SR-A on macrophage cell membrane and cytoplasm, including the ER and Golgi. Treatment with fucoidan, a ligand of SR-A, can promote recruitment of MVP to lipid rafts to form an SR-A-MVP complex. This in turn activates p38 and JNK signaling pathways causing TNF-α synthesis and cell apoptosis.

cal functions in drug resistance, intracellular transport, cellular differentiation, innate immunity, virus infections, and cell survival (25, 26). The ability of MVP to modulate cell function is largely derived from its virtue of being a scaffolding protein that

coordinates intracellular signaling transduction. For instance, MVP is known to influence EGF signaling (27) and PI3K/Akt signaling (28) by interacting with and amending the activity of SHP2 and PTEN, respectively. Our results indicate that in fucoidan-treated macrophages, MVP is necessary for the activation of p38 and JNK kinases but not ERK. To our best knowledge, this is heretofore the first demonstration that MVP is specifically tied to some, but not all, of the MAPK pathways in macrophages. Previous investigations have shown that MVP can attenuate Src- and YPEL4-induced ERK activation (29, 30). Clearly, this discrepancy reflects on not only the cell types, but also the complicated nature of intracellular signaling of which MVP is an intimate part. Future investigations employing high throughput proteomic tools should shed additional light on the precise role MVP plays in regulating signal transduction.

Our previous study has demonstrated that fucoidan-SR-A internalization occurs through two endocytic routes, namely the clathrin-dependent and the caveolae-dependent pathways, the latter of which is linked to p38/JNK signaling pathway and is responsible for SR-A-indulged apoptosis (18). Our new findings extend this line of investigation and allude to an MVP-containing complex that couples to SR-A-mediated endocytosis to the release of TNF-α and apoptosis. Interestingly, MVP can be rapidly recruited to lipid rafts when human lung epithelial cells are infected with *Pseudomonas aeruginosa* (31), supporting a role for MVP in promoting the formation of membra-

nous structures. On the other hand, Dortet *et al.* have suggested that MVP may, by interacting with the bacterial protein InlK, facilitate the evasion of opportunistic bacterium by impairing the formation of autophagous vesicles (32). Similarly, we demonstrate here that a SR-A-MVP-caveolin complex serves as a prerequisite for apoptosis in macrophages whereas Ryu *et al.* have reported that MVP affords senescent fibroblast cells the resistance to oxidative stress induced apoptosis (23). The two seemingly distinct aspects of MVP activity are probably indications of its flexibility in mediating cellular events. By interacting with different factors, MVP may adapt and tailor to the specific needs of its binding partners.

Atherosclerosis is a chronic inflammatory disorder inflicted by excessive pro-inflammatory mediators such as TNF-α. A recent investigation led by the Zhu group suggests that MVP is required for the activation of an anti-viral transcriptional program in hepatocyte (33). Of note, MVP overexpression leads to the nuclear accumulation of NF-κB, the master regulator of pro-inflammatory transcription, highlighting its role in initiating and/or sustaining cellular inflammatory response, a notion that is strengthened by our findings.

Atherogenesis is shaped by multiple factors including oxidative stress, chronic inflammation, ER stress, and excessive nutrition. Of intrigue, there appears to be a strong correlation between the reorganization of lipid raft and one or more of the pro-atherogenic stress signals in macrophages (34–36). Therefore, we propose that in response to these various pro-atherosclerotic cues, MVP is recruited together with SR-A to the lipid raft wherein MVP facilitates the assembly of an SR-A-centered signaling complex to promote a series of downstream events including enhanced TNF-α synthesis and macrophage apoptosis. Future studies employing an animal model harboring macrophage-specific knock-in or knock-out of MVP will potentially provide additional rationale for targeting MVP in the intervention of atherosclerosis.

REFERENCES

1. Rader, D. J., and Daugherty, A. (2008) Translating molecular discoveries into new therapies for atherosclerosis. *Nature* **451,** 904–913
2. Tabas, I. (2010) Macrophage death and defective inflammation resolution in atherosclerosis. *Nat. Rev. Immunol.* **10,** 36–46
3. Klingenberg, R., and Hansson, G. K. (2009) Treating inflammation in atherosclerotic cardiovascular disease: emerging therapies. *Eur. Heart J.* **30,** 2838–2844
4. Moore, K. J., and Tabas, I. (2011) Macrophages in the pathogenesis of atherosclerosis. *Cell* **145,** 341–355
5. Moore, K. J., and Freeman, M. W. (2006) Scavenger receptors in atherosclerosis: beyond lipid uptake. *Arterioscler. Thromb. Vasc. Biol.* **26,** 1702–1711
6. Kzhyshkowska, J., Neyen, C., and Gordon, S. (2012) Role of macrophage scavenger receptors in atherosclerosis. *Immunobiology* **217,** 492–502
7. Hsu, H. Y., Chiu, S. L., Wen, M. H., Chen, K. Y., and Hua, K. F. (2001) Ligands of macrophage scavenger receptor induce cytokine expression via differential modulation of protein kinase signaling pathways. *J. Biol. Chem.* **276,** 28719–28730
8. Coller, S. P., and Paulnock, D. M. (2001) Signaling pathways initiated in macrophages after engagement of type A scavenger receptors. *J. Leukoc. Biol.* **70,** 142–148
9. Brown, J. M., Swindle, E. J., Kushnir-Sukhov, N. M., Holian, A., and Metcalfe, D. D. (2007) Silica-directed mast cell activation is enhanced by scavenger receptors. *Am. J. Respir. Cell Mol. Biol.* **36,** 43–52
10. Usui, H. K., Shikata, K., Sasaki, M., Okada, S., Matsuda, M., Shikata, Y.,

11. Ogawa, D., Kido, Y., Nagase, R., Yozai, K., Ohga, S., Tone, A., Wada, J., Takeya, M., Horiuchi, S., Kodama, T., and Makino, H. (2007) Macrophage scavenger receptor A-deficient mice are resistant against diabetic nephropathy through amelioration of microinflammation. *Diabetes* **56,** 363–372
11. Oz, H. S., Zhong, J., and de Villiers, W. J. (2009) Pattern recognition scavenger receptors, SR-A and CD36, have an additive role in the development of colitis in mice. *Dig. Dis. Sci.* **54,** 2561–2567
12. Xu, Y., Qian, L., Zong, G., Ma, K., Zhu, X., Zhang, H., Li, N., Yang, Q., Bai, H., Ben, J., Li, X., and Chen, Q. (2012) Class A scavenger receptor promotes cerebral ischemic injury by pivoting microglia/macrophage polarization. *Neuroscience* **218,** 35–48
13. Nakamura, T., Hinagata, J., Tanaka, T., Imanishi, T., Wada, Y., Kodama, T., and Doi, T. (2002) HSP90, HSP70, and GAPDH directly interact with the cytoplasmic domain of macrophage scavenger receptors. *Biochem. Biophys. Res. Commun.* **290,** 858–864
14. Ben, J., Gao, S., Zhu, X., Zheng, Y., Zhuang, Y., Bai, H., Xu, Y., Ji, Y., Sha, J., He, Z., and Chen, Q. (2009) Glucose-regulated protein 78 inhibits scavenger receptor A-mediated internalization of acetylated low density lipoprotein. *J. Mol. Cell. Cardiol.* **47,** 646–655
15. Li, D., Tang, H. Y., and Speicher, D. W. (2008) A structural model of the erythrocyte spectrin heterodimer initiation site determined using homology modeling and chemical cross-linking. *J. Biol. Chem.* **283,** 1553–1562
16. Anderson, H. A., Chen, Y., and Norkin, L. C. (1996) Bound simian virus 40 translocates to caveolin-enriched membrane domains, and its entry is inhibited by drugs that selectively disrupt caveolae. *Mol. Biol. Cell* **7,** 1825–1834
17. Scherer, P. E., Lisanti, M. P., Baldini, G., Sargiacomo, M., Mastick, C. C., and Lodish, H. F. (1994) Induction of caveolin during adipogenesis and association of GLUT4 with caveolin-rich vesicles. *J. Cell Biol.* **127,** 1233–1243
18. Zhu, X. D., Zhuang, Y., Ben, J. J., Qian, L. L., Huang, H. P., Bai, H., Sha, J. H., He, Z. G., and Chen, Q. (2011) Caveolae-dependent endocytosis is required for class A macrophage scavenger receptor-mediated apoptosis in macrophages. *J. Biol. Chem.* **286,** 8231–8239
19. Devries-Seimon, T., Li, Y., Yao, P. M., Stone, E., Wang, Y., Davis, R. J., Flavell, R., and Tabas, I. (2005) Cholesterol-induced macrophage apoptosis requires ER stress pathways and engagement of the type A scavenger receptor. *J. Cell Biol.* **171,** 61–73
20. Hansson, G. K., and Libby, P. (2006) The immune response in atherosclerosis: a double-edged sword. *Nat. Rev. Immunol.* **6,** 508–519
21. Hu, Y., Stephen, A. G., Cao, J., Tanzer, L. R., Slapak, C. A., Harrison, S. D., Devanarayan, V., Dantzig, A. H., Starling, J. J., Rome, L. H., and Moore, R. E. (2002) A very early induction of major vault protein accompanied by increased drug resistance in U-937 cells. *Int. J. Cancer* **97,** 149–156
22. Ikeda, R., Iwashita, K., Sumizawa, T., Beppu, S., Tabata, S., Tajitsu, Y., Shimamoto, Y., Yoshida, K., Furukawa, T., Che, X. F., Yamaguchi, T., Ushiyama, M., Miyawaki, A., Takeda, Y., Yamamoto, M., Zhao, H. Y., Shibayama, Y., Yamada, K., and Akiyama, S. (2008) Hyperosmotic stress up-regulates the expression of major vault protein in SW620 human colon cancer cells. *Exp. Cell Res.* **314,** 3017–3026
23. Ryu, S. J., An, H. J., Oh, Y. S., Choi, H. R., Ha, M. K., and Park, S. C. (2008) On the role of major vault protein in the resistance of senescent human diploid fibroblasts to apoptosis. *Cell Death Differ.* **15,** 1673–1680
24. Yi, C., Li, S., Chen, X., Wiemer, E. A., Wang, J., Wei, N., and Deng, X. W. (2005) Major vault protein, in concert with constitutively photomorphogenic 1, negatively regulates c-Jun-mediated activator protein 1 transcription in mammalian cells. *Cancer Res.* **65,** 5835–5840
25. Berger, W., Steiner, E., Grusch, M., Elbling, L., and Micksche, M. (2009) Vaults and the major vault protein: novel roles in signal pathway regulation and immunity. *Cell Mol. Life Sci.* **66,** 43–61
26. Lara, P. C., Pruschy, M., Zimmermann, M., and Henríquez-Hernández, L. A. (2011) MVP and vaults: a role in the radiation response. *Radiat. Oncol.* **6,** 148
27. Kolli, S., Zito, C. I., Mossink, M. H., Wiemer, E. A., and Bennett, A. M. (2004) The major vault protein is a novel substrate for the tyrosine phosphatase SHP-2 and scaffold protein in epidermal growth factor signaling. *J. Biol. Chem.* **279,** 29374–29385

28. Yu, Z., Fotouhi-Ardakani, N., Wu, L., Maoui, M., Wang, S., Banville, D., and Shen, S. H. (2002) PTEN associates with the vault particles in HeLa cells. *J. Biol. Chem.* **277,** 40247–40252

29. Kim, E., Lee, S., Mian, M. F., Yun, S. U., Song, M., Yi, K. S., Ryu, S. H., and Suh, P. G. (2006) Crosstalk between Src and major vault protein in epidermal growth factor-dependent cell signalling. *FEBS J.* **273,** 793–804

30. Liang, P., Wan, Y., Yan, Y., Wang, Y., Luo, N., Deng, Y., Fan, X., Zhou, J., Li, Y., Wang, Z., Yuan, W., Tang, M., Mo, X., and Wu, X. (2010) MVP interacts with YPEL4 and inhibits YPEL4-mediated activities of the ERK signal pathway. *Biochem. Cell Biol.* **88,** 445–450

31. Kowalski, M. P., Dubouix-Bourandy, A., Bajmoczi, M., Golan, D. E., Zaidi, T., Coutinho-Sledge, Y. S., Gygi, M. P., Gygi, S. P., Wiemer, E. A., and Pier, G. B. (2007) Host resistance to lung infection mediated by major vault protein in epithelial cells. *Science* **317,** 130–132

32. Dortet, L., Mostowy, S., Samba-Louaka, A., Gouin, E., Nahori, M. A., Wiemer, E. A., Dussurget, O., and Cossart, P. (2011) Recruitment of the major vault protein by InlK: a *Listeria monocytogenes* strategy to avoid autophagy. *PLoS Pathog.* **7,** e1002168

33. Liu, S., Hao, Q., Peng, N., Yue, X., Wang, Y., Chen, Y., Wu, J., and Zhu, Y. (2012) Major vault protein: a virus-induced host factor against viral replication through the induction of type-I interferon. *Hepatology* **56,** 57–66

34. Cuschieri, J., and Maier, R. V. (2007) Oxidative stress, lipid rafts, and macrophage reprogramming. *Antioxid. Redox Signal.* **9,** 1485–1497

35. Cheng, A. M., Handa, P., Tateya, S., Schwartz, J., Tang, C., Mitra, P., Oram, J. F., Chait, A., and Kim, F. (2012) Apolipoprotein A-I attenuates palmitate-mediated NF-κB activation by reducing Toll-like receptor-4 recruitment into lipid rafts. *PLoS One* **7,** e33917

36. Han, S., Liang, C. P., DeVries-Seimon, T., Ranalletta, M., Welch, C. L., Collins-Fletcher, K., Accili, D., Tabas, I., and Tall, A. R. (2006) Macrophage insulin receptor deficiency increases ER stress-induced apoptosis and necrotic core formation in advanced atherosclerotic lesions. *Cell Metab.* **3,** 257–266