The scavenger receptor SCARA1 (CD204) recognizes dead cells through spectrin

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Running title: SCARA1/CD204 recognizes dead cells through spectrin

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Abstract
Scavenger receptor class A member 1 (SCARA1 or CD204) is an immune receptor highly expressed on macrophages. It forms homotrimers on cell surface and plays important roles in regulating immune responses via its involvement in multiple pathways. However, both the structure and the functional roles of SCARA1 are not fully understood. Here, we determined the crystal structure of the C-terminal SRCR domain of SCARA1 at 1.8 Å resolution, revealing its Ca\(^{2+}\)-binding site. Results from cell-based assays revealed that SCARA1 can recognize dead cells, rather than live cells, specifically through its SRCR domain and in a Ca\(^{2+}\)-dependent manner. Furthermore, by combining MS and biochemical assays, we found that cellular spectrin is the binding target of SCARA1 on dead cells and that the SRCR domain of SCARA1 recognizes the SPEC repeats of spectrin in the presence of Ca\(^{2+}\). We also found that macrophages can internalize dead cells or debris from both erythrocytes and other cells through the interaction between SCARA1 and spectrin, suggesting that SCARA1 could function as a scavenging receptor that recognizes dead cells. These results suggest that spectrin, which is one of the major components of the cytoskeleton, acts as a cellular marker that enables the recognition of dead cells by the immune system.

Introduction
Scavenger receptors (SRs) were discovered in late 1970s through the uptake of the modified low density lipoprotein (LDL) by macrophages (1,2). Over the past decades, a large number of cell surface receptors have been identified as SRs and are categorized into more than 10 subfamilies or classes based on the sequence and structural similarities (3,4). SRs are typically expressed on antigen presenting cells such as macrophages and dendritic cells and involved in regulation of both innate and adaptive immune pathways (5,6). The ligands of SRs are quite diverse including both self and non-self targets (3,7), which is not surprising as numerous substances such as metabolites and dead cells need to be removed promptly to maintain body homeostasis, and evidence over the past years has shown that SRs are widely associated with diseases including autoimmunity, cardiovascular diseases and cancer (4,8,9)

SR class A (SR-A) has five known members, including SCARA1 (CD204, SR-A, SR-A1, MSR, etc.), SCARA2 (MARCO), SCARA3 (CSR), SCARA4 (SRCL) and SCARA5 (10). These molecules are type II transmembrane proteins and share similar structural features including a small N-terminal cytoplasmic region, a transmembrane helix and a large C-terminal extracellular portion (Fig. 1A), but their functions appear to be diverse based on the published data (11-13). The ectodomains of SR-A members have been predicted to form homotrimers on cell surface and may contain coiled-coil regions (CC), a collagen-like region (CL) and a C-terminal globular domain (Fig. 1A). Among them, SR class A member 1, SCARA1/CD204, is highly expressed...
on macrophages and dendritic cells (14). The ectodomain of SCARA1 contains a CC region, a CL region and a scavenger receptor cysteine-rich (SRCR) domain (15) (Fig. 1A). The SRCR domain is typically found in SRs and may contain cation binding sites, which are usually associated with ligand recognition (16). Previous studies have shown that SCARA1 is involved in multiple biological pathways and associated with diseases including cancer (17), cardiovascular disease (18) and Alzheimer's disease (19). A number of ligands of SCARA1 have been reported (14,20) and evidence has also shown that it might be involved in phagocytosis of dead cells by macrophages (21-23). However, the mechanisms of SCARA1 in these pathways are not fully understood.

In order to maintain body homeostasis, dead cells (apoptotic and necrotic cells) need to be removed promptly to prevent autoimmunity and other potential diseases (24-26). Phagocytes such as macrophages and dendritic cells play critical roles in the clearance of dead cells, which recognize the specific markers on dead cells by the surface receptors (27). Among the known dead cell markers, phosphatidylserine (PS) has been identified decades ago as a typical marker on the surface of apoptotic cells and can be recognized by several receptors and mediates apoptotic cell clearance (26,28). Recently, it has been shown that other cellular markers can also mediate the recognition of dead cells through different receptors on macrophages or dendritic cells (29-31), suggesting that the efferocytosis pathways could be more complex than previous thought (25,27,32,33).

Spectrin was initially identified in red blood cells and known as “ghost protein” as it locating on the medial side of erythrocyte membrane (34) and is important for maintaining the biconcave shape of erythrocytes (35,36). Later it was found that spectrin was also widely expressed in non-erythrocytes as a general component of cytoskeleton (37,38). Spectrin has an α chain and a β chain, and the sequence of α chain contains a number of repetitive domains (SPEC domains) as well as a SH3 domain and an EF hand domain (37,39,40). As a part of cytoskeleton, spectrin interacts with other cytoskeleton components such as actin, ankyrin, adducin, myosin and flotillin, thus forming a cytoskeleton network in cytoplasm, which is important for cell growth, tissue patterning and organ development (37,41-43), and it has also been linked to a number of diseases (44). But the functional roles of spectrin other than cytoskeleton remain unclear.

Here we determined the structure of the C-terminal SRCR domain of SCARA1 by crystallography and explored the function of SCARA1 using biochemical and biophysical methods, and found that SCARA1 could recognize dead cells specifically through cellular spectrin in a Ca\(^{2+}\)-dependent manner, suggesting a novel pathway for the immune recognition of dead cells.

**Results**

*Crystal structure of the C-terminal SRCR domain of SCARA1*
Since the intact ectodomain of SCARA1 contains three regions with flexible linkers in between (Fig. 1A), the structural determination of the whole ectodomain might be difficult. Therefore we expressed the C-terminal SRCR domain of mouse SCARA1 (residues 347–454) in insect cells and purified by Ni-NTA affinity chromatography and size-exclusion chromatography (SEC) (Fig. 1B). The purified SRCR domain was crystalized and the structure was solved by molecular replacement using the SRCR domain of Mac-2 binding protein (M2BP) (PDB entry: 1BY2) as a search model and refined to 1.8 Å resolution (Supplementary Table 1). The SRCR domain of SCARA1 has the typical SRCR fold (Fig. 1C), similar to the SRCR domains from other SRs (13,15,45). The SRCR domain of SCARA1 has a cation binding site coordinated with residues D379, D380 and E446 (Fig. 1D), which is modeled as Ca$^{2+}$ as it is included in the crystallization buffer. The residues around the Ca$^{2+}$ binding site are conserved for the mouse and the human SRCR domain of SCARA1, and a similar cation binding site has also been identified in the SRCR domain of SCARA2 (13). Although some other SRCR domains, for example, the SRCR domain of Mac-2 binding protein (M2BP) (PDB entry: 1BY2) (15) and the SRCR domain of CD6 (PDB entry: 5A2E) (45), do not contain the similar Ca$^{2+}$ binding site, the local conformations around the site are relatively conserved, suggesting that the Ca$^{2+}$ binding is largely contributed by the coordinating residues on protein surface.

Ca$^{2+}$-dependent recognition of dead cells by SCARA1

To explore the potential scavenging function of SCARA1, the ectodomains of both mouse and human SCARA1 fused with GFP were expressed in insect cells and purified by Ni-NTA affinity chromatography and SEC. The purified proteins were used for cell binding assays and monitored by flow cytometry. The results showed that mouse SCARA1 had no binding to live Jurkat cells or NIH 3T3 cells (Fig. 2A and S1A-C). However, when these cells were treated with actinomycin D (ActD) to induce apoptosis and necrosis, mouse SCARA1 exhibited binding activities to the dead cells in the presence of Ca$^{2+}$ (Fig. 2A and S1A-C), and the binding is pH-independent (Fig. 2A and S1A-C). Similar binding characteristics were also observed for human SCARA1 with Jurkat cells (Fig. 2B and S1D), and ELISA data also showed that SCARA1 could bind to Jurkat cell lysates (Fig. 2C). Moreover, confocal microscopy showed that SCARA1 could bind the permeabilized NIH 3T3 and HEK293 cells in the presence of Ca$^{2+}$ (Fig. 2D-E, S1E-F), suggesting that the ligands of SCARA1 might be the naturally existed cellular components, rather than cell death induced products. In addition, the binding data showed that human and mouse SCARA1 appeared to have cross activities in recognizing dead cells from human and mouse, this is not unexpected as the sequence identity between human and mouse SCARA1 is higher than 70% and the Ca$^{2+}$ binding site is also conserved for the SRCR domain from human and
mouse. Furthermore, Ca²⁺ was replaced by Mg²⁺, Na⁺ or K⁺ in the binding assays, and the results showed that Mg²⁺ could also mediate the recognition of SRCR with dead cells, but no binding was detected in the cases of Na⁺ and K⁺ (Fig.S1G), suggesting that other divalent cations may also induce the dead cell recognition by the SRCR domain of SCARA1.

To further characterize the binding of SCARA1 with dead cells, both viable and the ActD treated Jurkat cells were stained by the GFP-fused SCARA1, Annexin V-APC and propidium iodide (PI) in the presence of Ca²⁺ (Fig. 2F), and the FACS results showed that SCARA1 had no binding to viable cells (Annexin V-PI⁻), but was able to recognize both apoptotic (Annexin V⁺PI⁻) and necrotic cells (Annexin V⁺PI⁺), implying that the cellular ligands of SCARA1 were exposed at apoptotic and necrotic stages during cell death.

**SCARA1 recognizes dead cells through its C-terminal SRCR domain**

Based on the sequence of SCARA1, the ectodomain of SCARA1 is predicted to have a CC region, a CL region and a SRCR domain (Fig. 1A). To locate the dead cell recognizing region of SCARA1, we expressed a series of truncation mutants for binding assays. The results showed that the fragment containing the CC and the CL regions of SCARA1 had no binding to dead cells either in the presence or absence of Ca²⁺ (Fig. 3A and S2A). By contrast, a truncation mutant containing both the CL region and the SRCR domain showed strong binding to dead cells in the presence of Ca²⁺ at both acidic and basic pH (Fig. 3B and S2B). These results were also confirmed by dot blot assays with cell lysates (Fig. 3D) and confocal microscopy (Fig. 3F-G and S2C-F).

Since the Ca²⁺ binding site on the SRCR domain is conserved for human and mouse, we mutated the Ca²⁺ binding sites of the mouse SRCR domain by generating a single mutant (E446S) and a double mutant (D379S/D380S) (Fig. 1D), which were expressed in insect cells and purified similarly. The binding data showed that both mutants exhibited no binding affinities to the ActD treated Jurkat cells in the presence of Ca²⁺ (Fig. 1E and S3C). Similar results were obtained for the mutants with the frozen-thawed NIH 3T3 cells (Fig. 1F and S3D), confirming that the cation binding site is indispensable in recognizing dead cells by the SRCR domain.

Moreover, ELISA data showed that the CL-SRCR fragment had similar binding affinities to dead cells with Ca²⁺ concentration ranging from 2 mM to 20 mM (Fig. S1I), whereas no obvious binding was detected at 2 μM Ca²⁺ (Fig. S1H), which roughly corresponds to the Ca²⁺ concentration at endosomes (46).

**The trimeric form of SCARA1 increases the binding affinity to dead cells**

Previous structural prediction (14) suggested that the ectodomain of SCARA1 might form a homophilic trimer on cell surface, therefore we expressed the ectodomain of mouse SCARA1 (88-454) in insect cells (Fig. S3A), and the purified SCARA1 ectodomains were cross-linked by
glutaraldehyde and loaded onto SDS/PAGE, and indeed, it showed a band with the triple molecular weight (Fig. S3B), suggesting the formation of homotrimers. Since the SRCR domain alone is a monomer as shown in the crystal structure, the SCARA1 homotrimer is formed through the CC and the CL regions (Fig. 1A). Interestingly, FACS data showed that the monomeric SRCR domain only had weak binding to dead cells (Fig. 3H and S2I; Fig. 3I and S2J), whereas the trimeric SCARA1 ectodomain showed stronger binding to dead cells (Fig. 3H and S2I; Fig. 3I and S2J).

To validate the effect of trimerization of SCARA1 on dead cell recognition, we expressed an artificial homotrimer of SRCR by using a de novo designed trimeric tag 5J0J (PDB entry: 5J0J) (47) to replace the CC-CL region of SCARA1 (Fig. S2G). The results showed that the artificial SRCR trimer was able to recognize dead cells efficiently (Fig. 3C and S2H) and had similar binding activities to the trimeric SCARA1 ectodomain (Fig. 2A-B and S1A-D) and the CL-SRCR fragment (Fig. 3B and S2B), thus supporting that the formation of SCARA1 homotrimer could increase the binding affinity to dead cells significantly.

**SCARA1 recognizes protein ligands on dead cells**

To identify the ligands of SCARA1 on dead cells, we treated dead cells with a number of enzymes including protease K, DNase I, and RNase A. The results showed that only the treatment of protease K could block the binding of SCARA1 to dead cells (Fig. 3J), suggesting that SCARA1 might recognize dead cells through protein ligands. We also tested the potential lipid binding activities of SCARA1 by dot-blot assays, and no obvious binding was detected (Fig. 3E). Furthermore, we treated HEK293 cell lysates with protease K at different concentrations and with different incubation time in the dot-blot assays (Fig. S4A). The results showed that the binding of SCARA1 to dead cells diminished gradually as the protease K concentration and incubation time increased, confirming that SCARA1 may recognize protein ligands on dead cells.

**SCARA1 recognizes dead cells through cellular spectrin**

To isolate the protein ligands of SCARA1, purified human SCARA1 ectodomain was incubated with Jurkat cell lysates in the presence of Ca\(^{2+}\) and then pulled down with Ni-NTA beads. The pull-down assays were also performed in the absence of Ca\(^{2+}\) in parallel as controls. The eluates from Ni-NTA beads were analyzed by mass spectrometry (MS). Although hundreds of proteins were detected by MS, the potential ligands of SCARA1 could be identified by comparing the abundance of different proteins found in the eluates in the presence of Ca\(^{2+}\) with the controls in the presence EDTA. Indeed, the MS results showed that cellular spectrins could be pulled down by SCARA1 in the presence of Ca\(^{2+}\), and spectrin \(\alpha\)-chain was the major protein showing large differences over the controls (Fig. S4B), suggesting that spectrin might be the binding target of SCARA1 on dead cells.

Furthermore, we did dot blot assays using the purified
GFP-hCL-SRCR fragment (fused with a His-tag) with the cell lysates from Jurkat cells. The cell lysates were incubated with the Ni-NTA beads bound with the purified GFP-hCL-SRCR fragments in the presence of Ca\(^{2+}\) or EDTA, and the pull-down products were spotted onto nitrocellulose membranes and detected by anti-spectrin or anti-GFP antibodies. The results showed that spectrin could be pulled down by the GFP-hCL-SRCR fragment from the cell lysates only in the presence of Ca\(^{2+}\) (Fig. 3K).

To verify the interactions between SCARA1 and spectrin, we expressed the \(\alpha\)-chain of human non-erythrocytic spectrin in three fragments: F1 (resides 1-785), F2 (resides 784-1549) and F3 (resides 1544-2476) (Fig. 4A) in Escherichia coli and purified by Ni-NTA affinity chromatography and SEC. The ELISA data showed that all three spectrin fragments were able to bind to SCARA1 in a Ca\(^{2+}\)-dependent manner with similar binding profiles (Fig. 4B). Moreover, all three spectrin fragments were able to inhibit the binding of the CL-SRCR fragment of SCARA1 to dead cells efficiently (Fig. 4F-G, S5A-B), suggesting that spectrin is the binding target of SCARA1 on dead cells. In addition, the binding data also showed that both human and mouse SCARA1 could bind to human \(\alpha\)-spectrin, which is not surprising as \(\alpha\)-spectrins are well conserved between human and mouse.

To assess to exposure of spectrin during cell death, the binding of antibodies against spectrin with dead cells was monitored by FACS at different stages of cell death, and the results showed that the antibodies had no binding to viable cells (Annexin V\(^-\)PI\(^-\)), but could recognize both apoptotic (Annexin V\(^-\)PI\(^+\)) and necrotic cells (Annexin V\(^+\)PI\(^+\)) (Fig. 3L), similar to the SCARA1 binding results shown above (Fig. 2F), suggesting that cellular spectrins could be exposed at both apoptotic and necrotic stages. This seems not surprising as spectrins locate at the intracellular side of the plasma membrane, therefore could be exposed when membrane asymmetry is lost during apoptosis.

**The SRCR domain of SCARA1 recognizes the SPEC domain of spectrin specifically**

To identify the spectrin binding domain of SCARA1, three fragments of SCARA1, including the SRCR, the CL-SRCR and the CC-CL fragment, were expressed in insect cells and applied for dot blot assays (Fig. 4C-E). The results showed that both the SRCR and the CL-SRCR fragments could bind to the all three spectrin fragments in the presence of Ca\(^{2+}\), whereas the CC-CL fragment had no binding to the three fragments, suggesting that the SRCR domain might be the spectrin binding domain of SCARA1. Sequence analyses suggest that the \(\alpha\)-chain of human spectrin contains 19 SPEC domains (SPEC1 to SPEC19), a SH3 domain and an EF-hand domain (Fig. 4A) (37,48). The sequence identity among the individual SPEC domains is around 30%. To narrow down the binding region of SCARA1 on spectrin, we randomly chose three representative SPEC domains, including SPEC1, SPEC11 and SPEC9 (SPEC9 was chosen as it has a longer sequence among the SPEC domains),
as well as the SH3 domain and the EF hand domain for binding assays (Fig. 4A). The ELISA results showed that both mouse and human SCARA1 could bind to SPEC9 and SPEC11 in the presence of Ca\(^{2+}\), rather than the SH3 or the EF-hand domain of spectrin (Fig. 5A and S6A). Furthermore, the FACS data showed that both SPEC9 and SPEC11 could block the binding of SCARA1 to dead Jurkat cells almost completely (Fig. 5B and S6B-D), but the SH3 and the EF hand domains had no inhibition to the dead cell recognition, confirming that the SPEC domains of spectrin are the binding targets of SCARA1 on dead cells. The binding/inhibition experiments were also repeated using HEK293 and NIH 3T3 cells, and similar results were obtained (Fig. 5C-D, S6E-F). Moreover, two mutants of the CL-SRCR fragment, D379S/D380S and E446S, where the Ca\(^{2+}\) binding site on the SRCR domain were mutated, were also applied for ELISA assays, and the results showed that both mutants had no detectable binding to the SPEC domains (Fig. 5E), thus validating the Ca\(^{2+}\)-dependent binding of the SPEC domain by the SRCR domain of SCARA1. Furthermore, the binding affinities between SRCR (monomer), CL-SRCR (trimer) and 5J0J-SRCR (artificial trimer, Fig. S2G) with the SPEC domain were estimated based on the ELISA assays (Fig. 5H). The results showed that the trimerized SRCR domains, CL-SRCR and 5J0J-SRCR, had higher affinities for spectrin than the monomeric SRCR, consistent with the FACS results shown above.

Since spectrins are usually categorized as erythrocytic spectrins and non-erythrocytic spectrins (49), therefore we also expressed the SPEC domains, SPEC9\(_e\) and SPEC11\(_e\), from human erythrocytes for binding assays. The ELISA results showed that both human and mouse SCARA1 could bind to SPEC9\(_e\) and SPEC11\(_e\), similar to the SPEC domains from non-erythrocytic spectrin (Fig. 5F). The FACS data also showed that both SPEC9\(_e\) and SPEC11\(_e\) could block the binding of SCARA1 to dead cells efficiently as the non-erythrocytic SPEC domains (Fig. 5G, S6G-H), suggesting that SCARA1 might be a generic spectrin receptor for both erythrocytes and non-erythrocytes.

**SCARA1 transfected cells recognize spectrin though the SPEC domain**

To test the recognition of spectrin by SCARA1 at cellular level, the SCARA1 transfected HEK293 cells were incubated with either the SPEC11\(_e\) from erythrocytes or the SPEC17-18 fragment from non-erythrocytes. The FACS data showed that the HEK293 cells transfected with the full-length SCARA1 could recognize SPEC11\(_e\) and SPEC17-18 specifically and similar results were obtained for both human and mouse SCARA1 (Fig. 6A and B), whereas the non-transfected cells had no binding to SPEC11\(_e\) and SPEC17-18 (Fig. 6A), suggesting that SCARA1 expressed on the cell surface can recognize spectrin in the presence of Ca\(^{2+}\). Furthermore, the fluorescent images of the SCARA1 transfected cells by confocal microscopy showed that the SPEC domains and SCARA1 were well co-localized on cell surface and cytoplasm in the presence of Ca\(^{2+}\).
(Fig. 6C), suggesting that the SPEC domains can be internalized by SCARA1.

**Macrophages internalize dead cells from both erythrocytes and non-erythrocytes through SCARA1-spectrin interactions**

Before endocytosis assays, we tested the binding of SCARA1 with the purified mouse erythrocytes (>99%) (Fig. S7). The results showed that mouse SCARA1 had no binding to the healthy erythrocytes (Fig. S7A-B), but the frozen-thawed erythrocytes could be recognized by both mouse and human SCARA1 in a Ca^{2+}-dependent manner (Fig. S7A-B). The confocal images of the permeabilized erythrocytes stained by mouse or human SCARA1 revealed the recognizable pattern of spectrin on erythrocytes (Fig. S7C-D) (36,50). Moreover, FACS data showed that SPEC11e could block the binding of SCARA1 to dead erythrocytes almost completely (Fig. S7E-F), suggesting that the SPEC domains of spectrin are the binding targets of SCARA1 on dead erythrocytes.

To examine the internalization of dead cells by the interaction between SCARA1 and spectrin, macrophages were applied for endocytosis assays. The expression of SCARA1 on the surface of macrophages was confirmed by the binding of anti-SCARA1 antibodies using flow cytometry (Fig. S8), which is consistent with the published data showing that SCARA1 is highly expressed on macrophages (14). Then either the GFP-tagged spectrin fragments or the ultra-sonication treated erythrocytes or non-erythrocytes (HEK293 cells or Jurkat cells) were fed to macrophages for internalization. The FACS data showed that both the spectrin fragments and the dead cells could bind to macrophages (Fig. 6D and E), and the binding could be blocked by the CL-SRCR fragment of SCARA1 almost completely (Fig. 6D and E). Moreover, the confocal images showed that both the GFP-tagged spectrin fragments and the spectrins from the dead cells could be identified inside macrophages, and the internalization could be inhibited by the pre-incubation with the CL-SRCR fragment of SCARA1 (Fig. 6F), thereby validating the endocytosis of dead cells via the SCARA1-spectin interaction by macrophages.

**Discussion**

Although SRs were initially identified as receptors for modified LDL (2), evidence has shown that the ligands of SRs could be rather diverse, which is not surprising as numerous substances such as dead cells and metabolites need to be cleaned promptly to maintain homeostasis. Despite that PS receptors are widely known for recognizing apoptotic cells, recent evidence shows that several SRs are able to recognize dead cells through different ligands. For example, Clec9A binds F-actin of the damaged cells (30,31) and DEC205/CD205 recognizes dead cells through keratins at acidic environment (29). Previous studies have shown that SCARA1 might be involved in the phagocytosis of dead cells (14), but the mechanisms remain unclear. Here we find that SCARA1 can recognize dead cells specifically though spectrin with its
C-terminal SRCR domain, representing a novel pathway of dead cell recognition. The biochemical data show that the trimerization of SCARA1 enhances the dead cell binding affinity significantly, this is not entirely unexpected as spectrins are assembled into long fibrous structures and integrated into the cytoskeleton network, thus the trimeric SCARA1 may have advantages in spectrin uptake by providing higher binding affinities and larger mechanic force during dead cell clearance (Fig. 6G).

SRCR domains are commonly found in SRs with relatively conserved fold (16), and the cation binding sites on SRCR are usually associated with ligand recognition. For example, the cation binding sites on the SRCR domain of SCARA2, another member of SR-A, are involved in the binding with modified LDL (13); and the Ca$^{2+}$ binding site on the SRCR domain of CD163 is important for recognizing the haemoglobin-haptoglobin complexes (51). Therefore it is not surprising that Ca$^{2+}$ is required for the ligand binding of the SRCR domain of SCARA1, and it might be involved in ligand recognition directly at the active site. In addition, cell death is often associated with the change of Ca$^{2+}$ levels (52,53). The extracellular Ca$^{2+}$ is usually at millimolar level (46,54), which would allow the spectrin recognition by SCARA1. On the other hand, it has been shown that the Ca$^{2+}$ levels at endosomes could be much lower (46), implying that the ligands would be released from SCARA1 at endosomes, which is consistent with the binding data shown above.

Spectrin is an essential component of cytoskeleton and expressed in most of the eukaryotes (37,41). Spectrin usually locates near the intracellular side of the plasma membrane and forms a network by associating with other cytoskeleton components in the cytoplasm. It is known that during apoptosis, the plasma membrane asymmetry is lost and leads to the exposure of the inner leaflet components such as PS, therefore it would be expected spectrin could also be exposed at this stage, as has been confirmed by the antibody binding data shown above. This is in contrast with other known intracellular dead cell markers such as keratins and F-actins, which are largely exposed at necrotic stage of cell death (29-31). Considering the wide distribution of spectrin in eukaryotes and the high expression of SCARA1 on macrophages, SCARA1-spectin interaction might be a generic pathway for dead cell recognition and clearance under physiological conditions (Fig. 6G). Moreover, the finding of spectrin, together with actins and keratins as cellular markers for dead cells suggest that cytoskeleton is not only a scaffold for cellular structures, but also functions as universal markers for dead cells.

Erythrocytes have a lifespan of about 120 days, the removal of aged or dead erythrocytes is largely controlled by macrophages (55), but it is not entirely clear how erythrocytes are cleaned by the interactions with macrophages, as erythrocytes are lack of the organelles normally found in eukaryotes and do not have the usual apoptotic pathways. Spectrin is one of the major proteins expressed in
erythrocytes and critical for maintaining the membrane structure of erythrocytes. It has been shown that the loss of membrane asymmetry also occurs for erythrocytes during eryptosis (56), therefore spectrin could be exposed and mediates the interactions with macrophages. In addition, it has been shown that during eryptosis, erythrocytes would undergo membrane blebbing and Ca\textsuperscript{2+} leakage (57), which would allow the binding of spectrin by SCARA1 on macrophages during the clearance of erythrocytes.

Since billions of cells are renewed every day in human body, dead cell clearance is critical for homeostasis. In the past decades, researches have been largely focused on the recognition and clearance of apoptotic cells, especially the interactions between the PS receptors and apoptotic cells (26,28). The emerging evidence has shown that efferocytosis could be much more complex than previous thought, the receptors such as Clec9A, DEC205/CD205 and SCARA1/CD204 are involved in the clearance of dead cells by recognizing intracellular protein markers, which could be exposed at apoptotic or necrotic stages. This is not surprising as more cell death pathways such as necroptosis and pyroptosis have started to be characterized (58,59), suggesting that a large number of dead cells could be generated under different circumstances including inflammation or infection, which often lead to the exposure or release of intracellular components. Therefore, the receptors recognizing intracellular components would be necessary to remove dead cells or cell debris efficiently in these cases. On the other hand, since the expression of the intracellular dead cell markers could vary for different cell types, these receptors may play different roles in the clearance of dead cells in different tissues or environments. Therefore, clarifying the mechanisms of these pathways would not only improve the understanding of efferocytosis, but also would facilitate the therapeutic strategies against the related diseases in the future.

**Experimental Procedures**

**Protein expression and purification**

The ectodomain of SCARA1 contains a coiled-coil (CC) region (residue 88-262 for mouse SCARA1; residue 84-262 for human SCARA1), a collagen-like (CL) region (residue 262-352 for mouse SCARA1; residue 263-347 for human SCARA1) and a SRCR domain (residue 348-454 for mouse SCARA1; residue 348-451 for human SCARA1). Constructs encoding the ectodomain of SCARA1, CL-SRCR, CC-CL, SRCR and 5JOJ-SRCR were all sub-cloned into the pFastBac vectors with a melittin signal sequence and an N-terminal 6xHis-tag, respectively and expressed in insect cells (Invitrogen). The GFP-tagged SCARA1 and its truncation mutants were also sub-cloned with a melittin signal sequence and an N-terminal 8xHis-tag into the pFastBac vectors for expression. The Sf9 cells were used for generating recombinant baculoviruses and High Five cells were used for protein production (Invitrogen). The infected cells were cultured for 3 days in a 27 °C humidified incubator. The
supernatants of the infected High Five cells were buffer exchanged with 50 mM Tris, 150 mM NaCl at pH 8.0, then applied to Ni-NTA chromatography following the manufacture’s instruction (Ni-NTA Superflow, GE Healthcare). The imidazole eluates were further purified by gel filtration chromatography with a Superdex 200 column or a Superdex 6 column (GE Healthcare).

The full length of SCARA1 (mouse SCARA1, residue 1-454; human SCARA1, residue 1-451) were sub-cloned into the pTT5 expression vectors with mCherry inserted either between the ectodomain and the transmembrane domain of hSCARA1 or at the N-terminus of mSCARA1. The constructs were transiently transfected into HEK293F cells with FreeStyle 293 Expression Medium (Gibco). The SCARA1 transfected cells were cultured in a humidified CO2 incubator at 37 °C for 24 hrs before flow cytometry and confocal microscopy.

The truncation mutants of human spectrin F1, F2 and F3 were expressed in E.coli BL21 DE3 cells (Novagen) using the pET28a expression vector with a N-terminal Sumo-tag and a 6xHis-tag, and then purified as soluble proteins from the supernatants of cell lysates by Ni-NTA chromatography followed by gel filtration chromatography with a Superdex 200 column (GE Healthcare). The individual domains or fragments of human spectrin, including SPEC9e and SPEC11e from erythrocytes and SPEC9, SPEC11, SPEC17-18, SH3 and EF hand from non-erythrocytes, fused with an N-terminal Sumo or GFP-tag and a 6xHis-tag were also expressed and purified similarly.

**Cross-linking experiment**

The purified mSCARA1 ectodomain (25 mM Hepes, 150 mM NaCl, pH 7.4) were treated with 0.75% (w/v) glutaraldehyde for 45 min at room temperature, then 2 mM glycine was added to terminate the cross-linking reaction. The samples were re-suspended in 25 μL SDS loading buffer and boiled for 10 min and loaded onto SDS/PAGE (Bio-Rad Laboratories) for separation and detection.

**Crystallization and structural determination**

The SRCR domain of mSCARA1 purified from insect cell supernatants were buffer exchanged into 10 mM Tris, 150 mM NaCl, 10 mM CaCl2 (pH 7.4) at 10 mg/mL. Crystal screening was performed by the hanging-drop vapor diffusion method and crystals were obtained at 4 °C in a solution containing 20% (v/v) polyethylene glycol 300, 5% (w/v) polyethylene glycol 8000, 10% Glycerol and 0.1 M Tris, pH 8.5. Diffraction data were collected at BL18U beamline at Shanghai Synchrotron Radiation Facility (SSRF) and processed using the HKL-3000 package (60). The structure was solved by molecular replacement using the structure of M2BP (PDB entry: 1BY2) as a search model. The iterative-build OMIT map procedure is applied for removing model bias (61). Coot (62) and PHENIX (63) were used for structural refinement. The crystallographic statistics are listed in Table S1. Figures were made using UCSF Chimera (64).
**preparation**

Jurkat cells were cultured in 1640 medium (Gibco) supplemented with 10% (v/v) FBS (HyClone Laboratories). NIH 3T3 cells were cultured in DMEM medium (Gibco) supplemented with 10% (v/v) FBS (HyClone Laboratories). To induce apoptosis and necrosis, Jurkat cells or NIH 3T3 cells were both incubated in tissue culture flasks for 12~16 hrs with 1 μg/mL actinomycin D (ActD) until use. For inducing apoptosis and necrosis of HEK293 cells, the cells were cultured in FreeStyle 293 medium (Gibco) including apoptosis inducers A (Apopida) (1:1,000 (v/v)) (Beyotime) for 16 hrs. The frozen-thawed cells (3T3 cells or HEK293 cells) were prepared by incubating in a dry-ice ethanol bath for 10 min and then transferring immediately into a 37 °C water bath for 10 min and repeated for three times.

**Flow cytometry**

Apoptotic and necrotic cells were monitored using Annexin V Apoptosis Detection Kit APC (eBioscience, Inc.). Briefly, cells were washed three times with the washing buffer (10 mM Hepes at pH 7.4, 150 mM NaCl) and then with the binding buffer (10 mM Hepes at pH 7.4, 150 mM NaCl, 10 mM CaCl₂), and then re-suspended in the binding buffer including 5 μL Annexin V-APC at 1-5 × 10⁶ cells/mL and incubated at 4 °C for 20 min. Then the cells were washed in the binding buffer and re-suspended in 400 μL binding buffer including 5 μL propidium iodide (PI) staining solution and analyzed by flow cytometry.

For the triple staining assays, the cells were washed three times in the buffer (25 mM Hepes, 150 mM NaCl, 10 mM CaCl₂, pH 7.4), then re-suspended in the binding buffer (10 mM Hepes at pH 7.4, 150 mM NaCl, 10 mM CaCl₂) including the GFP-tagged mSCARA1 and 5μL Annexin V-APC solution and incubated at 4 °C for 30 min. Then the cells were washed in the binding buffer and stained with PI similarly as described above for flow cytometry.

For GFP staining, the cells were washed as described above and then washed three times with the corresponding buffers (25 mM Hepes, 150 mM NaCl, 10 mM CaCl₂ or 1mM EDTA at pH 7.4 or 25 mM Bis-tris, 150 mM NaCl, 10 mM CaCl₂ or 1mM EDTA at pH 6.0) for different assays. Buffers with different Ca²⁺ concentrations (2 μM, 2-10 mM) were used for Ca²⁺ gradient assays. The cells were incubated with the GFP-tagged mouse or human SCARA1 fragments in the buffers (pH 7.4 or 6.0) at room temperature for 20 min and then washed by the buffers (pH 7.4 or 6.0) for three times, and stained with PI for flow cytometry.

For the cation assays, the ActD treated Jurkat cells were washed three times with the buffer A (25 mM Hepes, 150 mM NaCl, 1 mM EDTA, pH 7.4) and three times with buffer B (25 mM Hepes, 150 mM NaCl, pH 7.4). Then the buffers (25 mM Hepes, 150 mM NaCl, pH 7.4) containing Ca²⁺ or Mg²⁺ or Na⁺ or K⁺ at 5mM concentration were added to the cells for binding assays. The cells were incubated with the GFP-tagged human SCARA1 in the corresponding buffers at room temperature for 20 min and then
washed by the corresponding buffers for three times, and stained with PI for flow cytometry.

For the enzymatic treatment assays, the cells were washed three times with the buffer (25 mM Hepes, 150 mM NaCl, pH 7.4) and then treated with DNase I, RNase A, or protease K at concentrations of 10 μg/mL for 30 min, respectively. After washing three times with the binding buffer (25 mM Hepes, 150 mM NaCl, 10 mM CaCl₂ at pH7.4), the cells were incubated with the GFP-tagged mouse SCARA1 in the binding buffer at room temperature for 20 min. After washing three times with the binding buffer, then the cells were stained with PI for flow cytometry.

For the spectrin inhibition assays, the cells were washed three times with the buffers (25 mM Hepes, 150 mM NaCl, 10 mM CaCl₂ or 1 mM EDTA at pH 7.4 or 25 mM Bis-tris, 150 mM NaCl, 10 mM CaCl₂ or 1mM EDTA at pH6.0) and incubated with the GFP-tagged mouse or human SCARA1 fragments (10 μg/mL) with or without the fragments of spectrin (20 μg/mL) at room temperature for 20 min. After washing three times with the corresponding buffer, the cells were stained with PI for flow cytometry.

For the endocytosis assay, HEK293 cells were transiently transfected with the full-length SCARA1 fused with a mCherry-tag. After 24 hours, 10 μg GFP or GFP-tagged SPEC fragments (SPEC11-17 or SPEC17-18) were added to the culture medium containing 10 mM Ca²⁺. Similar conditions were applied for the non-transfected HEK293 cells as controls. After 2-4 hours, cells were washed twice with the washing buffer (25 mM Hepes, 150 mM NaCl, 10 mM CaCl₂) and stained with PI for flow cytometry.

For the spectrin exposure assays, ActD treated what Jurkat cells blocked in blocking buffer (25 mM Hepes, 150 mM NaCl, 5% (w/v) BSA, 0.1% Tween 20, pH 7.4) for 1 hr. And stained with anti-spectrin antibody (Abcam, ab11755) at room temperature for 1 hr. After wash three times with washing buffer (25 mM Hepes, 150 mM NaCl, 1% (w/v) BSA, 0.1% Tween 20, pH 7.4), the anti-mouse IgG (H+L), F(ab')2 Fragment (Alexa Fluor® 647 Conjugate) (Cell Signaling, 4410S) were added and incubated for 1 hr. After washing three times with the washing buffer, the cells were stained with PI for flow cytometry.

FACS data were acquired using a Becton Dickenson FACSCaliber flow cytometer with CELLQuest software. Data analysis was performed using FlowJo software (Tree Star, Inc.).

**Dot-blot assay**

Cell lysates were prepared by ultra-sonication and spotted onto nitrocellulose membranes (Whatman) according to the manufacturer’s instruction. The membranes were air-dried at room temperature for 2 hrs and blocked in blocking buffer (25 mM Hepes, 150 mM NaCl, 5% (w/v) BSA, 0.1% Tween 20, pH 7.4) for at least 1 hr. Then the GFP-tagged SCARA1 fragments (10 μg/mL) was applied to the membranes and incubated with the mouse anti-GFP antibody (Abcam, ab184601) for 1 hr, then incubated with the goat
anti-mouse IgG secondary antibody HRP conjugates (SAB, L3032-2) for 1 hr and detected with the DAB reagent. Between every two steps, the membranes were washed six times with the washing buffer (25 mM Hepes, 150 mM NaCl, 10 mM CaCl$_2$, 0.1% Tween 20, pH 7.4) for 5 min each.

For the lipid strip dot-blot assays, the phospholipids were purchased from Echelon Biosciences. About 2 µg Jurkat cell lysates were also spotted as a positive control. The strips were incubated overnight in a blocking buffer (25 mM Hepes, 150 mM NaCl, 5% (w/v) BSA, 0.1% Tween 20, pH 7.4) at 4 °C and then transferred into a blocking buffer containing the GFP-tagged SCARA1 (10 µg/mL) for 2 hrs at room temperature, then the similar staining procedures were carried out as described above.

For the SCARA1 ligand detection, about 2 µg of HEK293 cell lysates prepared by ultra-sonication were treated with or without protease K at different conditions and different time, then applied for dot-blot assays with similar procedures and reagents as described above.

For the dot-blot assays of the SCARA1 fragment pull-down products, the Jurkat cell lysates were incubated with the Ni-NTA beads bound with the purified GFP-hCL-SRCR fragments (fused with a His-tag) in the presence of Ca$^{2+}$ or EDTA. Then the pull-down products were spotted onto nitrocellulose membranes (Whatman) and blocked similarly as described above. The pull-down products from the empty Ni-NTA beads as well as the GFP-hCL-SRCR fragment alone were spotted as controls. Then the membranes were incubated with mouse anti-GFP antibody (Abcam, ab184601) or mouse anti-spectrin antibody (Abcam, ab11755) for 1 hr at room temperature, then incubated with goat anti-mouse IgG secondary antibody HRP conjugates (SAB, L3032-2) for 1 hr following the similar staining procedures described above.

For the binding between the purified SCARA1 fragments and spectrin fragments, SCARA1 fragments were spotted onto nitrocellulose membranes (Whatman) and blocked similarly as described above. Then spectrin fragments (10 µg/mL) were applied to the membranes in the binding buffer (25 mM Hepes, 150 mM NaCl, 10 mM CaCl$_2$ or 1mM EDTA, 0.1% Tween 20, pH 7.4) and incubated with mouse anti-GFP antibody (Abcam, ab184601) or mouse anti-spectrin antibody (Abcam, ab11755) for 1 hr, then stained with the similar procedures described above.

**Pull-down experiment and ligand isolation**

HEK293 cells, Jurkat cells or NIH 3T3 cells (~5 × 10$^7$ cells) were lysed in 2 mL buffer (25 mM Hepes, 150 mM NaCl, 10 mM CaCl$_2$ or 1mM EDTA, pH 7.4) by ultra-sonication (Scientz). The cell lysates were centrifuged at 6,000 x g for 10 min, and the insoluble material was discarded. The supernatant was split into two halves and incubated with 10 µL Ni-sepharose excel beads (GE/Amersham/whatman, 17371201) pre-absorbed with SCARA1 in 25 mM Hepes, 150 mM NaCl, 10 mM CaCl$_2$, 0.1% Tween 20, pH 7.4 or 25 mM Hepes, 150 mM NaCl, 1mM EDTA, 0.1% Tween 20 at 4 °C for 5 hrs. The
beads were washed six times with the washing buffer (25 mM Hepes, 150 mM NaCl, 10 mM CaCl$_2$ or 1mM EDTA, pH 7.4), then washed with the washing buffers containing 8 mM and 20 mM imidazole, respectively before eluted with 50 μL elution buffer (250 mM imidazole, 25 mM Hepes, 150 mM NaCl, pH 7.4). The eluates were used for mass spectrometry and dot-blot assays.

**Mass spectrometry**

The solution sample from the pull-down experiments were precipitated and resolved by 8 M urea, and then treated with 5 mM TCEP and 10 mM IAA to reduce the disulfide bonds and alkylate the resulting thiol groups, sequentially. The mixture was digested for 16 hrs at 37 °C by trypsin at an enzyme-to-substrate ratio of 1:50 (w/w). The trypsin-digested peptides were loaded on an in-house packed capillary reverse-phase C18 column (15 cm length, 100-μm i.d. × 360-μm o.d. 3 μM particle size, 100 Å pore diameter) connected to a Thermo Easy-nLC1000 HPLC system. The samples were analyzed with a 90 min-HPLC gradient from 0 to 80% of buffer B (buffer A: 0.1% formic acid in water; buffer B: 0.1% formic acid in acetonitrile) at 300 nL/min. The eluted peptides were ionized and directly introduced into a Q-Exactive mass spectrometer using a nano-spray source. Survey full-scan MS spectra (from m/z 300–1,800) was acquired in the Orbitrap analyzer with resolution r = 70,000 at m/z 200. Protein identification was done with Proteome Discoverer2.1 (65,66). PSM value represents the number of secondary mass spectrum identified in the protein group, which can be used to estimate the relative content of the protein.

**ELISA experiments**

For the interaction of mSCARA1 with cell lysates, Jurkat cell lysates were prepared by ultra-sonication and coated onto 96-well plates with ~2 μg protein per well at 4 °C overnight. The plates were then blocked with the blocking buffers (25 mM Hepes, 150 mM NaCl, 0.1% Triton X-100, 2% (w/v) BSA, pH 7.4) at 37 °C for 3 hrs. The purified mSCARA1 was serially diluted and added to each well in the binding buffer (25 mM Hepes, 150 mM NaCl, 10 mM CaCl$_2$ or 1mM EDTA, 0.1% Triton X-100, 2mg/ml BSA, pH 7.4). In the calcium concentration gradient experiment, different calcium concentrations (2 mM, 5 mM, 10 mM, 20 mM) are used to replace 10 mM of calcium. After 2 hrs of incubation at 37 °C, the plates were washed five times with the washing buffer (25 mM Hepes, 150 mM NaCl, 10 mM CaCl$_2$ or 1mM EDTA, 0.1% Triton X-100, pH 7.4), then incubated with the mouse anti-GFP antibody (Abcam, ab184601) for 1 hr. After washing, the plates were incubated with the goat anti-mouse IgG secondary antibody HRP conjugates (SAB, L3032-2) for 1 h. After washing, 100 μL of chromogenic substrate (1 μg/mL tetramethylbenzidine, 0.006% H$_2$O$_2$ in 0.05 M phosphate citrate buffer, pH 5.0) was added to each well and incubated for 30 min at 37 °C. Then, 50 μL H$_2$SO$_4$ (2.0 M) was added to each well to stop the reactions. The plates were read at 450 nm on a Synergy Neo machine (BioTek Instruments). For the Ca$^{2+}$ gradient assays, 2 to 20 mM
CaCl$_2$ were used in the ELISA experiments.

For the interactions between the SCARA1 fragments and spectrin fragments, about 1 μg of the purified SCARA1 fragments were coated onto each well of 96-well plates and the spectrin fragments were serially diluted and added to each well. Mouse anti-spectrin antibody (Abcam, ab11755) and goat anti-mouse IgG secondary antibody HRP conjugates (SAB, L3032-2) were used for binding detection following the similar procedures described above.

For the interactions between the SCARA1 fragments (mSRCR, mCL-SRCR and and 5J0J-mSRCR) with the SPEC domain of spectrin, about 1 μmol of the purified SPEC1 were coated onto each well of 96-well plates and the SCARA1 fragments were serially diluted and added to each well. Mouse anti-GFP antibody (Abcam, ab184601) and goat anti-mouse IgG secondary antibody HRP conjugates (SAB, L3032-2) were used for binding detection following the similar procedures described above. The $K_D$ values were calculated based on the fitting of the sigmoidal curves using software GraphPad Prism 6 (67,68).

**Confocal Microscopy**

HEK293 or NIH 3T3 cells grown on coverslips were fixed by 4% paraformaldehyde in TBS (pH 7.4). After washing with the buffer (25 mM Heps, 150 mM NaCl, 10 mM CaCl$_2$ or 1mM EDTA at pH 7.4 or 25 mM Bis-tris, 150 mM NaCl, 10 mM CaCl$_2$ or 1mM EDTA at pH 6.0), the cells were permeabilized with 0.25% Triton X-100 in the corresponding buffers mentioned above. After washing twice with 0.1% Triton X-100 in the buffers, the cells were stained with the GFP-tagged SCARA1 fragments for 2 hrs in the binding buffer (25 mM Hepes, 150 mM NaCl, 10 mM CaCl$_2$ or 1mM EDTA, 0.1% Triton X-100, at pH 7.4 or 25 mM Bis-tris, 150 mM NaCl, 10 mM CaCl$_2$ or 1mM EDTA, 0.1% Triton X-100, at pH 6.0). Then the cells were washed twice with the buffers and incubated with 5 μM DAPI for 30 min, and then washed twice again for confocal microscopy. The confocal images were taken on a Leica SP8 microscope.

For cell binding assays of spectrin, HEK293 were transfected with the full-length SCARA1 fused with a mCherry tag in six well plates. After 24 hours of transfection, 10 μg of GFP-tagged SPEC domains (SPEC11 from erythrocyte, SPEC17-18 from non-erythrocyte) were added to the plates with 10 mM Ca$^{2+}$. After 2-4 hours of incubation, the cells were washed three times with the buffer (25 mM Hepes, 150 mM NaCl, 10 mM CaCl$_2$) and incubated with 5 μM DAPI for 30 min. Then the plates were washed again for confocal microscopy with a Leica SP8 microscope.

**Erythrocyte assays**

Isolated fresh mouse erythrocytes (99%) were purchased (Beres, Beijing) and stored in the Archer's fluid. Before experiment, the erythrocyte cells were cultured in 1640 medium (Gibco) supplemented with 10% (v/v) FBS (HyClone Laboratories) overnight. The frozen-thawed cells were prepared by incubating cells in a dry ice ethanol bath for 10 min and then transferring immediately into a 37 °C water bath.
for 10 min and repeated for three times. For GFP staining, the cells were washed three times with the washing buffer (10 mM Hepes at pH 7.4, 150 mM NaCl) and then washed three times with the binding buffers (25 mM Hepes, 150 mM NaCl, 2 mM CaCl2, pH 7.4). Then cells were blocked with blocking buffer (25 mM Hepes, 150 mM NaCl, 2 mM CaCl2, 5% (v/v) FBS, 3% BSA, 0.1% Tween 20, pH 7.4) for 1 hr and stained with the GFP-tagged mouse or human SCARA1 fragments, anti-Ly76 antibody (Abcam, ab91113) and goat anti-rat IgG H&L (Abcam, ab150160) in the binding buffer at room temperature for 1h, and then washed with the buffers (pH 7.4) for three times and analyzed by flow cytometry. For inhibition experiments, both mouth and human SCARA1 are blocked with SPEC11e for about 4 hours in 4 °C.

For confocal microscopy assay, erythrocytes on coverslips were fixed by 4% paraformaldehyde in TBS (pH 7.4). After washing with the buffer (25 mM Hepes, 150 mM NaCl, 2 mM CaCl2 or 1mM EDTA at pH 7.4), the cells were permeabilized with 0.25% Triton X-100 in the corresponding buffers mentioned above. After washing twice with 0.1% Triton X-100 in the buffers, the cells were stained with the GFP-tagged SCARA1 fragments for 2 hrs in the binding buffer (25 mM Hepes, 150 mM NaCl, 2 mM CaCl2 or 1mM EDTA, 0.1% Triton X-100, at pH 7.4). Then the cells were washed twice with the buffers and incubated with 5 μM DAPI for 30 min, and then washed twice again for confocal microscopy. The confocal images were taken on a Leica SP8 microscope.

**Macrophage endocytosis assays**

The macrophages (RAW264.7) were cultured in DMEM medium (Hyclone) supplemented with 10% (v/v) FBS (HyClone Laboratories). To test the expression of SCARA1 on the surface of macrophages, the cells were washed three times with the washing buffer (10 mM Hepes at pH 7.4, 150 mM NaCl) and then blocked with blocking buffer (25 mM Hepes, 150 mM NaCl, 10% (v/v) FBS, 3% BSA, 0.1% Tween 20, pH 7.4) for 2 hr and stained with anti-SCARA1 antibody (Abcam, ab217843) and goat anti-rabbit IgG H&L Alexa Fluor 594 (Abcam, ab150080) in the binding buffer at room temperature for 1h, and then washed three times with the buffers (pH 7.4), and viable cells were selected for flow cytometry assays.

Mouse erythrocytes, HEK293 cells or Jurkat cells were treated by ultra-sonication with low intensity and centrifuged briefly to remove fractions that may interfere with imaging, and then incubated with anti-spectrin antibody (Abcam, ab11755) and goat anti-mouse IgG H&L Alexa Fluor 488 (Abcam, ab150113) before feeding to macrophages blocked with 10% (v/v) FBS, 3% BSA for 1 hr, and incubated in the binding buffer (DMEM medium supplemented with 10% (v/v) FBS) at 37 °C for 4 h before flow cytometry and confocal microscopy. The GFP-tagged spectrin fragments (SPEC11e and SPEC 17-18) were also applied for endocytosis assays. For flow cytometry, the macrophages were washed three times with the washing buffer (25 mM Hepes, 150 mM NaCl, 10 mM CaCl2) and stained with PI.
similarly as described above. For confocal microscopy, the macrophages on coverslips were fixed by 4% paraformaldehyde in TBS (pH 7.4). After washing with the buffer (25 mM Hepes, 150 mM NaCl, 2 mM CaCl$_2$), the cells were permeabilized with 0.25% Triton X-100. After washing twice with 0.1% Triton X-100 in the buffers, the cells were incubated with 5 μM DAPI for 30 min, and then washed once again for confocal microscopy. The confocal images were taken on a Leica SP8 microscope. For endocytosis inhibition assays, the GFP-tagged spectrin fragments or the ultra-sonication treated cells were incubated with mCL-SRCR fragment of SCARA1 at 4 °C for 4 hours before feeding to macrophages.

Author contributions

C.C. and Y.H. designed the experiments. C.C., Z.H., L.C. and C.P. performed the experiments. C.C. and Y.H. analyzed the data and wrote the manuscript.

Data availability

The structure of the SRCR domain of mouse SCARA1 is deposited in PDB (www.rcsb.org) with accession number 6J02.

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Conflict of interest
The authors declare no conflict of interest.

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Figures
Fig. 1. Crystal structure and the mutagenesis studies of the SRCR domain of mSCARA1

(A) Schematic representation of SCARA1 domain arrangement.

(B) The SEC profile and SDS/PAGE of the SRCR domain of mouse SCARA1.

(C) Crystal structure of the SRCR domain of mouse SCARA1. Ca$^{2+}$ is shown as a gray ball.

(D) The Ca$^{2+}$ binding site on the SRCR domain. The residues and water molecules (green) at the binding site are labeled.

(E) The CL-SRCR fragment of mSCARA1 binds to the ActD-treated Jurkat cells in the presence of Ca$^{2+}$, whereas the fragments with the Ca$^{2+}$ binding site mutations do not bind to the dead cells. GFP is applied as a control.

(F) The CL-SRCR fragment of mSCARA1 binds to the frozen-thawed NIH 3T3 cells in the presence of Ca$^{2+}$, whereas the fragments with the Ca$^{2+}$ binding site mutations do not bind to the dead cells. GFP is applied as a control.
Fig. 2. Ca\textsuperscript{2+}-dependent recognition of dead cells by SCARA1

(A) mSCARA1 binds to the ActD-treated NIH 3T3 cells in the presence of Ca\textsuperscript{2+}. Mock represents untreated viable cells.

(B) hSCARA1 binds to the ActD-treated Jurkat cells in the presence of Ca\textsuperscript{2+}.

(C) ELISA data show that mSCARA1 binds to the Jurkat cell lysates in the presence of Ca\textsuperscript{2+}.

(D) A confocal image of the permeabilized NIH 3T3 cells stained by GFP-mSCARA1 and DAPI with Ca\textsuperscript{2+} (Scale bar, 25 μm).

(E) A confocal image of the permeabilized NIH 3T3 cells stained by GFP-mSCARA1 and DAPI with EDTA (Scale bar, 25 μm).

(F) Staining of viable (left) and the ActD-treated (middle) Jurkat cells by Annexin V-APC, PI, and GFP-mSCARA1 in the presence of Ca\textsuperscript{2+}. The binding of GFP-mSCARA1 to the gated subsets of viable and the ActD-treated Jurkat cells are shown on the right. Mock represents untreated viable cells.
Fig. 3. SCARA1 recognizes dead cells through cellular spectrin.

(A) The CC-CL fragment of mSCARA1 does not bind to the ActD-treated Jurkat cells. Mock represents untreated viable cells.

(B) The CL-SRCR fragment of mSCARA1 binds to the ActD-treated Jurkat cells in the presence of Ca$^{2+}$. GFP is applied as a control.

(C) The trimeric 5J0J-SRCR binds to the ActD-treated Jurkat cells in the presence of Ca$^{2+}$.

(D) Dot blot assays show that the ectodomain and the CL-SRCR fragment of mSCARA1 bind to the Jurkat cell lysates in the presence of Ca$^{2+}$, whereas the CC-CL fragment has no detectable binding to the cell lysates. Serial diluted cell lysates were spotted on the nitrocellulose membranes.

(E) Mouse SCARA1 shows no binding to the lipids on the strip. Jurkat cell lysates were spotted as a positive control.

(F) A confocal image of the permeabilized HEK293 cells stained by the CL-SRCR fragment of hSCARA1 and DAPI in the presence of Ca$^{2+}$ (Scale bar, 25 μm).

(G) A confocal image of the permeabilized NIH 3T3 cells stained by the CL-SRCR fragment of mSCARA1 and DAPI in the presence of Ca$^{2+}$ (Scale bar, 25 μm).

(H) Monomeric SRCR domain of mSCARA1 shows weaker binding to the ActD-treated Jurkat cells than the trimeric mSCARA1 ectodomain. GFP is applied as a control.

(I) Monomeric SRCR domain of hSCARA1 shows weaker binding to the ActD-treated Jurkat cells than the trimeric hSCARA1 ectodomain.
(J) The binding of mSCARA1 to dead cells is abolished by the protease K treatment.

(K) Dot blot assays shows that spectrin can be pulled down by GFP-hCL-SRCR fragment from the Jurkat cell lysates. The cell lysates were incubated with the Ni-NTA beads bound with the purified GFP-hCL-SRCR fragments in the presence of Ca^{2+} or EDTA, and the pull-down products were spotted onto nitrocellulose membranes and detected by anti-spectrin or anti-GFP antibodies. The pull-down products from the empty Ni-NTA beads as well as the GFP-hCL-SRCR fragment alone were also spotted as controls.

(L) Staining of viable (left) and the ActD-treated (middle) Jurkat cells by Annexin V-APC, PI, and anti-spectrin antibodies. The binding of anti-spectrin antibodies to the gated subsets of viable and the ActD-treated Jurkat cells are shown on the right. Mock represents untreated viable cells.
Fig. 4. The SRCR domain of SCARA1 recognizes cellular spectrin specifically

(A) Diagram of the domain arrangement of the α-chain of human spectrin. The fragments (F1, F2, F3) expressed for binding assays are labeled.

(B) ELISA assays show the binding of the spectrin fragments to hSCARA1. Sumo is applied as a control.

(C) Dot blot assays show the binding of spectrin F1 fragment to the SRCR and the CL-SRCR fragments of mSCARA1 or hSCARA1, while the F1 fragment has no detectable binding to the CC-CL fragment of SCARA1. SCARA1 fragments were spotted onto nitrocellulose membranes, then the spectrin F1 fragment was incubated with the membranes in the binding buffer including Ca$^{2+}$ or EDTA. Anti-GFP or anti-spectrin antibodies were used for detection. Similar procedures were followed for F2 (D) and F3 (E) fragments.

(D) Dot blot assays show the binding of spectrin F2 fragment to the SRCR and the CL-SRCR fragments of mSCARA1 or hSCARA1, while the F2 fragment has no detectable binding to the CC-CL fragment of SCARA1.

(E) Dot blot assays show the binding of spectrin F3 fragment to the SRCR and the CL-SRCR fragments of mSCARA1 or hSCARA1, while the F3 fragment has no detectable binding to the CC-CL fragment of SCARA1.

(F) Spectrin fragments (F1, F2, F3) block the binding of the CL-SRCR fragment of mSCARA1 to dead cells. Sumo is applied as a control.

(G) Spectrin fragments (F1, F2, F3) block the binding of the CL-SRCR fragment of hSCARA1 to dead cells.
Fig. 5. The SRCR domain of SCARA1 recognizes the SPEC domain of spectrin

(A) The SPEC domain of spectrin binds to hSCARA1 in the presence of Ca\(^{2+}\), while the SH3 and the EF hand domain of spectrin have no binding to hSCARA1. Sumo is applied as a control.

(B) The SPEC domain of spectrin block the binding of the CL-SRCR fragment of hSCARA1 to the dead Jurkat cells, whereas the SH3 and the EF hand domain have no inhibition to the binding.

(C) The SPEC domain of spectrin block the binding of the CL-SRCR fragment of hSCARA1 to the dead HEK293 cells, whereas the SH3 and the EF hand domain have no inhibition to the binding.

(D) The SPEC domain of spectrin block the binding of the CL-SRCR fragment of mSCARA1 to the dead NIH 3T3 cells, whereas the SH3 and the EF hand domain have no inhibition to the binding.

(E) The CL-SRCR fragments with the Ca\(^{2+}\) binding site mutations have no binding to the SPEC domain of spectrin.

(F) The SPEC9e and SPEC11e from erythrocytic spectrin bind to SCARA1 in the presence of Ca\(^{2+}\). The SH3 domain and Sumo are applied as controls.

(G) The SPECe domains from erythrocytic spectrin block the binding of the CL-SRCR fragment of hSCARA1 to dead Jurkat cells.

(H) The binding affinities between the SCARA1 fragments (SRCR, CL-SRCR and 5J0J-SRCR) with SPEC1 in the presence of Ca\(^{2+}\). GFP is applied as a control.
A hSCARA1 transfected HEK293 cells

B mSCARA1 transfected HEK293 cells

C

D Macrophages

E Macrophages

F

G Viable cells

Dead cells
Fig. 6. Recognition and internalization of spectrin fragments or dead cells by the SCARA1 transfected cells or macrophages.

(A) HEK293 cells transfected with the full-length hSCARA1 fused with mCherry recognize the SPEC domains of spectrin in the presence of Ca$^{2+}$. SPEC11$^e$ is from erythrocytes and SPEC17-18 is from non-erythrocytes. The mCherry positive cells were selected for the experiments with GFP, GFP-SPEC11$^e$ and GFP-SPEC17-18. Non-transfected HEK293 cells (Mock) were used for experiments with GFP-SPEC11$^e$ and GFP-SPEC17-18.

(B) HEK293 cells transfected with the full-length mSCARA1 fused with mCherry recognize the SPEC domains of spectrin in the presence of Ca$^{2+}$. The mCherry positive cells were selected for the experiments.

(C) Confocal images show that HEK293 cells transfected with the full-length mSCARA1 fused with mCherry can recognize and internalize the GFP-tagged SPEC domains of spectrin in the presence of Ca$^{2+}$ (Scale bars, 10 μm).

(D) Macrophages can bind both SPEC11$^e$ and SPEC17-18 fragments of spectrin, and the binding can be inhibited by the mCL-SRCR fragment of SCARA1.

(E) Macrophages can bind dead cells or debris (ultrasonic-treated) from both erythrocytes and HEK293 cells, and the binding can be inhibited by the mCL-SRCR fragment of SCARA1. Dead cells or debris are pre-stained by anti-spectrin antibodies.

(F) Confocal images show the internalization of spectrin fragments (SPEC11$^e$ and SPEC17-18), dead erythrocytes and Jurkat cells by macrophages. Dead cells or debris are pre-stained by anti-spectrin antibodies. The internalization can be blocked by the
mCL-SRCR fragment of SCARA1 (Scale bars, 5 μm).

(G) A cartoon representation of dead cell recognition by SCARA1/CD204 via spectrin.
The scavenger receptor SCARA1(CD204) recognizes dead cells through spectrin
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