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Scavenger Receptor-A Functions in Phagocytosis of *E. Coli* by Bone Marrow Dendritic Cells

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Abstract

Class A scavenger receptors (SR-A) are cellular pattern recognition receptors that bind and traffic a variety of endogenous and microbial ligands. However, despite an emerging role for SR-A as a contributor to the innate immune system, little is known of the regulation or function of SR-A on dendritic cells (DCs). Here we show that SR-A expression is upregulated during murine DC differentiation and that SR-A expression levels correlate with the expression of the murine DC marker CD11c. Using bone marrow-derived DCs (BMDCs) from SR-A knockout (SR-A$^{-/-}$) mice, we investigated the contribution of SR-A to BMDC particulate phagocytosis. Functional analyses demonstrated that SR-A is a critical phagocytic receptor for BMDC internalization of the gram-negative bacteria *E. coli*. SR-A$^{-/-}$ BMDCs were impaired in their ability to phagocytose bacteria, and this deficit varied with the bacteria:BMDC cell ratio. Microscopic and biochemical analyses revealed that SR-A is broadly distributed on the surface of BMDCs and is not physically associated with lipid rafts. However, cholesterol depletion demonstrated dependence of SR-A –mediated phagocytosis upon lipid rafts. These data demonstrate a functional contribution for SR-A in the BMDC phagocytic pathway.

Keywords

dendritic cell; bacteria; phagocytosis; trafficking; scavenger receptor; SR-A

Introduction

Scavenger receptors are a family of pattern recognition receptors (PRR) originally defined by their ability to mediate the binding and uptake of modified low-density lipoprotein (LDL) into macrophages [1,2]. Subsequently, these receptors were found to bind and traffic a broad range of polyanionic ligands and, additionally, to contribute to cellular adhesion [3,4]. The Class-A scavenger receptors (SR-A) are trimeric transmembrane glycoproteins [5-7] consisting of SRAI/II, MARCO [8,9], and the recently identified SCARA4 and SCARA5 [10,11]; in this manuscript we will use SR-A to refer to the SR-A I/II isoforms. The SR-A family of genes has been extensively studied in the context of atherosclerosis and for their role in macrophage foam cell formation [12,13]. Additionally, SR-A has an emerging role as a modulator of immune responses by functioning as an endocytic receptor for binding and trafficking of inflammatory

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microbial products including lipopolysaccharide (LPS) and lipoteichoic acid (LTA) [14,15], and as a phagocytic receptor for the cellular internalization, clearance and neutralization of particulates and microbes [14,16-18]. However, while SR-A expression and function has been extensively investigated for macrophages, the role of this molecule on dendritic cells (DCs) is only beginning to become known.

Dendritic cells are professional antigen presenting cells (APCs) that facilitate the critical transition from the innate immune system to adaptive immune responses [19,20]. Endocytic and phagocytic processes are highly efficient in DCs [21], and these cells are proposed to serve as the key initiators of immune responses by constant sampling of the local environment for foreign antigens. DCs recognize and internalize foreign pathogens via a variety of pattern recognition receptors [22,23]. These PRRs function by recognizing and binding distinct and often specific molecular motifs, referred to as pathogen-associated molecular patterns (PAMPs) [22,23]. Scavenger receptors mediate pathogen recognition in macrophages [16], however the role of the class-A scavenger receptor family in DC function has only recently been implicated in both human and murine systems [24,25]. Recent reports that SR-A is specifically expressed by a subset of splenic DCs in the mouse [24], coupled with the observation that SR-A$^{-/-}$ mice are more susceptible to bacterial infections [26,27], implicate a physiological role for class-A scavenger receptors in the phagocytic functions of dendritic cells. Thus, we sought to elucidate the contribution of SR-A to phagocytosis by dendritic cells.

In these studies we report that SR-A expression is up-regulated during differentiation of BMDCs from bone marrow precursors and that SR-A expression levels correlate with the DC surface marker CD11c during the differentiation process. We then used biochemical and genetic approaches to determine the contribution of SR-A to the BMDC phagocytic pathway. Functional analyses revealed that SR-A$^{-/-}$ BMDCs are impaired in their ability to phagocytose and accumulate both heat-killed and live *E. coli* bacteria. SR-A dependence for the phagocytosis of bacteria varied with the bacterial:BMDC cellular ratio, and SR-A expression enabled both a higher percentage of BMDCs to accumulate bacteria and for BMDCs to accumulate more bacterial cells. Finally, we examined the requirement for cell surface lipid rafts to SR-A – mediated phagocytosis by DCs. While SR-A was found to be unassociated with lipid rafts at the cell membrane, SR-A-dependent phagocytic internalization of *E. coli* was abrogated by cell surface cholesterol depletion using β-cyclodextrin.

Materials and Methods

Mice

C57BL/6 and Balb/C mice were obtained from the NCI, Frederick, MD. SR-A null (SR-A $^{-/-}$) mice (C57BL/6 background) were a generous gift of Drs. T. Kodama (Tokyo University) and M. W. Freeman (Massachusetts General Hospital) [28,29]. CB6F1 mice were F1 mice generated by crossing Balb/C mice with C57Bl/6 wildtype or SR-A$^{-/-}$ mice.

Bone Marrow-Derived Dendritic Cell Culture

The DC culture protocol is described previously [30] and is a modification of Inaba *et al.* [31]. In summary, C57BL/6, BalbC, and CB6F1 mouse (Charles River Laboratories, Raleigh, NC) bone marrow-derived cells were resuspended at 10^6 cells/ml in DC culture media (RPMI 1640 medium, 10% heat-inactivated fetal/bovine serum, 100 units/ml penicillin/streptomycin, 50 mM B-mercaptoethanol, 5% cell culture supernatant from X63 cells secreting GM-CSF [32]) and plated at 1 ml/well in a 24-well tissue culture plate. On days 2 and 4, the cells were washed and re-fed, and non-adherent cells removed. On day 6, wells were vigorously washed with culture medium to collect semi-adherent cells which were then phenotypically confirmed to be immature DCs.

Antibodies

Anti-mSR-A antibody used for Western Blot was purchased from R&D Research Systems (Minneapolis, MN); 2F8 anti-SR-A antibody from Serotec (Raleigh, NC); anti-CD11c from eBioscience (San Diego, CA); anti-CD16/CD32 from Pharmingen BD Biosciences (San Jose, CA); and FITC-wheat germ agglutinin and DAPI were purchased from Molecular Probes (Eugene, OR).

Endocytic/Phagocytic Uptake Assays

Binding, uptake and trafficking assays were performed as previously described [33-35]. Alexa488-AcLDL was from Molecular Probes (Eugene, OR); rabbit IgG, fucoidan, carrageenan, chondroitin B sulfate were purchased from Sigma (St. Louis, MO). *E. coli* were heat-killed prior to labeling by incubation for 1 hour at 90° C and fluorescently labeled as previously described [35]. Endocytosis and phagocytosis assays were performed by incubating cells in the presence of indicated ligands at 37° C for 20 minutes, washing cells three times with PBS and assaying for fluorescence by FACS.

Phagocytosis of Live E. coli (Gentamycin protection assay)

Phagocytosis of live *E. coli* was performed as previously described [36] with some modifications. Overnight DH5α *E. coli* cultures were washed twice in 10 ml serum-free RPMI and centrifuged at 3000 rpm. The bacterial pellet was resuspended in RPMI and concentration was determined by spectrophotometry at O.D. 600. 2×10^5 BMDCs were incubated with the indicated MOI's of bacteria for 45 minutes at 37°C. Cells were washed 3 times in serum-free RPMI and incubated for 30 minutes in 5 mM gentamycin for 30 minutes at 37°C. Cells were washed and resuspended in 200 μl 0.1% Triton-X 100 in PBS. 20 μl of resuspended cells/bacteria were plated on LBagar/ampicillin plates and incubated overnight at 37°C. Colonies were counted the following morning and colony forming units (CFUs) were calculated based on fraction of total sample plated. For analysis of lipid raft contribution to phagocytosis of live *E. coli*, BMDCs were pretreated with 5 mM β-cyclodextrin (Sigma, St. Louis, MO) for 30 minutes at 37°C and washed twice in serum-free RPMI prior to incubation with bacteria. Cholesterol depletion by β-cylodextrin was quantified with the Amplex Red Cholesterol Assay Kit (Molecular Probes, Eugene, OR) per manufacturer's directions. Briefly, β-cyclodextrin treated and untreated cells were washed three times in serum-free media. Cell pellets were resuspended in 2:1 choloroform:methanol and vigorously vortexed for 1 minute. The suspension was then centrifuged for 10 minutes at 8,000 rpm. The organic phase was collected and air-dried. The lipid extracts were then assayed against a standard curve of known cholesterol concentrations.

Western Analysis, FACS Analysis, and Confocal Microscopy

Cells were analyzed by flow cytometry at the NCCC Englert Cell Analysis Laboratory using a FACSCaliber cytometer, and subsequently analyzed using CellQuest. For confocal microscopy, cells affixed to coverslips were mounted in buffered glycerol containing 1mg/ml phenylenediamine. Microscopy was performed on a Zeiss LSM510 Meta microscope taking single optical sections with a 63x lens, followed by viewing on LSM5 Image Browser. Western analysis: cells were washed, and then lysed in SDS-sample buffer containing B-mercaptoethanol, followed by brief sonication. Cell lysates were separated by SDS-PAGE, and subsequently transferred to PVDF (Millipore Corp.). Westerns were developed using ECL (Amersham Biosciences) of HRP-conjugated secondary antibodies (Jackson Immuno Research).

Scanning Electron Microscopy

Cells were incubated with 2F8 antibody, washed, and stained with gold-conjugated goat-anti-rat antibody (Jackson ImmunoResearch Laboratories, Westgrove, PA). Stained cells were fixed and dried for SEM as previously described [37]. Briefly, cells were fixed with 3.5% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA) in phosphate buffer (100mM $NaPO_4$, pH 7.2). Cells on coverslips were washed with phosphate buffer and post-fixed in 1% osmium tetroxide (Electron Microscopy Sciences) for 1 hour. Coverslips were again washed with phosphate buffer, dehydrated in graded ethanol and hexamethyldisilazane (HMDS, Sigma Aldrich) washes, and then desiccated overnight under vacuum. Coverslips were mounted onto 12mm round aluminum SEM stubs (Electron Microscopy Sciences) and plasma-coated with 3nm osmium (OPC-60 Osmium Plasma Coater, Nippon Laser and Electronics). Cells were viewed on an XL-30 ESEM FEG (FEI Company) at 10-15kV, 7.5mm working distance using both SE and BSE detection modes.

Biochemical Separation of Lipid Rafts from BMDC Cell Membrane

Lipid rafts were purified from BMDC membranes as previously described [36,38] with some modifications. 2.5×10^6 BMDCs were labeled with 10 μg/ml FITC-conjugated cholera toxin B subunit [39] (CT-B; Sigma Aldrich) on ice, washed, and subsequently resuspended in Raft Buffer (25 mM Tris-HCL pH 7.5, 0.15 M NaCl) containing 1% Triton-X 100. Suspensions were mixed with an equal volume of 90% sucrose and 2 ml of suspension added to ultracentrifuge tubes. Samples were overlaid with a discontinuous sucrose gradient (4 ml 30%, 2 ml 5%, 4 ml 0%) and centrifuged overnight at 39,000 rpm in a SW41 rotor (Beckman Instruments, Palo Alto, CA). 1 ml fractions were collected and run on a 12% SDS-PAGE gel which was transferred to a nitrocellulose membrane. CT-B fractionation was determined by scanning the membrane for FITC fluorescence using a Typhoon 9410 Variable Mode Imager (GE Healthcare, Piscataway, NJ). The scanned imaged was analyzed using ImageQuant v5.2 software (GE Healthcare, Piscataway, NJ). Western analysis was then performed for SR-A.

Results

SR-A expression correlates with CD11c expression and is induced during BMDC differentiation

Primary murine and primate dendritic cells (DCs) and murine bone-marrow –derived dendritic cells (BMDCs) all express SR-A [24,25]. Here we used murine BMDCs to permit examination of SR-A expression during DC differentiation and to obtain sufficient numbers for biochemical analyses. The majority of murine BMDCs were $CD11c^+$ (murine DC marker [40]) and SR-A^+ (Figure 1A), and these cells expressed low levels of MHC II that were dramatically upregulated upon maturation of BMDCs with LPS (data not shown). Growth rate and dendritic cell surface markers determined by cell surface staining and FACS analysis exhibited no differences between SR-$A^{+/+}$, SR-$A^{+/-}$ and SR-$A^{-/-}$ BMDCs derived from mice with the corresponding genotypes (data not shown), indicating that SR-A expression is not necessary for proper BMDC differentiation and growth (consistent with Becker *et al*. [24]).

SR-A heterozygous mice (SR-$A^{+/-}$, CB6F1 background) were bred to control for any incongruities in the knockout background, and to allow for staining with the 2F8 monoclonal antibody, which does not recognize SR-A in C57BL/6 mice due to a genetic polymorphism [41]. SR-A expression, and specificity of staining, was confirmed by Western analysis and FACS analysis, with SR-A reactivity observed in SR-$A^{+/-}$, but not SR-$A^{-/-}$, BMDCs (Figure 1B, 1D). We then asked whether SR-A expression on DCs is regulated during cellular differentiation. Analysis of CD11c and SR-A expression during BMDC differentiation revealed an intrinsic correlation between CD11c and SR-A expression levels. Bone marrow precursors expressed undetectable levels of SR-A protein at Day 0 of BMDC culture as

determined by both Western blot and FACS analysis (Figure 1B, and data not shown). BMDC cultures were then harvested at Day 0, 2, 4, and 6 of the GM-CSF-induced BMDC differentiation process. Western blot analysis shows that SR-A expression is initially detected at Day 2 of culture in GM-CSF and increases incrementally through day 6 (Figure 1B). Correspondingly, FACS analysis of CD11c and SR-A expression during the differentiation process confirms the Western blot data and shows that SR-A levels are induced with the same expression kinetics as CD11c during BMDC development (Figure 1C). LPS-induced maturation of the BMDCs did not affect SR-A expression significantly as determined by both Western blot and FACS analysis (data not shown). These data support that SR-A expression is regulated during DC differentiation, and that SR-A expression correlates with that of the murine cell-surface dendritic cell marker CD11c.

SR-A$^{-/-}$ BMDCs are impaired in the phagocytosis of heat-killed E. coli

Since scavenger receptors are expressed on DCs [24,25] and are previously described to function in bacterial uptake by macrophages [14,16] we tested their contribution to BMDC phagocytosis using *E. coli* bacteria. Heat-killed *E. coli* bacteria were labeled with Alexa-488 or Alexa-647 to permit quantification of phagocytosis by fluorescence analysis. BMDCs were incubated with the *E. coli* at varied bacteria:BMDC cellular ratios for 20 minutes at 37°C to determine the linear range of the assay (Figure 2A). Cellular association with the fluorescently-labeled bacteria increased at higher ratios, and no difference was observed between the BMDC uptake of Alexa488 and Alexa647 -labeled bacteria (Figure 2A). To determine the contribution of scavenger receptors to the BMDC phagocytic pathway, we assayed for the competition of bacterial uptake by scavenger receptor ligands (Figure 2B). We chose fucoidan and carrageenan, which are both polyanions that bind to a broad array of scavenger receptors [42]; as a negative control we used chondroitin-B sulfate, similarly a polyanionic molecule, that does not bind to scavenger receptors (Figure 2B) [43]. Fucoidan competed the BMDC phagocytosis of Alexa488-labeled *E. coli* by ∼50%, while a 75% competition of bacterial uptake was observed in the presence of carrageenan (Figure 2B). These data support a contribution of scavenger receptors to the DC phagocytic process, and therefore we proceeded to specifically test the contribution of SR-A which has previously been implicated in phagocytic processes by other cell types [14,16].

We assessed the specific contribution of SR-A by comparing SR-A$^{+/-}$ and SR-A$^{-/-}$ immature BMDCs for their ability to phagocytose Alexa488-*E. coli* at varying concentrations of bacteria. SR-A$^{-/-}$ BMDCs were impaired in their ability to phagocytose *E. coli* bacteria, and the discrepancy in bacterial uptake between SR-A$^{+/-}$ and SR-A$^{-/-}$ cells increased at higher bacteria:BMDC ratios (Figure 2C). BMDCs incubated with Alexa488-*E. coli* were examined by microscopy to confirm that the acquired fluorescence was due to uptake of bacteria and not shed microbial products. Confocal images demonstrated that BMDCs bound the labeled *E. coli* at the cell surface and engulfed labeled bacteria, as observed by punctuate intracellular fluorescence (Figure 2D). To control that loss of SR-A does not lead to pleiotropic endocytic defects we tested SR-A-expressing and SR-A-deficient cells for their ability to endocytose IgG, an SR-A – independent process. No difference was observed in the receptor-mediated endocytosis of IgG between SR-A$^{+/-}$ and SR-A$^{-/-}$ BMDCs (Figure 2E, left panel). Accordingly, no difference was observed by FACS analysis between the cell-surface expression of Fc-receptors on CD11c-positive SR-A$^{+/-}$ and SR-A$^{-/-}$ BMDCs, nor was a difference in expression observed for the phagocytic receptors DEC205 and LOX-1 (Figure 2F) [44-48]. However, consistent with the established role of SR-A as a receptor for modified LDL, SR-A$^{-/-}$ BMDCs exhibited a 4-fold impairment in the endocytic uptake of AcLDL as compared to SR-A$^{+/-}$ cells (Figure 2E, right panel). These data demonstrate that SR-A functionally mediates phagocytic as well as endocytic events in BMDCs, that loss of SR-A

expression impairs the phagocytic ability of DCs, and that loss of SR-A function is not fully compensated by other dendritic cell receptors.

The phagocytic defect in SR-A$^{-/-}$ BMDCs is characterized by impairment along two parameters

SR-A$^{-/-}$ BMDCs consistently exhibited less uptake of Alexa488-*E. coli* than SR-A$^{+/-}$ cells following the phagocytosis assays (Figures 2C and 3A). Therefore, we examined the contribution of SR-A to phagocytic uptake of bacteria by BMDCs more rigorously to determine the nature of the phagocytic defect. To assess the phagocytic impairment in SR-A$^{-/-}$ BMDCs, we analyzed phagocytic uptake by two different parameters: the percent of dendritic cells that phagocytosed bacteria, and the total number of bacteria phagocytosed per dendritic cell. BMDCs were incubated with bacteria at a ratio within the linear range of phagocytic uptake, whereupon the Alexa488-positive BMDC population was analyzed (gate illustrated in Figure 3B). From this we identified that the phagocytic defect derived from the loss of SR-A was the result of a combination of two parameters: only half as many SR-A$^{-/-}$ BMDCs acquired bacteria when compared to percent bacterial uptake in SR-A$^{+/-}$ cells (Figure 3C). Secondly, upon analysis of only those dendritic cells that acquired bacteria (the Alexa488^{+} dendritic cells, as gated in Figure 3B), the average fluorescence of those cells following the phagocytosis assay was reduced by 30% in SR-A$^{-/-}$ BMDCs relative to SR-A$^{+/-}$ BMDCs (Figure 3D). The latter observation demonstrates that on a per-cell basis, SR-A$^{-/-}$ BMDCs phagocytose fewer bacteria than SR-A – expressing cells. These data show that loss of SR-A affects BMDC phagocytosis both by reducing the number of phagocytosis-competent cells and by reducing the efficiency of bacterial uptake by the BMDCs.

SR-A$^{-/-}$ BMDCs are impaired in the phagocytic uptake of live E. coli

Having identified that SR-A contributes to the DC phagocytic uptake of heat-killed bacteria, we extended our investigation to test the physiologically relevant contribution of SR-A to the phagocytosis of live bacteria. To perform this analysis we used the gentamycin protection assay [36]: BMDCs were incubated with live *E. coli* for 45 minutes at 37°C, followed by incubation in media containing gentamycin. Bacteria phagocytosed by BMDCs are protected from the lethal effects of gentamycin, and can be quantified as colony-forming units (CFUs). Consistent with the results with heat-killed bacteria, we observed that SR-A$^{-/-}$ BMDCs are deficient in the phagocytosis of live *E. coli* and that this deficit increased at higher multiplicities of infection (MOIs) (Figure 4). Moreover, there was no difference in the phagocytic capacity between BMDCs homozygous for SR-A and those heterozygous for SR-A, suggesting that a single copy of the SR-A gene is sufficient to provide full SR-A-dependent phagocytic function (Figure 4). These experiments demonstrate that SR-A is a functional and necessary receptor on BMDCs for the efficacious phagocytosis of live bacteria.

SR-A localizes outside lipid raft domains but requires rafts to mediate phagocytosis

Multiple cell-surface receptors as well as cholesterol-rich lipid rafts have been implicated to play important roles in phagocytosis processes [49-51]. Moreover, previous work has demonstrated that lipid rafts are important for SR-A -dependent infection of macrophages by the intracellular bacteria *Brucella abortus* [52]. To determine the cell-surface localization of SR-A on BMDCs we performed electron microscopy. Scanning Electron Microscopy (SEM) of immature SR-A -expressing BMDCs revealed the expected cellular architecture of elongated dendritic processes and lamellapodial extensions (Figure 5A, top panel). SEM was then used to identify gold-decorated SR-A on the murine BMDCs. Immature BMDCs were stained with anti-SR-A mAb 2F8 followed by gold-conjugated goat-anti-rat antibody. Qualitative assessment of gold decoration revealed that SR-A protein is broadly distributed on the cell surface, and localizes to characteristic morphological features of DCs, with SR-A staining

observed on both the dendrites of BMDCs as well as on the surface of the lamellapodia (Figure 5A, bottom panel). Gold was not observed on cells unstained with 2F8 (data not shown). The cell-surface distribution of SR-A observed by SEM was confirmed by confocal microscopy, as BMDCs stained with FITC-conjugated 2F8 antibody also exhibited a broad, slightly punctuate, distribution (Figure 5B).

Due to the distribution of SR-A observed in the confocal microscopy, we biochemically tested if SR-A associates with lipid raft microdomains in the cellular plasma membrane. Lipid rafts were extracted from non-raft membrane components using low-detergent treatment of BMDCs, and the raft and non-raft –associated proteins were separated using a discontinuous sucrose gradient [36,38]. Detergent-soluble fractions from the sucrose gradient represent non-raft components of the cell membrane, while detergent-insoluble fractions represent lipid raft-associated proteins. Western blot analysis of the sucrose gradient demonstrates that SR-A is found exclusively in the detergent-soluble fractions of the gradient and is thus not associated with lipid rafts on the plasma membrane of immature BMDCs (Figure 5C). Fluorescent cholera toxin-B subunit (CT-B), which binds the glycosphingolipid GM1 at the cell membrane and is commonly used to label lipid rafts [39,53-55], was used as a positive control for the fidelity of raft separation within the gradient. Detection of fluorescent CT-B on the blot fractions verified separation of lipid rafts from the detergent-soluble fractions (Figure 5C).

Since SR-A localization to lipid raft domains on immature BMDCs was not observed, we then asked whether SR-A-dependent phagocytosis and lipid raft-mediated phagocytosis were independent or interrelated processes in BMDCs. To perform this analysis, we used β-cyclodextrin (β-CD) which is commonly employed to extract cholesterol from cell membranes [56,57]. After pre-treatment of SR-$A^{+/-}$ and SR-$A^{-/-}$ BMDCs with 5 mM β-CD, the phagocytosis of live *E. coli* was compared in treated and untreated cells using the gentamycin protection assay. Treatment of BMDCs with 5 mM β-CD resulted in 71 ± 4.5 (S.D., n=4) percent cholesterol depletion, consistent with previous reports [58,59]. We observed the characteristic phagocytic deficiency in untreated SR-$A^{-/-}$ BMDCs relative to untreated SR-$A^{+/-}$ BMDCs. Importantly, β-CD treatment further and equally impaired the phagocytic capability of both cell types to ~30% the levels of untreated SR-A-expressing BMDCs (Figure 5D). We note that treatment of BMDCs with β-CD did not remove SR-A from cell membranes (by Western analysis; data not shown). To test whether β-CD treatment of BMDCs nonspecifically abolished all phagocytic activity, we compared uptake of live *E. coli* in β-CD -treated BMDCs to untreated cells left on ice (Figure 5E). These data show that a substantial amount of phagocytic activity remains in cholesterol-depleted cells, even in the absence of SR-A expression (Figures 5D and 5E). This indicates that the cells are still functional and, moreover, that there is a phagocytic activity that is independent of both SR-A and cholesterol depletion. Additionally, we note that treatment with β-cyclodextrin does not affect BMDC viability in these cells under the aforementioned experimental conditions, as determined by propidium iodide staining of untreated and treated BMDCs (Figure 5F). These functional analyses indicate that SR-A substantially contributes to bacterial phagocytosis by BMDCs, that SR-A –mediated phagocytosis requires lipid raft activity, and that SR-A function augments that conferred by lipid rafts.

Discussion

A critical aspect of dendritic cell function is their ability to constantly sample the local microenvironment [19,20,22]. Since scavenger receptors recognize pathogens and microbe-derived molecules and facilitate the uptake of these ligands [22] we asked what the contribution of these receptors, SR-A in particular, were to the DC phagocytic process. Here we report that SR-A participates in the phagocytosis of gram-negative bacteria by dendritic cells. SR-A expression is upregulated during DC differentiation and SR-A expression levels during

differentiation correlate with CD11c expression. Using the SR-A knockout mouse as a genetically-defined source of dendritic cells, we show that SR-A$^{-/-}$ BMDCs exhibit impaired phagocytic uptake of the gram-negative bacteria *E. coli* relative to SR-A-expressing BMDCs. The phagocytic defect observed in SR-A$^{-/-}$ BMDCs is a function of both a decrease in the percentage of cells that can phagocytose bacteria as well as a decrease in the bacterial uptake efficiency per BMDC. Moreover, mechanistic analysis of the phagocytic defect in SR-A –deficient cells identified that, although SR-A does not appear to be integrated into cell-surface lipid rafts, there is a functional dependence of SR-A -mediated phagocytosis upon the presence of lipid rafts.

Using the SR-A$^{-/-}$ mouse as a tool to study the functional contribution of SR-A to BMDC phagocytosis, a 50% decrease was observed in the ability of SR-A$^{-/-}$ BMDCs to phagocytose heat-killed fluorescently labeled *E. coli* as compared to SR-A$^{+/-}$ BMDCs. Moreover, we demonstrate that SR-A$^{-/-}$ BMDCs are significantly impaired in their ability to phagocytose and accumulate live *E. coli*. The phagocytic deficiency for uptake of both heat-killed and live bacteria increased at higher bacteria:BMDC ratios. Interestingly, we note that BMDCs that lack SR-A are deficient in phagocytosis of bacteria along two parameters. First, SRA$^{-/-}$ BMDCs are less efficient at the phagocytosis of bacteria, taking up 30% fewer particles-per-DC than heterozygous controls. Second, the percentage of the total BMDC population that is able to phagocytose bacteria is drastically reduced in the SRA$^{-/-}$ BMDCs as compared to controls. This latter observation could be the result of several different possibilities. One explanation of the data is that due to heterogeneity of BMDCs produced during the differentiation process, a proportion of these cells rely solely on SR-A for the phagocytosis of *E. coli* and that disruption of SR-A expression renders this percentage of the BMDC population incompetent for bacterial uptake. This possibility requires that SR-A is uniquely responsible for bacterial uptake in a sub-population of DCs. We consider that a more likely scenario is that DCs express a variety of scavenger receptors which work in concert to coordinate the recognition and internalization of foreign pathogens. This is supported by our observation that ligands that compete for the binding of multiple scavenger receptors, carrageenan and fucoidin, inhibit BMDC bacterial uptake to a greater extent than the phagocytic defect due to the specific loss of SR-A. We propose that the loss of SR-A disrupts the receptor-to-pathogen stoichiometry of these DCs. We suggest that a critical number of receptors need to be engaged to initiate the phagocytosis of bacteria and that the loss of SR-A in BMDCs decreases the rate at which this critical number is reached during DC-pathogen interactions. In this manner, we hypothesize that SR-A disruption decreases the percent of BMDCs capable of phagocytosis and the number of phagocytic events that a given DC is able to achieve.

Phagocytic processes can be mediated by several membrane receptors and involve dramatic restructuring of plasma membrane morphology and composition [49,50]. Previous work indicates that both SR-A and lipid raft domains at the cell surface can contribute to phagocytic internalization [14,16,51,52]. Thus, we asked whether SR-A localizes to lipid raft domains on BMDCs and whether SR-A -mediated phagocytosis in BMDCs is dependent on the presence of lipid rafts. Our biochemical and microscopic analyses did not reveal a physical association of SR-A with lipid rafts. However, functional analyses of phagocytosis by SR-A$^{+/-}$ and SR-A$^{-/-}$ BMDCs following treatment with β-cyclodextrin revealed that cholesterol sequestration substantially impairs the phagocytosis of live bacteria and reduces phagocytosis in both SR-A$^{+/-}$and SR-A$^{-/-}$ BMDCs to the same level (∼30% of untreated SR-A$^{+/-}$levels). These results demonstrate that SR-A functions cooperatively with lipid rafts to maximize phagocytic activity and, moreover, that SR-A -dependent phagocytosis of *E. coli* requires lipid raftstructures. The relationship between SR-A and lipid raft activity at the cell membrane during phagocytosis remains to be characterized. However, previous work in macrophages has demonstrated that specific engagement of SR-A can produce signaling events [18], and that SRA –dependent interactions can significantly affect responses to microbial products [18,60]. We speculate that

SR-A may functionally enhance cellular phagocytic capability both through its established role of binding microbes and through potentiating the uptake of microbes through cellular signaling. Further studies into SR-A –dependent signaling processes in dendritic cells will provide novel insight into how this scavenger receptor influences immune functions.

In this work, we show that SR-A plays a significant role in the phagocytosis of the gram-negative bacteria *E. coli* by dendritic cells. SR-A has traditionally been regarded as a macrophage receptor, however our data now describes an important function for this molecule in dendritic cells. Since dendritic cell phagocytic activity is critical both for pathogen clearance and for immune response activation, this work provides insight into the impaired pathogen defense of SR-A –deficient mice [17] and their increased susceptibility to microbial infection [26,27].

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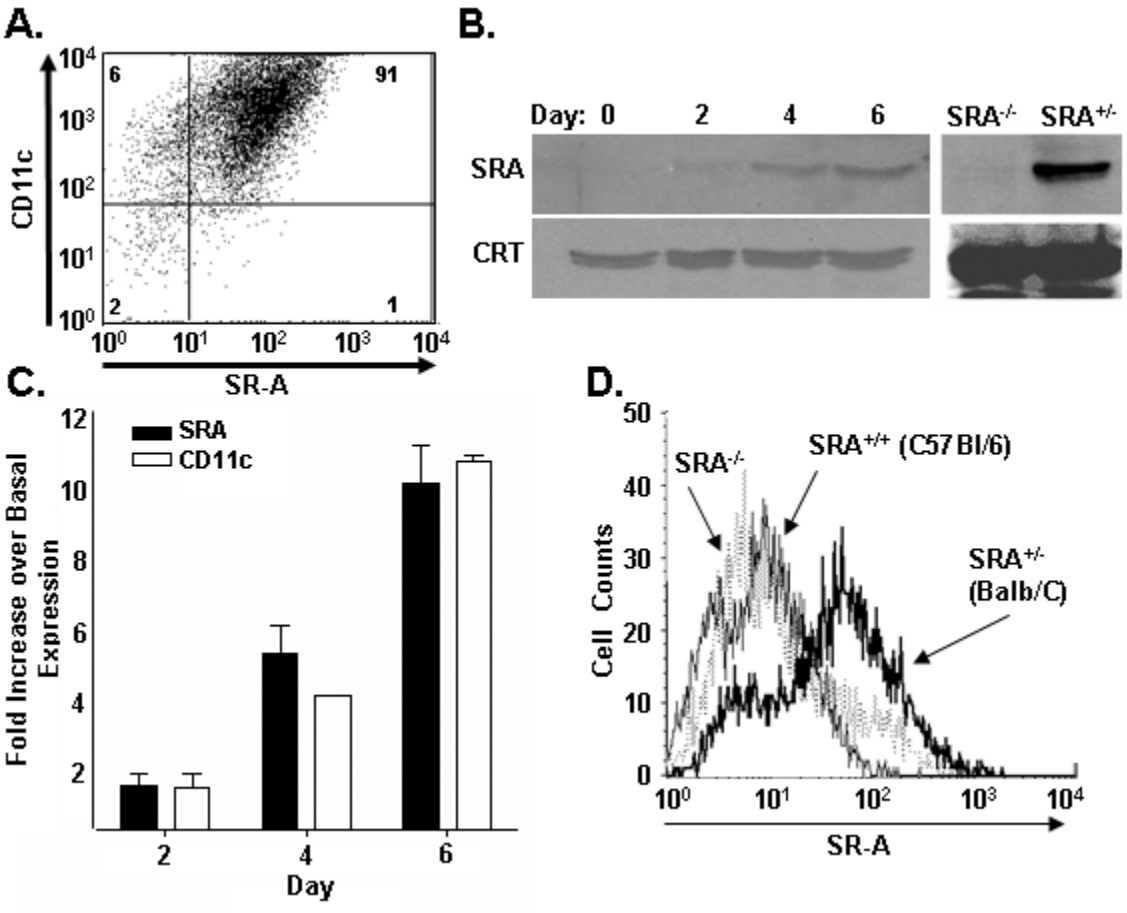

Figure 1.

SR-A expression is induced during BMDC differentiation and correlates with CD11c expression levels. (A) BMDCs from SR-A$^{+/-}$ mice were assayed for CD11c and SR-A expression by FACS analysis. Analysis shows that >90% of Day 6 BMDCs are CDllc^{+}SR-A^{+}. Cells were gated on viable, differentiated BMDCs by forward and side scatter profile. (B) CB6F1 SR-A$^{+/-}$ BMDCs were harvested on the indicated days and lysates from these harvests were assayed for SR-A expression by Western Blot (left). Cell lysates from Day 6 BMDCs of SR-A$^{+/-}$ and SR-A$^{-/-}$ mice were prepared and assayed by Western blot for total cellular SR-A expression (right). Calreticulin (CRT) was used as a loading control. (C) BMDC cultures were prepared and harvested on indicated days as in (B) and analyzed for CD11c and SR-A expression by FACS analysis. Graph is represented as fold-increase over time, with standard deviation of triplicate samples shown. (D) Anti- SR-A staining specificity was confirmed by FACS analysis using 2F8 mAb to stain Day 6 BMDCs from CB6F1 SR-A$^{+/-}$, C57Bl/6, and SR-A$^{-/-}$ mice. SR-A$^{+/-}$ BMDCs show robust staining, SR-A staining is not detected in the SR-A$^{-/-}$ cells, while C57BL/6 -derived SR-A$^{+/+}$ BMDCs serve as a negative control since 2F8 does not recognize the C57BL/6-derived SR-A gene product.

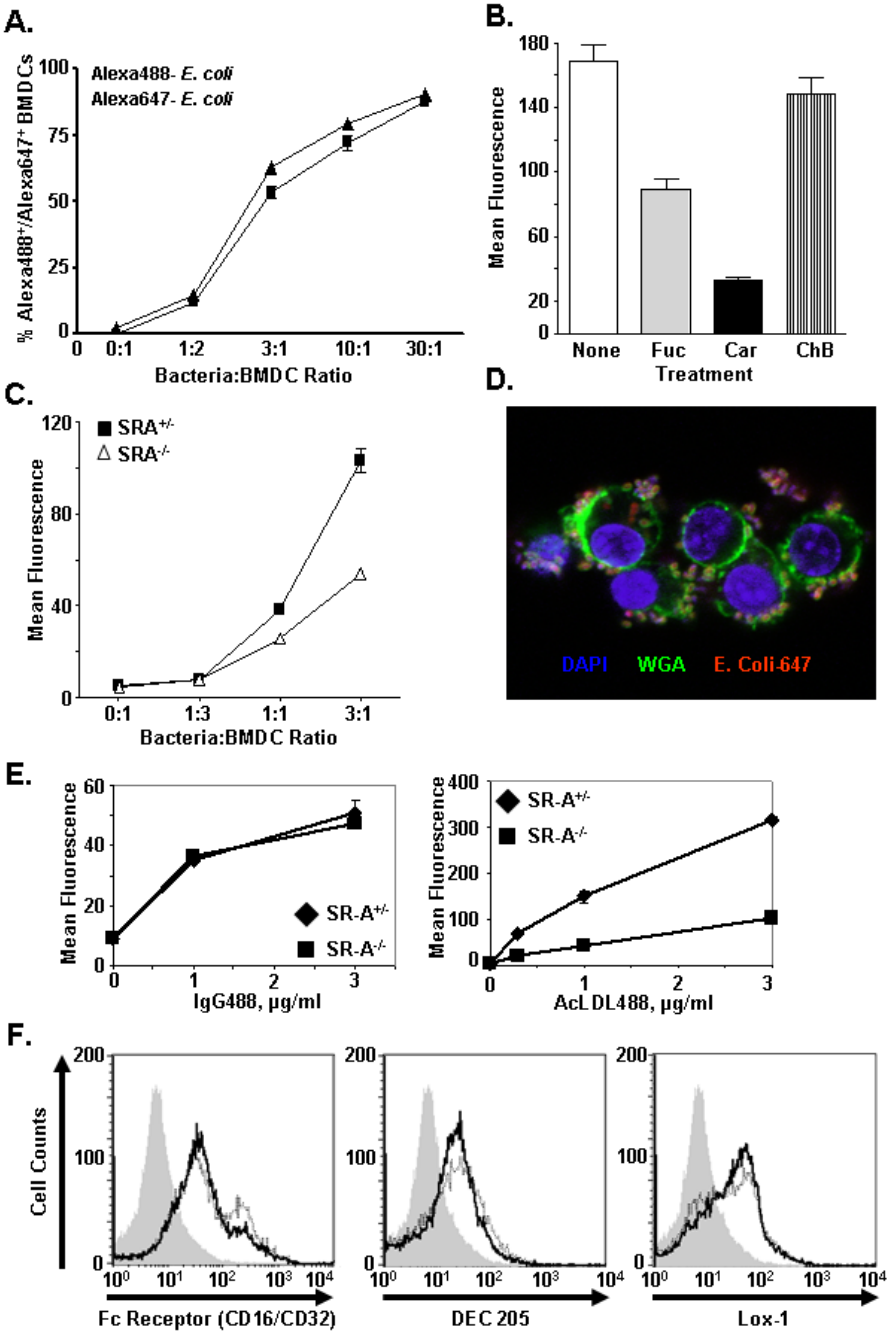

Figure 2.

SR-A$^{-/-}$ BMDCs exhibit defective phagocytosis of heat-killed *E. coli*. (A) Alexa488-labeled and Alexa647-labeled *E. coli* were incubated at the indicated bacteria:BMDC ratios with SR-A$^{+/-}$ BMDCs to determine the linear range of BMDC phagocytic activity. The percent of BMDCs that acquired bacteria, as assessed by fluorescence, was used as the analysis parameter. (B) FACS analysis of BMDCs incubated with Alexa488-labeled *E. coli* at a 3:1 bacteria:BMDC ratio for 20 minutes at 37°C in the absence (None) or presence of the scavenger receptor ligands fucoidan (Fuc, 250 µg/ml) or carrageenan (Car, 75 µg/ml). Chondroitin-B sulfate (ChB, 250 µg/ml) was used as a negative control for the scavenger receptor competitive ligands. (C) SRA$^{+/-}$ and SRA$^{-/-}$ BMDCs were incubated with Alexa488-labeled *E. coli* as in (A) at the

indicated bacteria:BMDC ratios, and assessed for relative uptake of *E. Coli* by FACS analysis. (D) Confocal microscopy of $SRA^{+/-}$ BMDCs incubated with Alexa647-labeled *E. coli* (viewed as red) at a 3:1 bacteria:BMDC ratio for 20 minutes at 37°C, followed by counter-staining with DAPI (blue) and FITC-conjugated wheat germ agglutinin (WGA; viewed as green), show that fluorescence conferred to BMDCs by incubation with fluorescently labeled *E. coli* is due to binding and uptake of the bacteria. (E) SR-$A^{+/-}$ or SR-$A^{-/-}$ BMDCs were incubated with the indicated concentrations of Alexa488-labeled IgG (left) or Alexa488-labeled acetylated-LDL (right) for 20 minutes at 37°C, followed by washing and FACS analysis to assess endocytic uptake of each ligand. Standard deviation is shown. (F) FACS analyses of SR-$A^{+/-}$ (black lines) or SR-$A^{-/-}$ (grey lines) BMDCs stained for the phagocytic receptors Fc receptors CD16/CD32, DEC205, and Lox-1. The grey-filled histograms represent negative controls.

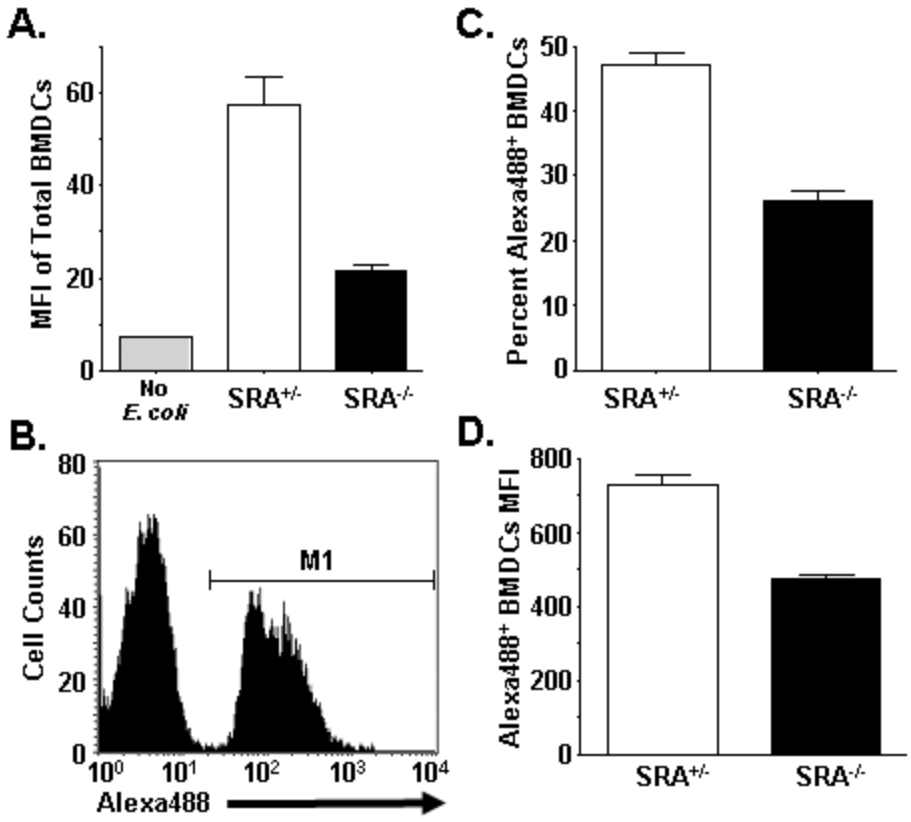

Figure 3.

The phagocytosis defect in SRA$^{-/-}$ BMDCs is a function of multiple parameters. (A) SRA$^{+/-}$ and SRA$^{-/-}$ BMDCs were untreated or were incubated with Alexa488-*E. coli* at a 3:1 bacteria:BMDC ratio. Total mean fluorescence intensity (MFI) was assayed by FACS, with SRA$^{-/-}$ BMDCs exhibiting impairment in bacterial uptake. (B) FACS histogram of SRA$^{+/-}$ BMDCs incubated with Alexa488-*E. coli* at a 3:1 bacteria:BMDC ratio. Gate (M1) indicates BMDCs that accumulated Alexa488-*E. coli*. The phagocytic impairment of SR-A$^{-/-}$ BMDCs was then analyzed by two different parameters, percent of dendritic cells that phagocytose bacteria (C) and the relative number of bacteria phagocytosed per dendritic cell (D). (C) The percent of BMDCs that accumulate *E. coli* was calculated by the number of cells within gate M1 (as in (B)) divided by the total number of cells analyzed. Twice the percentage of SR-A -expressing BMDCs acquired bacteria in comparison to SR-A –deficient BMDCs. (D) The relative number of bacteria phagocytosed per dendritic cell within gate M1 was assessed by mean Alexa488 fluorescence. Amongst the dendritic cells that accumulated bacteria, SR-A$^{-/-}$ BMDCs acquired fewer bacteria per cell than SR-A –expressing BMDCs. Standard deviation is shown.

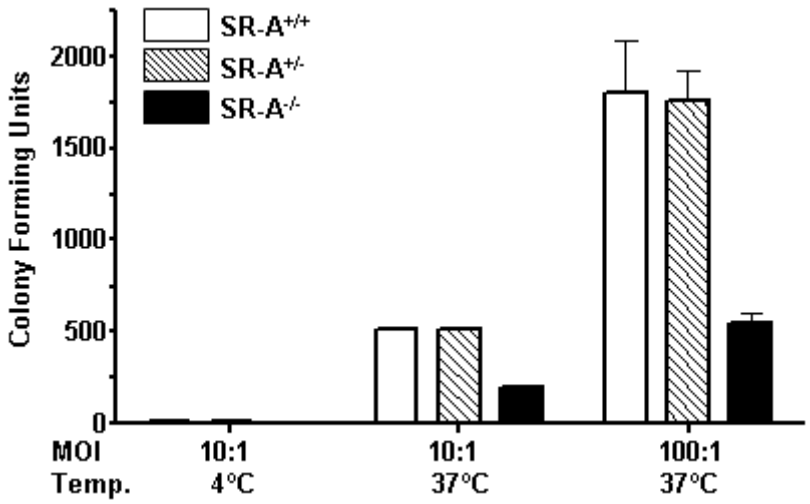

Figure 4.

$SRA^{-/-}$ dendritic cells are impaired in their ability to phagocytose live *E. coli*. $SRA^{+/+}$, $SRA^{+/-}$ and $SRA^{-/-}$ BMDCs were incubated with live *E. coli* bacteria at the indicated multiplicity of infection (MOI) for 45 minutes either on ice, or at 37°C**.** Gentamycin was then used to kill non-internalized bacteria. Bacteria phagocytosed (protected from antibiotic) was quantified as the number of colony-forming units (CFU) following gentamycin treatment. Standard deviation is shown.

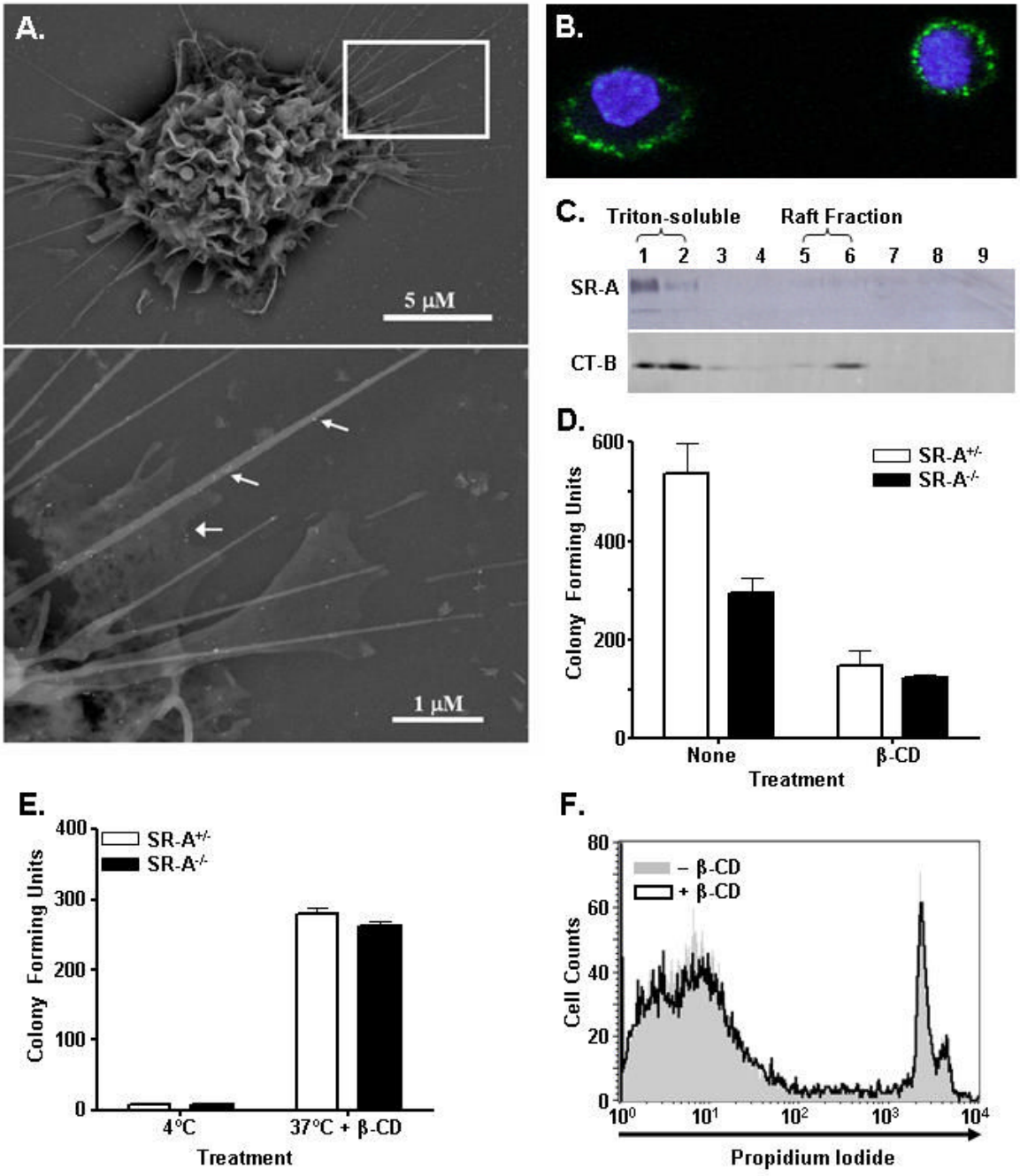

Figure 5.

SR-A –mediated phagocytosis is dependent on lipid rafts although SR-A does not physically associate with lipid rafts at the cell membrane. (A) Scanning Electron Microscopy of CB6F1 BMDCs shows the distinct morphological features of dendritic cells (top panel). Magnification of the section outlined in the white box (shown in the bottom panel) reveals gold-decorated SR-A on both dendritic extensions (small arrows) and lamellapodia (large arrows). 18 nM gold particles were used to decorate the 2F8-stained BMDCs. (B) Confocal microscopy of SR-$A^{+/-}$ BMDCs stained for SR-A (green) shows broad surface expression of SR-A. Cells were counter-stained with DAPI (blue). (C) Detergent-insoluble BMDC membranes (lipid rafts) were separated from detergent-soluble membrane components using a discontinuous sucrose

gradient as described in Methods. 1 ml fractions from the gradient were collected and assessed by Western blot for SR-A expression. SR-A was observed in the detergent-soluble membrane fractions (fractions 1-2) but not in the detergent-insoluble lipid raft fractions (fractions 5-6) of the gradient, while raft-associated cholera toxin-B subunit (CT-B, FITC-conjugated) migrated to fractions 5 and 6 of the gradient. (D) $SRA^{+/-}$ and $SRA^{-/-}$ BMDCs were incubated in the presence or absence of 5 mM β-Cyclodextrin (β-CD) for 30 minutes at 37°C, followed by washing. Cells were then incubated with live *E. coli* bacteria at an MOI of 10:1, followed by incubation in gentamycin to kill non-internalized bacteria. Phagocytosis of bacteria was evaluated as the number of colony-forming units (CFU) per treatment. Standard deviation is shown. (E) $SRA^{+/-}$ and $SRA^{-/-}$ BMDCs treated with or without 5 mM β-CD were subsequently incubated with a 10:1 MOI of live *E. coli* for 45 minutes at 37°C or 4°C as indicated, followed by gentamycin treatment to kill non-internalized bacteria. Phagocytosis of bacteria is represented by the number of colony-forming units (CFU) per treatment. Standard deviation is shown. (F) β-CD treated and untreated $SRA^{+/-}$ BMDCs were comparatively assessed for cell viability by FACS analysis of propidium iodide staining. With the described experimental conditions β-CD treatment did not affect the viability of BMDCs.