The scavenger receptor SR-A I/II (CD204) signals via the receptor tyrosine kinase Mertk during apoptotic cell uptake by murine macrophages

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Abstract: Apoptotic cells (AC) must be cleared by macrophages (Mø) to resolve inflammation effectively. Mertk and scavenger receptor A (SR-A) are two of many receptors involved in AC clearance. As SR-A lacks enzymatic activity or evident intracellular signaling motifs, yet seems to signal in some cell types, we hypothesized that SR-A signals via Mer receptor tyrosine kinase (Mertk), which contains a multisubstrate docking site. We induced apoptosis in murine thymocytes by dexamethasone and used Western blotting and immunoprecipitation to analyze the interaction of Mertk and SR-A in the J774A.1 (J774) murine Mø cell line and in peritoneal Mø of wild-type mice and SR-A−/− mice. Phagocytosis (but not adhesion) of AC by J774 was inhibited by anti-SR-A or function-blocking SR-A ligands. In resting J774, SR-A was associated minimally with unphosphorylated (monomeric) Mertk; exposure to AC induced a time-dependent increase in association of SR-A with Mertk in a direct or indirect manner. Anti-SR-A inhibited AC-induced phosphorylation of Mertk and of phospholipase Cγ2, essential steps in AC ingestion. Relative to tissue Mø of wild-type mice, AC-induced Mertk phosphorylation was reduced and delayed in tissue Mø of SR-A−/− mice, as was in vitro AC ingestion at early time-points. Thus, during AC uptake by murine Mø, SR-A is essential for optimal phosphorylation of Mertk and subsequent signaling required for AC ingestion. These data support the Mertk/SR-A complex as a potential target to manipulate AC clearance and hence, resolution of inflammation and infections. *J. Leukoc. Biol.* 84: 510–518; 2008.

Key Words: *apoptosis · phagocytosis · signal transduction · protein kinases/phosphatases · mice · inbred strains*

INTRODUCTION

Efficient clearance of apoptotic leukocytes in resolving inflammation is vital to minimize tissue injury and to avoid the risk of autoimmunity as a result of inappropriate presentation of self-antigens [1–3]. In some organs, apoptotic cells (AC) can clearly be ingested by cell types other than macrophages (Mø), especially certain specialized epithelial cells [4–7]. In the lungs, a frequent site of infection and inflammatory diseases, epithelial cells and fibroblasts ingest AC in vitro, albeit less efficiently than do Mø [8–10]. However, during steady-state in most organs and certainly during resolving inflammation, mononuclear phagocytes are believed to clear most AC. Importantly, Mø that contact AC assume a unique activation state [11–18] and elaborate TGF-β and PGE_2 [13, 19]. These immunosuppressive mediators hasten the resolution of inflammation from successfully managed infections [20] but also have the potential to compromise host defenses against subsequent pathogens and to promote fibrosis [21–25]. Thus, understanding the molecular mechanisms mediating Mø ingestion of AC has significant implications for multiple areas of biology and medicine.

No current model comprehensively explains Mø uptake of AC, a process initiated by changes on the AC surface (referred to as "eat me" signals) that induce its adhesion to the phagocyte. This interaction is mediated by phagocyte receptors that bind the AC directly or via a variety of opsonizing proteins, which bridge apoptotic ligands and the receptor. The best-characterized AC eat-me signal is the exposure of phosphatidylserine (PS) on the outer leaflet of the AC plasma membrane [26]. The "tether and tickle" model of AC ingestion [27] postulates that initial AC adhesion via multiple redundant receptors is followed by triggering via stereospecific recognition of the exposed PS [28]. Although it has since become evident that the original gene identified as this PS receptor [29] appears not to encode a cell-surface receptor (reviewed in ref. [30]), three groups have recently identified novel and unrelated PS-binding receptors: T cell Ig- and mucin domain-containing molecule [31], brain-specific angiogenesis inhibitor 1 [32], and stabilin-2 [33]. It appears likely that PS recognition will prove to be central to AC ingestion [34].

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However, a bewildering array of other phagocyte receptors of unrelated molecular classes has also been implicated in AC uptake [26, 35, 36], in some cases based on antibody-blocking not corroborated by more definitive experiments using gene-targeting or peptide-domain modification. Such putative AC receptors include lectins, the vitronectin receptor, scavenger receptors of several classes, CD14, and CD91 (reviewed in ref. [37]). The key question of how these many receptors may interact to induce AC ingestion and subsequent immunoregulatory effects is only beginning to be understood [15, 38, 39]. That some of these receptors may be nonessential for the decision by the phagocyte to ingest the AC is suggested by the finding that expression of individual receptors can increase AC adhesion with minimal effects on uptake [40]. One reason for this apparent redundancy might be that receptor expression and possibly use are tissue-, cell type-, and condition-dependent [41]. An alternative, not mutually exclusive, reason could be that efficient Mø ingestion of AC benefits from the integration of signals from multiple receptors, some possibly not specific for the apoptotic state.

Scavenger receptor A (SR-A) I/II is a multifunctional Mø receptor [42, 43] that is involved in AC clearance, although not obligatorily [44–46]. SR-A, also known as CD204, is a helically coiled, homotrimeric, type II integral membrane protein, which does not bind unmodified PS [47]. SR-A does bind a wide variety of ligands, most of which are covalently modified molecules [e.g., oxidized low-density lipoprotein (LDL)] distinguished from their native counterparts in part by electrostatic charge [48]. Such modifications include oxidation and glycation that may be particularly prevalent under conditions such as cellular stress and death. Importantly, the amino acid sequence of SR-A contains no evident motifs associated with intracellular signaling. Thus, despite evidence that SR-A ligation not only induces AC ingestion but also regulates cytokine and chemokine elaboration [49–51], how SR-A signals and whether it is yet another potentially redundant AC adhesion receptor are unknown.

By contrast, Mer receptor tyrosine kinase (Mertk; also known as Tyro12, c-mer proto-oncogene tyrosine kinase, Eyk, and NYK; Swissprot accession number Q12866) is essential for AC uptake by murine Mø in vivo and in vitro [3, 52, 53]. Mertk is a large (200-kDa) transmembrane receptor that binds PS indirectly [via the serum-derived protein growth arrest-specific gene 6 (Gas6)] and possesses well-described signaling capabilities [54]. Mertk contains extracellular Ig and fibronectin III domains (implying possible cell–cell interactions) and an intracellular, multisubstrate docking site that can bind Src homology 2-containing proteins such as the p85 chain of the enzyme PI-3K and the adapters Grb2 and Vav1 [55, 56]. As a receptor tyrosine kinase (RTK) of the Tyro3 family, Mertk can phosphorylate and activate multiple downstream signaling intermediaries. Stimulation of a chimeric receptor containing the Mertk intracellular domain phosphorylated PI-3K p85, Grb2, Raf-1, Shc, phospholipase C (PLC)γ, and ERK [57]. We have previously reported that Mertk is not required for AC adhesion by murine tissue Mø and that exposure of murine peritoneal Mø (PMø) to AC sequentially induces tyrosine phosphorylation of Mertk, association of Mertk with PLCγ2, the principal PLCγ isoform in murine Mø, and tyrosine phosphorylation of PLCγ2 [58, 59]. Collectively, these characteristics indicate that Mertk plays a central role in signaling during AC recognition.

We hypothesized that on Mø contact with AC, SR-A might interact with and signal via Mertk. To test this hypothesis, we examined the interactions of SR-A and Mertk in murine Mø cell line J774A.1 (J774), which avidly ingests AC in a Mertk-dependent manner [59, 60], and in resident murine tissue Mø of wild-type and SR-A gene-targeted mice. Using immunoprecipitation, Western blotting, and functional assays of in vitro phagocytosis and adhesion, we provide evidence that SR-A signaling during AC ingestion involves Mertk and PLCγ2.

MATERIALS AND METHODS

Reagents

The following reagents were purchased from the indicated vendors: PBS without cations, DMEM, FBS, and penicillin/streptomycin from Life Technologies (Rockville, MD, USA); sodium orthovandadate, sodium fluoride, octyl-β-D-glucopyranoside (OG), fucoidan, heparin, dextran sulfate, BSA Fraction V, dexamethasone, 2-ME, glycerol, NaCl, Tris HCl, and phosphatase inhibitor cocktail II from Sigma Chemical Co. (St. Louis, MO, USA); rat anti-SR-A antibody (clone 2F8) from Serotec (Raleigh, SC, USA); goat anti-SR-A antibody and polyclonal goat anti-Mertk antibody from R&D Systems (Minneapolis, MN, USA); anti-CD36 from BD PharMingen (San Jose, CA, USA); polyclonal rabbit anti-PLCγ2 antibody (sc-407), anti-actin antibody, rat IgG, mouse IgG, protein L-agarose, protein G-agarose, and HRP-conjugated anti-rabbit and anti-goat IgG from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-phospho (p)Mertk antibody from FabGennix Inc. (Frisco, TX, USA); SDS, 0.2 μm polyvinylidene difluoride (PVDF) membrane, nonfat dry milk blocker, and 7.5% acrylamide Ready Gels from BioRad (Hercules, CA, USA); Supersignal West Femto maximum sensitivity substrate and TBS, pH 7.2, from Pierce (Rockford, IL, USA); and Kodak X-Omat AR film and eight-well Lab-Tek slides from Fisher Scientific (Chicago, IL, USA).

Mice

SR-A−/− mice on a 129/SvJ strain background, the generous gift of Dr. Willem J. S. de Villiers (University of Kentucky Medical Center, Lexington, KY, USA), were bred locally at our facility. Specific pathogen-free, female mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA; 129P3/J strain) or Charles River Laboratories Inc. (Wilmington, MA, USA; C57BL/6 strain) at 7–8 weeks of age and were used at 8–14 weeks of age. Mice were housed in the Animal Care Facility at the Ann Arbor VA Medical Center (Ann Arbor, MI, USA), which is fully accredited by the American Association for Accreditation of Laboratory Animal Care. This study complied with the National Institutes of Health "Guide for the Care and Use of Laboratory Animals" (Department of Health, Education, and Welfare, Publication No. 80-32) and followed a protocol approved by the Animal Care Subcommittee of the local Institutional Review Board.

Preparation of AC

All thymocytes were obtained from unmanipulated C57BL/6 mice, which were killed humanely by rapid asphyxia in a high CO_2 environment. Thymuses were harvested and minced to yield a single-cell suspension. To induce apoptosis, thymocytes were resuspended in complete medium to a concentration of 1×10^6 cells/ml and incubated for 5 h in complete medium containing 1 μM dexamethasone. This treatment yields a population with a low degree of contamination by late apoptotic or necrotic cells [60, 61].

Preparation of activated Mø and PMø

129/SvJ SR-A−/− mice and wild-type 129P3/J mice were killed by asphyxia in a high CO_2 environment. PMø were collected by peritoneal lavage using a total of 10 ml PBS-EDTA, which was administered in 2 ml aliquots. PMø among the lavage cells were isolated by plastic adherence with an overnight incubation in complete medium in a 5% CO_2 environment at 37°C. Nonadherent cells were removed by gentle washing.

Cell line

The J774 cell line was obtained from American Type Culture Collection (Manassas, VA, USA). These Mø were grown in 150 cm^2 plastic flasks in DMEM high-glucose medium containing 10% heat-inactivated FCS and 100 units/ml penicillin/streptomycin. The cells were incubated at 37°C in air (95%)-CO_2 (5%) in a humidified incubator. Cells were removed from the flasks by scraping and plated at $10–15 \times 10^6$ in a 100-mm plate (for immunoprecipitation and Western blot) and used for experiments (immunoprecipitation, Western blot, phagocytosis, and adhesion assays) after 24 h adherence.

Immunoprecipitation

For immunoprecipitation studies, J774 cells or Mø from SRA−/− mice or wild-type 129P3/J mice were exposed to apoptotic thymocytes in a 1:5 ratio for various times. For blocking experiments, J774 were preincubated with anti-SR-A or rat IgG (20 μg/ml) for 45 min. For lysis, J774 were washed gently three times using cold PBS with 10 mM sodium vanadate and 50 mM sodium fluoride (phosphatase inhibitors) and then lysed for 30 min on ice using cold OG buffer, which contained 1.5% OG, 50 mM Tris HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 10% glycerol, protease inhibitor cocktail II (1:100, Sigma Chemical Co.), and phosphatase inhibitors, as above. Cells were drawn twice into a 3-ml syringe via a 25-G needle. The lysed cells were centrifuged at 13,000 *g* for 10 min at 4°C. To preclear lysed cell supernatants, one of the following combinations was added: 5 μg mouse IgG and 40 μl protein G agarose (for phosphorylation studies) or 5 μg goat IgG and 40 μl protein G-agarose (for association studies). Cell lysates were rocked for 30 min at 4°C and then were centrifuged at 2090 *g* for 5 min at 4°C. The pellets were washed twice in OG buffer, and the supernatants were combined. The immunoprecipitating antibody (6 μg) was added to the combined supernatants, and the mixture was rocked overnight at 4°C. Next, 40 μl protein G agarose was added, and the mixture was rocked for 2 h at 4°C. Finally, the protein bound to the agarose conjugate was centrifuged at 2090 *g* for 5 min at 4°C, and the pellet was washed twice using OG buffer.

Western analysis

To run samples on SDS-PAGE, 15 μl 5× SDS-PAGE sample buffer and 7 μl 1 M DTT were added to the pellet from immunoprecipitation, and samples were heated at 90°C for 5 min. The samples were centrifuged at 2090 *g* for 5 min at room temperature, and the supernatant was saved for SDS-PAGE. Protein from an equal number of cells (for immunoprecipitation and expression of pMertk) or an equal amount of protein (for expression of SR-A or Mertk) was loaded onto 7.5% acrylamide Ready Gels (Bio-Rad), run at 150 V, and transferred to 0.2 μm PVDF sequencing grade overnight at 30 V in 20% methanol, 25 mM Tris HCl, and 192 mM glycine. Blots were blocked in 5% milk, 0.1% Tween TBS (blocker) for 45 min at room temperature. Primary antibody was added in optimal dilution in blocker and incubated overnight at 4°C. Blots were washed five times for 15 min each using Tween-TBS. Secondary antibody was added in blocker, incubated for 45 min at room temperature, and washed five times for 15 min each using TBS-Tween. Blots were stained for 5 min at room temperature using Pierce Supersignal West Femto detection systems. Control blots stained with the secondary antibody alone showed no staining.

Flow cytometry

J774 were washed twice in staining buffer (Difco, Detroit, MI, USA), resuspended in 100 μl staining buffer, and incubated for 30 min at 4°C in the dark with labeled antibodies diluted in 100 μl staining buffer. Final antibody concentrations were 1–2 μg/10^6 cells. FcR was blocked using mAb 2.4G2 (anti-CD16/32) for all primary mAb. After incubation, cells were washed twice in staining buffer, resuspended in 0.5 ml staining buffer, and analyzed immediately. Flow cytometry was performed as described previously in detail [62] using a FACScan cytometer (Becton Dickinson, Mountain View, CA, USA) running CellQuest software on a PowerPC microcomputer (Apple, Cupertino, CA, USA) for data collection and analysis. A minimum of 10,000 viable cells was analyzed to determine cell-surface receptor expression.

Phagocytosis assay

AC phagocytosis in vitro was assayed by coincubation of 1.0×10^5 J774 with 2.0×10^6 apoptotic thymocytes for 120 min (J774) at 37°C in 5% CO_2 as described previously [60]. Results are expressed as percentage of J774 containing at least one ingested AC (percent phagocytosis) and as phagocytic index, which was generated by multiplying the percentage of phagocytosis by the mean number of ingested AC per Mø. Antibody (20 μg/ml) or SR-A ligands (150 μg/ml) were added 30 min before addition of AC at concentrations previously found to be inhibitory.

Adhesion assay

Adherence of AC to J774 in vitro was assayed in the same manner as phagocytosis, except that 1×10^7 apoptotic thymocytes were added to each well, yielding a 100:1 thymocyte:Mø ratio [58]. The slides were incubated for 15 min at 37°C and then washed in a standardized manner by dipping individual slides in each of two Wheaton jars filled with ice-cold PBS, and stained using H&E (Richard-Allan, Kalamazoo, MI, USA). These assay conditions have been found to be optimal (unpublished observation). In previously published experiments, we definitively excluded a contribution of ingested cells at this time-point using a fluorescence approach that permitted complete quenching of extracellular AC [58]. Additionally, we verified that under these conditions, interference by any viable thymocytes in the AC mixture is negligible [58]. Adhesion was evaluated by counting 200 J774 per well at 1000× magnification under oil immersion and scoring for bound thymocytes. Results are expressed as percentage of J774 binding at least one AC (percent adhesive Mø) and as adhesion index, which was generated by multiplying the percentage of adherence-positive Mø by the mean number of adherent AC per Mø.

Statistical analysis

Data are expressed as mean ± SEM. Statistical calculations were performed using Statview 5.0 (SAS Institute, Cary, NC, USA) on a Macintosh Power PC G4 computer. Continuous ratio scale data were evaluated by ANOVA with post-hoc analysis by the two-tailed Dunnett test, which specifically compares treatment groups with a control group [63]. Use of this parametric statistic was deemed appropriate, as phagocytosis of apoptotic thymocytes by PMø has been shown to follow a Gaussian distribution [64]. Significant differences were defined as $P < 0.05$.

RESULTS

SR-A is expressed strongly on the surface of J774 Mø but not by apoptotic thymocytes

To determine the relative expression of SR-A by J774 and apoptotic thymocytes, we used Western blot analysis of equal amounts of protein from each cell type (**Fig. 1A**). Results confirmed that J774 strongly expressed SR-A. Apoptotic thymocytes did not express SR-A (Fig. 1A), making it unlikely that any residual, adherent thymocytes remaining after washing would contribute to our analysis of SR-A. Flow cytometric analysis confirmed that J774 expressed SR-A on the cell surface (Fig. 1B).

Anti-SR-A and SR-A ligands significantly reduce J774 phagocytosis but not adhesion of AC

To verify the importance of SR-A in the phagocytosis of AC by the J774 cell line, we measured phagocytosis in the presence of anti-SR-A, control rat IgG, or functional SR-A ligands. Pretreatment with anti-SR-A or with the SR-A ligands dextran sulfate and fucoidan significantly inhibited phagocytosis of AC by J774, whereas pretreatment with the control compounds BSA, heparin, and anti-CD36 did not (**Fig. 2A**). By contrast, SR-A inhibition had no effect on adhesion of AC by J774 (Fig. 2B). Thus, J774 appears to use other receptors for AC adhesion but uses SR-A in signaling, which leads to AC uptake in agreement with previous reports using thymic Mø in vitro [44].

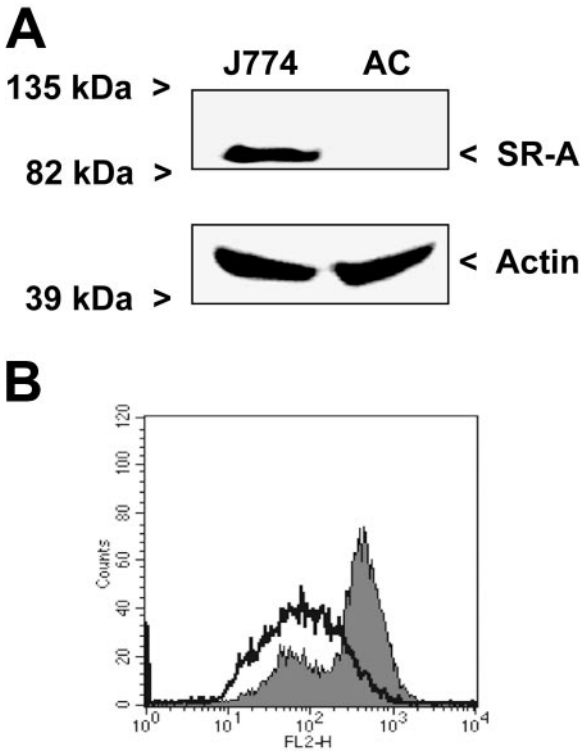

Fig. 1. SR-A is expressed strongly by J774 but not by AC. (A) Western blotting. The murine Mø cell line J774 and apoptotic thymocytes (AC) from normal C57BL/6 mice were lysed, and equal amounts of protein were run on a 7.5% polyacrylamide gel under reducing conditions and were transferred to PVDF. Blots were blocked and probed with the anti-SR-A mAb 2F8 diluted in blocker. Blots were then washed for 15 min using Tween-TBS and incubated with HRP-conjugated donkey, anti-rat IgG diluted in blocker, and the signal was detected by ECL. To demonstrate protein loading, blots were then stripped and reprobed using anti-actin antibody. (B) Flow cytometry. J774 were stained with anti-SR-A (filled profile) or with isotype-matched control rat IgG (open profile). Representative histograms are shown. FL2-H, Fluorescence 2-height.

SR-A and Mertk physically associate in response to AC

To determine whether SR-A and Mertk associate in response to exposure to AC, we used an immunoprecipitation approach (**Fig. 3**). We found that there was slight association of SR-A and Mertk in untreated J774 but that association increased after AC addition at 5, 10, and 30 min and regressed at 60 min (Fig. 3A). This finding was confirmed by reversing the IP and IB antibodies, which showed slight association of SR-A and Mertk in untreated J774 but increased association at 5 and 10 min after AC addition (Fig. 3B). Exposure to AC also induced increased association of Mertk and SR-A in PMø from wild-type mice (Fig. 3C); as expected, no band was detected regardless of exposure to AC in PMø of SR-A−/− mice, which verified that the band seen at ~89 kDa was indeed SR-A. There was no association of Mertk and SR-A in preparations of apoptotic thymocyte alone (data not shown), as that cell type lacks Mertk and SR-A. Therefore, exposure to AC induces the association of SR-A and Mertk (which could be direct or via another intermediary protein), potentially focusing the kinase on specific intracellular substrates.

SR-A inhibition blocks Mertk and PLCγ2 phosphorylation in J774 in response to AC

We previously reported that after AC, adhesion to Mø Mertk becomes tyrosine-phosphorylated (an event essential for development of its kinase activity as well as to activate its multisubstrate-docking site), followed by physical association with and tyrosine phosphorylation of PLCγ2 [59]. Using a molecular approach, the Birge laboratory [65] has recently demonstrated that Tyr867 of Mertk directly mediates the tyrosine phosphorylation of PLCγ2 in response to the AC-binding Mertk ligand Gas6. Therefore, having shown that SR-A and Mertk associate, we asked whether anti-SR-A could block these sequential phosphorylation events. Immunoprecipitation with anti-pTyr and Western blotting with anti-Mertk showed no detectable pMertk at baseline, but rapid induction of phosphorylation in J774 exposed to apoptotic thymocytes for 5, 10, and 20 min (**Fig. 4A**, upper gel). This AC-induced phosphorylation of Mertk was substantially inhibited by soluble anti-SR-A at 5 and 10 min and was strongly reduced at 20 min. To confirm these results, we performed Western blotting with

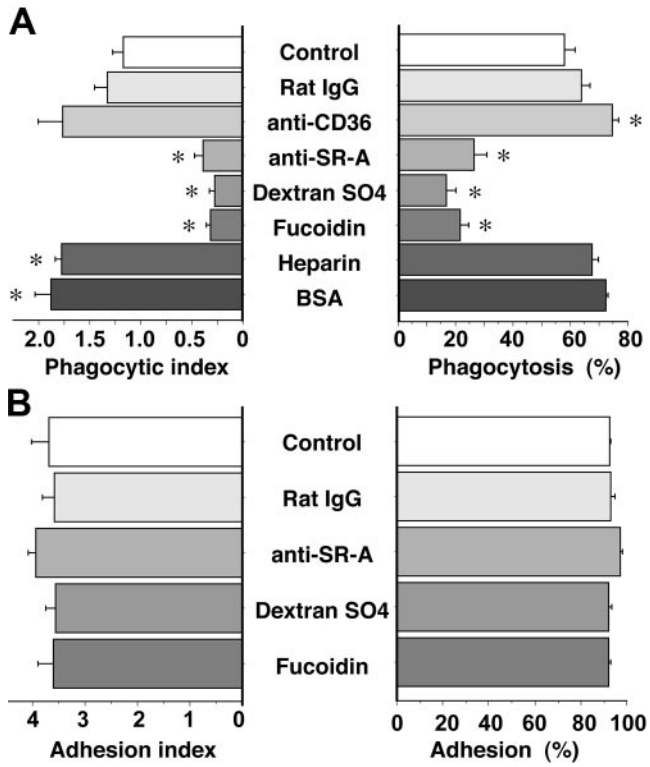

Fig. 2. Anti-SR-A and function-blocking SR-A ligands reduce phagocytosis but not adhesion of AC by the J774 Mø cell line. J774 (1×10^5 cells/well) were adhered overnight to eight-well chamber slides in medium containing 10% FBS and then were preincubated for 30 min at 37°C and 5% CO_2 in medium containing rat IgG (Control), anti-CD36, anti-SR-A (each at 20 μg/ml final concentration), or SR-A ligands (250 μg/ml final concentration). Next, without washing, AC were added as 4×10^6 cells/well for 120 min for phagocytosis assay (A) or as 2×10^7 cells/well for 15 min for adhesion assay (B). Phagocytosis and adhesion were determined by examining H&E-stained slides under oil immersion. (A) Phagocytic index (right panel) and percentage of phagocytic Mø (left panel). Data are mean ± SEM of three to six replicates. *, $P < 0.05$, compared with control, ANOVA with post-hoc testing by the two-tailed Dunnett test. (B) Adhesion index (right panel) and percentage of adhesion-positive Mø (left panel). Data are mean ± SEM of three replicates per condition in a single experiment.

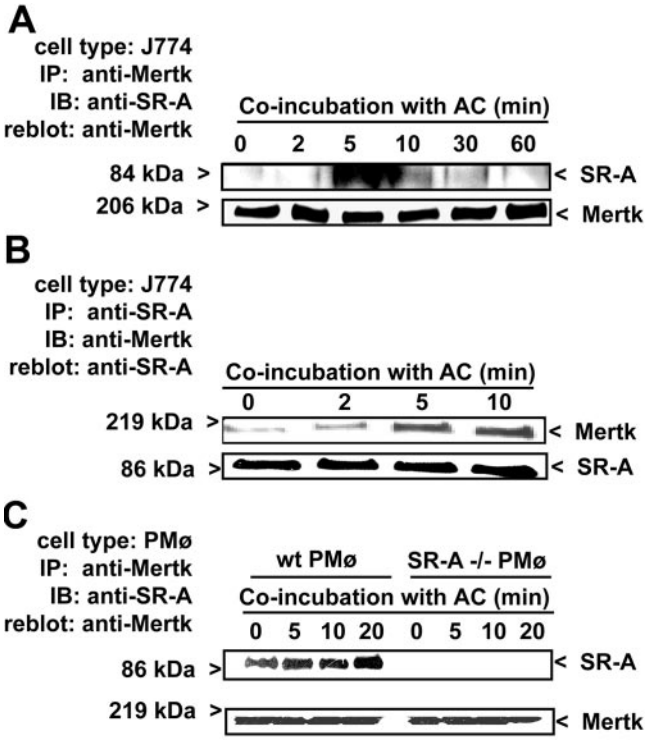

Fig. 3. Exposure of J774 to AC induces association of SR-A and Mertk. Equal numbers of J774 (A and B) or PMø from 129P3/J or SR-A−/− mice (C) were stimulated in vitro with a fivefold excess of AC for various times as indicated, washed extensively, and then lysed in OG buffer. After preclearing, Mø lysates were immunoprecipitated (IP) using anti-Mertk (A and C) or goat anti-SR-A (B) and protein G-agarose, run on a 7.5% acrylamide gel under reducing conditions, and transferred to PVDF. Blots [immunoblots (IB)] were then blocked and probed using rat anti-SR-A (A and C) or anti-Mertk (B) and washed for 15 min using Tween-TBS. After incubation with HRP-conjugated anti-rat or anti-goat IgG in blocker, signal was detected by ECL. Finally, to demonstrate protein loading, blots were stripped and reblotted with anti-Mertk (A and C) or rat anti-SR-A (B). In each case, similar results were obtained in an additional experiment of identical design. wt, Wild-type.

anti-pMertk of J774 cell lysates exposed to AC. These lysates showed limited pMertk at baseline but induction of phosphorylation at 5, 10, and 20 min (Fig. 4B, upper gel). Once again, AC-induced phosphorylation of Mertk was substantially inhibited by soluble anti-SR-A at 5 and 10 min and at 20 min. The rat IgG did seem to induce some Mertk phosphorylation in comparison with control conditions lacking any Ig, presumably as a result of Fc effects. Collectively, these results imply that SR-A function is essential for the prompt and optimal activation of Mertk by AC.

We next examined whether anti-SR-A also blocks PLCγ2 phosphorylation in response to AC, by reblotting with anti-PLCγ2. Some basal phosphorylation of PLCγ2 was detectable in J774 lysates without exposure to AC, but phosphorylation increased at 5 and 10 min after AC addition (Fig. 4A, lower gel). PLCγ2 phosphorylation in response to AC was almost entirely blocked by anti-SR-A preincubation. We have previously reported that apoptotic thymocytes themselves show undetectable phosphorylation of PLCγ2 [59].

Mertk phosphorylation in response to AC exposure is reduced in Mø of SR-A−/− mice

It was previously reported that SR-A−/− Mø show a 50% reduction in apoptotic thymocyte uptake in vitro [44]. To correlate this finding with changes in SR-A signaling through Mertk, we exposed PMø from wild-type mice or SR-A−/− mice to AC and measured Mertk tyrosine phosphorylation, which increased in PMø of wild-type mice at 10, 30, and 60 min after AC exposure, whereas this increase was reduced severely in PMø of SR-A−/− mice (**Fig. 5A**). Western blotting showed that this difference was not a result of inadequate expression of Mertk in SR-A−/− mice, in which the RTK was actually relatively increased (Fig. 5B). To confirm these results using an alternative design, we also exposed PMø of SRA−/− mice and wild-type mice to AC in vitro and performed Western blotting with anti-pMertk. Wild-type PMø lysates showed limited pMertk at baseline but induction of phosphorylation at 10 and 20 min. This AC-induced phosphorylation of Mertk was inhibited substantially in SR-A−/− PMø (Fig. 5C). Therefore, optimal tyrosine phosphorylation of Mertk in response to AC in vitro in mature murine PMø is dependent on SR-A expression.

Ingestion of AC by PMø of SR-A−/− mice is delayed but not abolished

Finally, we examined how genetic absence of SR-A influenced the kinetics of AC ingestion in vitro, using resident PMø from

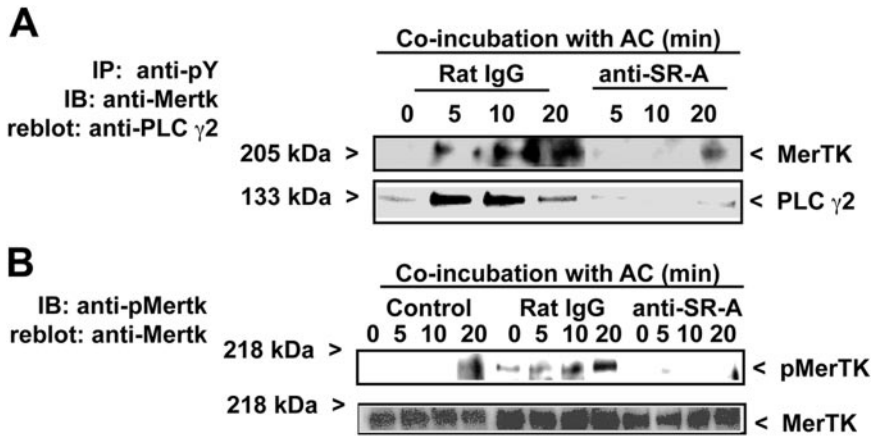

Fig. 4. Anti-SR-A inhibits the AC-induced tyrosine phosphorylation of Mertk and PLCγ2. Equal numbers of J774 were preincubated at 37°C and 5% CO_2 in medium containing 20 μg/ml rat IgG or anti-SR-A and after washing, were stimulated in vitro with a fivefold excess of AC for various times as indicated, washed extensively, and then lysed in OG buffer. After preclearing, Mø lysates were (A) immunoprecipitated using anti-pTyr antibody (anti-pY) and protein G-agarose and run on a 7.5% acrylamide gel under reducing conditions and transferred to PVDF or alternatively, (B) run directly on a 7.5% acrylamide gel. Blots were then blocked and probed using anti-Mertk (A) or anti-pMertk (B) and washed for 15 min using Tween-TBS. After incubation with HRP-conjugated anti-goat (for detection of Mertk) or anti-rabbit (for detection of PLCγ2) IgG in blocker, signal was detected by ECL. Finally, blots were stripped and reblotted with anti-PLCγ2 (A) or anti-Mertk (B). Similar results were obtained in an additional experiment of identical design.

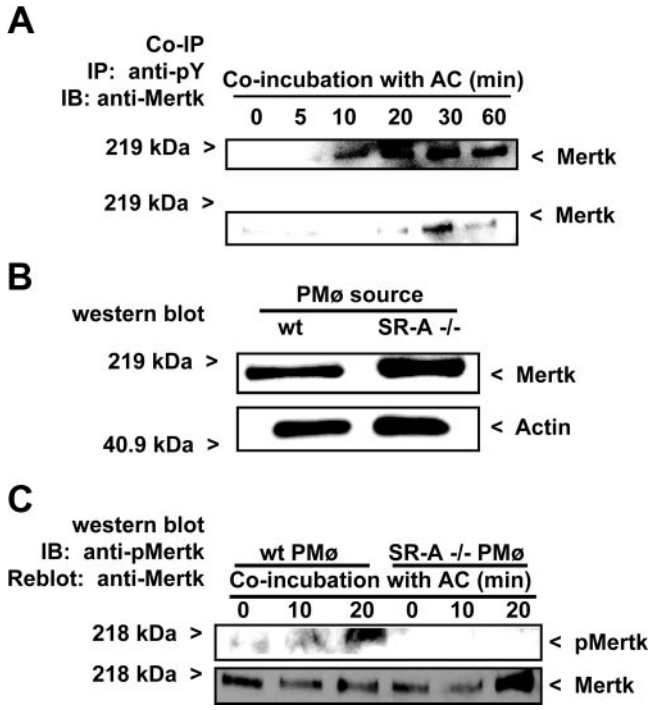

Fig. 5. Mertk phosphorylation is inhibited in tissue Mø of SR-A−/− mice. (A) Coimmunoprecipitation of PMø following AC exposure. PMø from 129P3/J mice (upper gel) or SR-A−/− mice (lower gel) were exposed to fivefold excess of AC for various times as indicated. Cells were lysed, pTyr immunoprecipitates formed, and gels were probed with anti-Mertk and developed as described in the legend to Figure 3. The experiments shown were repeated in two additional experiments with similar results. (B) Western blot. PMø lysates (40 μg protein) from wild-type 129P3/J mice or SR-A−/− mice were run on a 4–20% acrylamide gel, and gels were transferred and blocked as described in the legend to Figure 4 and probed with anti-Mertk, followed by stripping and reprobing with anti-actin. (C) Western blot. PMø lysates from wild-type 129P3/J mice or SR-A−/− mice were run on a 4–20% acrylamide gel, and gels were transferred and blocked as described in the legend to Figure 4 and probed with anti-pMertk, followed by stripping and reprobing with anti-Mertk.

untreated mice. Results showed that the phagocytic index was reduced significantly at early time-points (30 and 45 min) in PMø of SR-A−/− mice but that there was no significant difference at 60 min (**Fig. 6**). The eventual ability of Mø to ingest AC, despite the absence of SR-A in this kinetic experiment, correlates with the absence of an AC clearance defect in vivo [45], an example of the redundancy in AC clearance mechanisms.

DISCUSSION

The results of this study indicate that during AC-induced signaling and the resulting phagocytosis of AC by murine Mø, SR-A rapidly, physically associates with the receptor Mertk, directly or indirectly, and is essential for optimal Mertk activation. Soluble anti-SR-A blocked two known key early events during AC ingestion: the sequential tyrosine phosphorylation of Mertk and of PLCγ2 [59] as well as AC phagocytosis itself. AC-induced tyrosine phosphorylation of Mertk on tissue Mø of SR-A−/− mice was reduced in magnitude and duration relative to Mø of wild-type mice. We also found that SR-A, like Mertk itself, was not essential for Mø adhesion of AC, which is noteworthy given the known role of SR-A in mediating cation-independent adhesion to a wide range of substrates [66–68]. These results advance understanding of the earliest signaling steps in phagocytosis of AC by Mø, an event with important immunoregulatory consequences.

Defining how SR-A participates in Mø signal transduction is particularly important as a result of the central role of this highly versatile scavenger receptor in host defense, atherogenesis, and neoplasia [68–72]. The most common approach to this question has been to examine effects of nonselective SR ligands, such as fucoidan, polyinosinic:polycytidylic acid, or acetylated LDL using Mø [73–76] or cell lines transfected with SR-A [75, 77]. Interpretation of these studies is hampered by the lack of specificity of such ligands, which can bind to other scavenger receptors. This caveat remains even when they are tested on SR-A−/− Mø [78, 79] as a result of compensatory up-regulation of alternative receptors [46]. To avoid these issues, we, like other recent investigators [49], primarily used the specific function-blocking mAb 2F8. As this approach has potential problems as a result of FcR ligation, we used IgG as a control in the blocking experiments.

Our finding of a role for SR-A in Mø uptake of AC in vitro agrees with and extends previous results using mAb 2F8 on thymic and elicited Mø of wild-type mice and Mø from SR-A−/− mice [44]. The exact role of SR-A in AC clearance in vivo remains uncertain. SR-A−/− mice have no developmental abnormalities [46] and show normal clearance of AC within the thymus [45] (although clearance in other organs has not been reported). However, these observations might reflect compensatory changes in alternative AC receptors, such as CD36, which is up-regulated in fetal Mø of SR-A−/− mice [80], or efficient AC uptake by cell types other than Mø [81], as occurs in some organs [5, 7, 8, 82]. It is clear that SR-A is strongly expressed primarily by cell types that avidly engulf AC [46, 83]. These results are, to our knowledge, the first demonstration of an interaction (direct or indirect) between SR-A and a RTK. A variety of previous studies [75, 76, 84, 85] has provided indirect evidence that SR-A associates with tyrosine kinases, based in most cases on inhibitor studies and use of

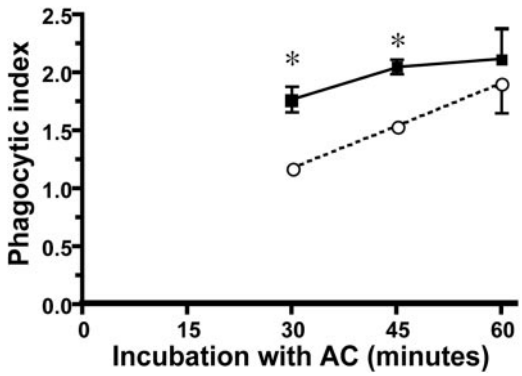

Fig. 6. AC phagocytosis by resident PMø is delayed in the absence of SR-A. In vitro phagocytosis of AC by resident PMø from wild-type mice (■) or SR-A−/− mice (○) was assayed at various times from 30 to 90 min after addition of a fivefold excess of AC. *, Significantly different; $P < 0.05$, ANOVA with post-hoc analysis by the two-tailed Dunnett test.

nonspecific SR-A ligands. Although we did find that one related RTK, STK/RON [86], was not activated on direct activation of SR-A using immobilized anti-SR-A (data not shown), it is unlikely that Mertk is unique in associating with SR-A; e.g., the cytoplasmic tyrosine kinase Lyn was coimmunoprecipitated using polyclonal rabbit anti-human SR-A antiserum within 30 s of exposing the Mø cell line THP-1 to acetyl LDL [87]. Conversely, SR-A is a target of serine phosphorylation by JNK2 [88], and Gas6, a Mertk ligand, can induce SR-A expression in human smooth muscle cells via a pathway requiring the serine/threonine kinase AKT [89].

Our findings imply that SR-A participates in optimal AC uptake by aggregating Mertk monomers to facilitate their autotransphosphorylation. Such an accessory action of SR-A could be important to initiate Mø signaling, which leads to phagocytosis of AC, as the Biacore assay has shown that Mertk interacts with low avidity with its soluble AC-binding intermediaries, Gas6 and protein S [90–92]. Activated Mertk provides multiple signals to initiate AC uptake, including release of VAV1 constitutively bound to inactive Mertk, freeing that guanine exchange factor to hydrolyze the RhoA family members [56]; activation of PLCγ2, which by releasing diacylglycerol and inducing Ca^{++} transients, recruits protein kinase C βII to the nascent AC phagosome [59, 93]; and Src-mediated tyrosine phosphorylation of focal adhesion kinase and its recruitment to the αvβ5 integrin [94]. This final step is particularly important, as it induces formation of the p130(CAS)/CrkII/Dock 180 signaling complex that activates Rac-1 and hence, actin polymerization. A recent paper identified Tyr867 of Mertk as required for activation of PI-3K and PLCγ2 [65]. In that study, PLCγ2 phosphorylation in response to the PS-binding Mertk agonist Gas6 was abrogated completely in the CD8-Mertk Y867F mutant or CD8-$Mertk^{KD}$ cells. The many consequences of Mertk activation place it at the center of the AC uptake process. Hence, data from this study and others, showing that SR-A is not obligatory for efficient AC uptake [44–46], imply that other receptors will be able to increase the efficiency of Mertk autotransphosphorylation during AC recognition.

A final aspect of these results is the insight they provide on the unique activation state induced by Mø contact with AC [11–20]. SR-A was the first pattern recognition receptor to be shown to counteract proinflammatory receptors [50, 95, 96], although the signaling pathways involved remain incompletely defined. SR-A−/− mice and gene-targeted mice with defective Mertk expression exhibit increased sensitivity to endotoxin-induced shock, related to increased TNF-α production [95, 97]. Significantly, two groups have recently shown an essential role for Mertk, along with other members of the Tyro3 family of RTKs, to which it belongs, for the ability of AC exposure to block subsequent proinflammatory cytokine production by dendritic cells (DC) [39, 98]. Whether immunosuppressive effects of SR-A activation by others of its many ligands depend on signaling via Mertk will require further study.

In summary, we provide evidence that SR-A, a receptor with broad ligand-binding properties and a central role in host defense, pairs with Mertk during signaling induced by AC, which leads to Mø ingestion of AC. This intermolecular interaction, likely induced when the two receptors bind their own cognate ligands on the AC surface, illustrates how a receptor with multiple ligands can participate in delivery of a specific signal through its binding partners. Defining the exact importance of SR-A in uptake of AC by Mø and DC in different organs will require additional in vivo experimentation. Understanding the signaling pathways induced during AC clearance is a first step to manipulate the process therapeutically.

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REFERENCES

1. Mohan, C., Adams, S., Stanik, V., Datta, S. K. (1993) Nucleosome: a major immunogen for pathogenic autoantibody-inducing T cells of lupus. *J. Exp. Med.* **177,** 1367–1381.
2. Burlingame, R. W., Boey, M. L., Starkebaum, G., Rubin, R. L. (1994) The central role of chromatin in autoimmune responses to histones and DNA in systemic lupus erythematosus. *J. Clin. Invest.* **94,** 184–192.
3. Cohen, P. L., Caricchio, R., Abraham, V., Camenisch, T. D., Jennette, J. C., Roubey, R. A., Earp, H. S., Matsushima, G., Reap, E. A. (2002) Delayed apoptotic cell clearance and lupus-like autoimmunity in mice lacking the c-mer membrane tyrosine kinase. *J. Exp. Med.* **196,** 135–140.
4. Fadok, V. A. (1999) Clearance: the last and often forgotten stage of apoptosis. *J. Mammary Gland Biol. Neoplasia* **4,** 203–211.
5. Hall, M. O., Prieto, A. L., Obin, M. S., Abrams, T. A., Burgess, B. L., Heeb, M. J., Agnew, B. J. (2001) Outer segment phagocytosis by cultured retinal pigment epithelial cells requires Gas6. *Exp. Eye Res.* **73,** 509–520.
6. Wiegand, U. K., Corbach, S., Prescott, A. R., Savill, J., Spruce, B. A. (2001) The trigger to cell death determines the efficiency with which dying cells are cleared by neighbors. *Cell Death Differ.* **8,** 734–746.
7. Nakanishi, Y., Shiratsuchi, A. (2004) Phagocytic removal of apoptotic spermatogenic cells by Sertoli cells: mechanisms and consequences. *Biol. Pharm. Bull.* **27,** 13–16.
8. Walsh, G. M., Sexton, D. W., Blaylock, M. G., Convery, C. M. (1999) Resting and cytokine-stimulated human small airway epithelial cells recognize and engulf apoptotic eosinophils. *Blood* **94,** 2827–2835.
9. Sexton, D. W., Blaylock, M. G., Walsh, G. M. (2001) Human alveolar epithelial cells engulf apoptotic eosinophils by means of integrin- and phosphatidylserine receptor-dependent mechanisms: a process upregulated by dexamethasone. *J. Allergy Clin. Immunol.* **108,** 962–969.
10. Hall, S. E., Savill, J. S., Henson, P. M., Haslett, C. (1994) Apoptotic neutrophils are phagocytosed by fibroblasts with participation of the fibroblast vitronectin receptor and involvement of a mannose/fucose-specific lectin. *J. Immunol.* **153,** 3218–3227.
11. Voll, R. E., Herrmann, M., Roth, E. A., Stach, C., Kalden, J. R., Girkontaite, I. (1997) Immunosuppressive effects of apoptotic cells. *Nature* **390,** 350–351.
12. Uchimura, E., Kodaira, T., Kurosaka, K., Yang, D., Watanabe, N., Kobayashi, Y. (1997) Interaction of phagocytes with apoptotic cells leads to production of pro-inflammatory cytokines. *Biochem. Biophys. Res. Commun.* **239,** 799–803.
13. Fadok, V. A., Bratton, D. L., Konowal, A., Freed, P. W., Westcott, J. Y., Henson, P. M. (1998) Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-β, PGE2, and PAF. *J. Clin. Invest.* **101,** 890–898.

14. Cocco, R. E., Ucker, D. S. (2001) Distinct modes of macrophage recognition for apoptotic and necrotic cells are not specified exclusively by phosphatidylserine exposure. *Mol. Biol. Cell* **12,** 919–930.
15. Lucas, M., Stuart, L. M., Zhang, A., Hodivala-Dilke, K., Febbraio, M., Silverstein, R., Savill, J., Lacy-Hulbert, A. (2006) Requirements for apoptotic cell contact in regulation of macrophage responses. *J. Immunol.* **177,** 4047–4054.
16. Weigert, A., Johann, A. M., von Knethen, A., Schmidt, H., Geisslinger, G., Brune, B. (2006) Apoptotic cells promote macrophage survival by releasing the anti-apoptotic mediator sphingosine-1-phosphate. *Blood* **108,** 1635–1642.
17. Johann, A. M., von Knethen, A., Lindemann, D., Brune, B. (2006) Recognition of apoptotic cells by macrophages activates the peroxisome proliferator-activated receptor-γ and attenuates the oxidative burst. *Cell Death Differ.* **13,** 1533–1540.
18. Patel, V. A., Longacre, A., Hsiao, K., Fan, H., Meng, F., Mitchell, J. E., Rauch, J., Ucker, D. S., Levine, J. S. (2006) Apoptotic cells, at all stages of the death process, trigger characteristic signaling events that are divergent from and dominant over those triggered by necrotic cells: implications for the delayed clearance model of autoimmunity. *J. Biol. Chem.* **281,** 4663–4670.
19. McDonald, P. P., Fadok, V. A., Bratton, D., Henson, P. M. (1999) Transcriptional and translational regulation of inflammatory mediator production by endogenous TGF-β in macrophages that have ingested apoptotic cells. *J. Immunol.* **163,** 6164–6172.
20. Fadok, V. A., Chimini, G. (2001) The phagocytosis of apoptotic cells. *Semin. Immunol.* **13,** 365–372.
21. Stuart, L. M., Lucas, M., Simpson, C., Lamb, J., Savill, J., Lacy-Hulbert, A. (2002) Inhibitory effects of apoptotic cell ingestion upon endotoxin-driven myeloid dendritic cell maturation. *J. Immunol.* **168,** 1627–1635.
22. Tridandapani, S., Wardrop, R., Baran, C. P., Wang, Y., Opalek, J. M., Caligiuri, M. A., Marsh, C. B. (2003) TGF-β 1 suppresses myeloid Fc γ receptor function by regulating the expression and function of the common γ-subunit. *J. Immunol.* **170,** 4572–4577.
23. Wang, L., Antonini, J. M., Rojanasakul, Y., Castranova, V., Scabilloni, J. F., Mercer, R. R. (2003) Potential role of apoptotic macrophages in pulmonary inflammation and fibrosis. *J. Cell. Physiol.* **194,** 215–224.
24. Aronoff, D. M., Canetti, C., Peters-Golden, M. (2004) Prostaglandin E2 inhibits alveolar macrophage phagocytosis through an E-prostanoid 2 receptor-mediated increase in intracellular cyclic AMP. *J. Immunol.* **173,** 559–565.
25. Wang, L., Scabilloni, J. F., Antonini, J. M., Rojanasakul, Y., Castranova, V., Mercer, R. R. (2006) Induction of secondary apoptosis, inflammation, and lung fibrosis after intratracheal instillation of apoptotic cells in rats. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **290,** L695–L702.
26. Lauber, K., Blumenthal, S. G., Waibel, M., Wesselborg, S. (2004) Clearance of apoptotic cells: getting rid of the corpses. *Mol. Cell* **14,** 277–287.
27. Somersan, S., Bhardwaj, N. (2001) Tethering and tickling: a new role for the phosphatidylserine receptor. *J. Cell Biol.* **155,** 501–504.
28. Kiss, R. S., Elliott, M. R., Ma, Z., Marcel, Y. L., Ravichandran, K. S. (2006) Apoptotic cells induce a phosphatidylserine-dependent homeostatic response from phagocytes. *Curr. Biol.* **16,** 2252–2258.
29. Fadok, V. A., Bratton, D. L., Rose, D. M., Pearson, A., Ezekewitz, R. A., Henson, P. M. (2000) A receptor for phosphatidylserine-specific clearance of apoptotic cells. *Nature* **405,** 85–90.
30. Williamson, P., Schlegel, R. A. (2004) Hide and seek: the secret identity of the phosphatidylserine receptor. *J. Biol.* **3,** 14.
31. Miyanishi, M., Tada, K., Koike, M., Uchiyama, Y., Kitamura, T., Nagata, S. (2007) Identification of Tim4 as a phosphatidylserine receptor. *Nature* **450,** 435–439.
32. Park, D., Tosello-Trampont, A. C., Elliott, M. R., Lu, M., Haney, L. B., Ma, Z., Klibanov, A. L., Mandell, J. W., Ravichandran, K. S. (2007) BAI1 is an engulfment receptor for apoptotic cells upstream of the ELMO/Dock180/Rac module. *Nature* **450,** 430–434.
33. Park, S. Y., Jung, M. Y., Kim, H. J., Lee, S. J., Kim, S. Y., Lee, B. H., Kwon, T. H., Park, R. W., Kim, I. S. (2008) Rapid cell corpse clearance by stabilin-2, a membrane phosphatidylserine receptor. *Cell Death Differ.* **15,** 192–201.
34. Pradhan, D., Krahling, S., Williamson, P., Schlegel, R. A. (1997) Multiple systems for recognition of apoptotic lymphocytes by macrophages. *Mol. Biol. Cell* **8,** 767–778.
35. Savill, J., Dransfield, I., Gregory, C., Haslett, C. (2002) A blast from the past: clearance of apoptotic cells regulates immune responses. *Nat. Rev. Immunol.* **2,** 965–975.
36. Gregory, C. D., Devitt, A. (2004) The macrophage and the apoptotic cell: an innate immune interaction viewed simplistically? *Immunology* **113,** 1–14.
37. Ravichandran, K. S., Lorenz, U. (2007) Engulfment of apoptotic cells: signals for a good meal. *Nat. Rev. Immunol.* **7,** 964–974.
38. Gardai, S. J., McPhillips, K. A., Frasch, S. C., Janssen, W. J., Starefeldt, A., Murphy-Ullrich, J. E., Bratton, D. L., Oldenborg, P. A., Michalak, M., Henson, P. M. (2005) Cell-surface calreticulin initiates clearance of viable or apoptotic cells through trans-activation of LRP on the phagocyte. *Cell* **123,** 321–334.
39. Sen, P., Wallet, M. A., Yi, Z., Huang, Y., Henderson, M., Mathews, C. E., Earp, H. S., Matsushima, G., Baldwin Jr., A. S., Tisch, R. M. (2007) Apoptotic cells induce Mer tyrosine kinase-dependent blockade of NF-{κ}B activation in dendritic cells. *Blood* **109,** 653–660.
40. Hoffmann, P. R., deCathelineau, A. M., Ogden, C. A., Leverrier, Y., Bratton, D. L., Daleke, D. L., Ridley, A. J., Fadok, V. A., Henson, P. M. (2001) Phosphatidylserine (PS) induces PS receptor-mediated macropinocytosis and promotes clearance of apoptotic cells. *J. Cell Biol.* **155,** 649–659.
41. Bratton, D. L., Henson, P. M. (2008) Apoptotic cell recognition: will the real phosphatidylserine receptor(s) please stand up? *Curr. Biol.* **18,** R76–R79.
42. Hughes, D. A., Fraser, I. P., Gordon, S. (1995) Murine macrophage scavenger receptor: in vivo expression and function as receptor for macrophage adhesion in lymphoid and non-lymphoid organs. *Eur. J. Immunol.* **25,** 466–473.
43. Krieger, M., Herz, J. (1994) Structures and functions of multiligand lipoprotein receptors: macrophage scavenger receptors and LDL receptor-related protein (LRP). *Annu. Rev. Biochem.* **63,** 601–637.
44. Platt, N., Suzuki, H., Kurihara, Y., Kodama, T., Gordon, S. (1996) Role for the class A macrophage scavenger receptor in the phagocytosis of apoptotic thymocytes in vitro. *Proc. Natl. Acad. Sci. USA* **93,** 12456–12460.
45. Platt, N., Suzuki, H., Kodama, T., Gordon, S. (2000) Apoptotic thymocyte clearance in scavenger receptor class A-deficient mice is apparently normal. *J. Immunol.* **164,** 4861–4867.
46. Komohara, Y., Terasaki, Y., Kaikita, K., Suzuki, H., Kodama, T., Takeya, M. (2005) Clearance of apoptotic cells is not impaired in mouse embryos deficient in class A scavenger receptor types I and II (CD204). *Dev. Dyn.* **232,** 67–74.
47. Gough, P. J., Gordon, S. (2000) The role of scavenger receptors in the innate immune system. *Microbes Infect.* **2,** 305–311.
48. Platt, N., Gordon, S. (2001) Is the class A macrophage scavenger receptor (SR-A) multifunctional? The mouse's tale. *J. Clin. Invest.* **108,** 649–654.
49. Jozefowski, S., Kobzik, L. (2004) Scavenger receptor A mediates H2O2 production and suppression of IL-12 release in murine macrophages. *J. Leukoc. Biol.* **76,** 1066–1074.
50. Cotena, A., Gordon, S., Platt, N. (2004) The class A macrophage scavenger receptor attenuates CXC chemokine production and the early infiltration of neutrophils in sterile peritonitis. *J. Immunol.* **173,** 6427–6432.
51. Becker, M., Cotena, A., Gordon, S., Platt, N. (2006) Expression of the class A macrophage scavenger receptor on specific subpopulations of murine dendritic cells limits their endotoxin response. *Eur. J. Immunol.* **36,** 950–960.
52. Scott, R. S., McMahon, E. J., Pop, S. M., Reap, E. A., Caricchio, R., Cohen, P. L., Earp, H. S., Matsushima, G. K. (2001) Phagocytosis and clearance of apoptotic cells is mediated by MER. *Nature* **411,** 207–211.
53. Lu, Q., Lemke, G. (2001) Homeostatic regulation of the immune system by receptor tyrosine kinases of the Tyro 3 family. *Science* **293,** 306–311.
54. Hafizi, S., Dahlback, B. (2006) Signaling and functional diversity within the Axl subfamily of receptor tyrosine kinases. *Cytokine Growth Factor Rev.* **17,** 295–304.
55. Georgescu, M. M., Kirsch, K. H., Shishido, T., Zong, C., Hanafusa, H. (1999) Biological effects of c-Mer receptor tyrosine kinase in hematopoietic cells depend on the Grb2 binding site in the receptor and activation of NF-κB. *Mol. Cell. Biol.* **19,** 1171–1181.
56. Mahajan, N. P., Earp, H. S. (2003) An SH2 domain-dependent, phosphotyrosine-independent interaction between Vav1 and the Mer receptor tyrosine kinase: a mechanism for localizing guanine nucleotide-exchange factor action. *J. Biol. Chem.* **278,** 42596–42603.
57. Ling, L., Templeton, D., Kung, H. J. (1996) Identification of the major autophosphorylation sites of Nyk/Mer, an NCAM-related receptor tyrosine kinase. *J. Biol. Chem.* **271,** 18355–18362.
58. Hu, B., Jennings, J. H., Sonstein, J., Floros, J., Todt, J. C., Polak, T., Curtis, J. L. (2004) Resident murine alveolar and peritoneal macrophages differ in adhesion of apoptotic thymocytes. *Am. J. Respir. Cell Mol. Biol.* **30,** 687–693.
59. Todt, J. C., Hu, B., Curtis, J. L. (2004) The receptor tyrosine kinase MerTK activates phospholipase C γ2 during recognition of apoptotic thymocytes by murine macrophages. *J. Leukoc. Biol.* **75,** 705–713.

60. Hu, B., Sonstein, J., Christensen, P. J., Punturieri, A., Curtis, J. L. (2000) Deficient in vitro and in vivo phagocytosis of apoptotic T cells by resident murine alveolar macrophages. *J. Immunol.* **165,** 2124–2133.
61. Hu, B., Punturieri, A., Todt, J., Sonstein, J., Polak, T., Curtis, J. L. (2002) Recognition and phagocytosis of apoptotic T cells by resident murine macrophages requires multiple signal transduction events. *J. Leukoc. Biol.* **71,** 881–889.
62. Curtis, J. L., Kim, S., Scott, P. J., Buechner-Maxwell, V. A. (1995) Adhesion receptor phenotypes of murine lung CD4+ T cells during the pulmonary immune response to sheep erythrocytes. *Am. J. Respir. Cell Mol. Biol.* **12,** 520–530.
63. Zar, J. H. (1974) *Biostatistical Analysis*, Englewood Cliffs, NJ, USA, Prentice-Hall.
64. Licht, R., Jacobs, C. W., Tax, W. J., Berden, J. H. (1999) An assay for the quantitative measurement of in vitro phagocytosis of early apoptotic thymocytes by murine resident peritoneal macrophages. *J. Immunol. Methods* **223,** 237–248.
65. Tibrewal, N., Wu, Y., D'Mello, V., Akakura, R., George, T. C., Varnum, B., Birge, R. B. (2008) Autophosphorylation docking site Tyr867 in Mer receptor tyrosine kianse allows for dissociation of multiple signaling pathways for phagocytosis of apoptotic cells and down-modulation of LPS-inducible NF-κ B transcriptional activation. *J. Biol. Chem.* **283,** 3618–3627.
66. Fraser, I., Hughes, D., Gordon, S. (1993) Divalent cation-independent macrophage adhesion inhibited by monoclonal antibody to murine scavenger receptor. *Nature* **364,** 343–345.
67. El Khoury, J., Hickman, S. E., Thomas, C. A., Cao, L., Silverstein, S. C., Loike, J. D. (1996) Scavenger receptor-mediated adhesion of microglia to β-amyloid fibrils. *Nature* **382,** 716–719.
68. Platt, N., Haworth, R., Darley, L., Gordon, S. (2002) The many roles of the class A macrophage scavenger receptor. *Int. Rev. Cytol.* **212,** 1–40.
69. Peiser, L., Mukhopadhyay, S., Gordon, S. (2002) Scavenger receptors in innate immunity. *Curr. Opin. Immunol.* **14,** 123–128.
70. Greaves, D. R., Gordon, S. (2005) Thematic review series: the immune system and atherogenesis. Recent insights into the biology of macrophage scavenger receptors. *J. Lipid Res.* **46,** 11–20.
71. Mytar, B., Woloszyn, M., Macura-Biegun, A., Hajto, B., Ruggiero, I., Piekarska, B., Zembala, M. (2004) Involvement of pattern recognition receptors in the induction of cytokines and reactive oxygen intermediates production by human monocytes/macrophages stimulated with tumor cells. *Anticancer Res.* **24,** 2287–2293.
72. Xu, J., Zheng, S. L., Komiya, A., Mychaleckyj, J. C., Isaacs, S. D., Hu, J. J., Sterling, D., Lange, E. M., Hawkins, G. A., Turner, A., et al. (2002) Germline mutations and sequence variants of the macrophage scavenger receptor 1 gene are associated with prostate cancer risk. *Nat. Genet.* **32,** 321–325.
73. Misra, U. K., Shackelford, R. E., Florine-Casteel, K., Thai, S. F., Alford, P. B., Pizzo, S. V., Adams, D. O. (1996) Maleylated-BSA induces hydrolysis of PIP2, fluxes of Ca2+, NF-κB binding, and transcription of the TNF-α gene in murine macrophages. *J. Leukoc. Biol.* **60,** 784–792.
74. Claus, R., Fyrnys, B., Deigner, H. P., Wolf, G. (1996) Oxidized low-density lipoprotein stimulates protein kinase C (PKC) and induces expression of PKC-isotypes via prostaglandin-H-synthase in P388D1 macrophage-like cells. *Biochemistry* **35,** 4911–4922.
75. Hsu, H. Y., Hajjar, D. P., Khan, K. M., Falcone, D. J. (1998) Ligand binding to macrophage scavenger receptor-A induces urokinase-type plasminogen activator expression by a protein kinase-dependent signaling pathway. *J. Biol. Chem.* **273,** 1240–1246.
76. Hsu, H. Y., Chiu, S. L., Wen, M. H., Chen, K. Y., Hua, K. F. (2001) Ligands of macrophage scavenger receptor induce cytokine expression via differential modulation of protein kinase signaling pathways. *J. Biol. Chem.* **276,** 28719–28730.
77. Post, S. R., Gass, C., Rice, S., Nikolic, D., Crump, H., Post, G. R. (2002) Class A scavenger receptors mediate cell adhesion via activation of G(i/o) and formation of focal adhesion complexes. *J. Lipid Res.* **43,** 1829–1836.
78. Peiser, L., De Winther, M. P., Makepeace, K., Hollinshead, M., Coull, P., Plested, J., Kodama, T., Moxon, E. R., Gordon, S. (2002) The class A macrophage scavenger receptor is a major pattern recognition receptor for *Neisseria meningitidis* which is independent of lipopolysaccharide and not required for secretory responses. *Infect. Immun.* **70,** 5346–5354.
79. Husemann, J., Obstfeld, A., Febbraio, M., Kodama, T., Silverstein, S. C. (2001) CD11b/CD18 mediates production of reactive oxygen species by mouse and human macrophages adherent to matrixes containing oxidized LDL. *Arterioscler. Thromb. Vasc. Biol.* **21,** 1301–1305.
80. Moodley, Y., Rigby, P., Bundell, C., Bunt, S., Hayashi, H., Misso, N., McAnulty, R., Laurent, G., Scaffidi, A., Thompson, P., Knight, D. (2003) Macrophage recognition and phagocytosis of apoptotic fibroblasts is critically dependent on fibroblast-derived thrombospondin 1 and CD36. *Am. J. Pathol.* **162,** 771–779.
81. Vucevic, D., Colic, M., Popovic, P., Gasic, S. (2002) Different roles of a rat cortical thymic epithelial cell line in vitro on thymocytes and thymocyte hybridoma cells: phagocytosis, induction of apoptosis, nursing and growth promoting activities. *Dev. Immunol.* **9,** 63–72.
82. Monks, J., Rosner, D., Jon Geske, F., Lehman, L., Hanson, L., Neville, M. C., Fadok, V. A. (2005) Epithelial cells as phagocytes: apoptotic epithelial cells are engulfed by mammary alveolar epithelial cells and repress inflammatory mediator release. *Cell Death Differ.* **12,** 107–114.
83. Lichanska, A. M., Browne, C. M., Henkel, G. W., Murphy, K. M., Ostrowski, M. C., McKercher, S. R., Maki, R. A., Hume, D. A. (1999) Differentiation of the mononuclear phagocyte system during mouse embryogenesis: the role of transcription factor PU.1. *Blood* **94,** 127–138.
84. Coller, S. P., Paulnock, D. M. (2001) Signaling pathways initiated in macrophages after engagement of type A scavenger receptors. *J. Leukoc. Biol.* **70,** 142–148.
85. Nikolic, D., Cholewa, J. M., Gass, C., Gong, M. C., Post, S. R. (2007) Class A scavenger receptor-mediated cell adhesion requires the sequential activation of Lyn and PI3-kinase. *Am. J. Physiol. Cell Physiol.* **292,** C1450–C1458.
86. Morrison, A. C., Wilson, C. B., Ray, M., Correll, P. H. (2004) Macrophage-stimulating protein, the ligand for the stem cell-derived tyrosine kinase/RON receptor tyrosine kinase, inhibits IL-12 production by primary peritoneal macrophages stimulated with IFN-γ and lipopolysaccharide. *J. Immunol.* **172,** 1825–1832.
87. Miki, S., Tsukada, S., Nakamura, Y., Aimoto, S., Hojo, H., Sato, B., Yamamoto, M., Miki, Y. (1996) Functional and possible physical association of scavenger receptor with cytoplasmic tyrosine kinase Lyn in monocytic THP-1-derived macrophages. *FEBS Lett.* **399,** 241–244.
88. Ricci, R., Sumara, G., Sumara, I., Rozenberg, I., Kurrer, M., Akhmedov, A., Hersberger, M., Eriksson, U., Eberli, F. R., Becher, B., Boren, J., Chen, M., Cybulsky, M. I., Moore, K. J., Freeman, M. W., Wagner, E. F., Matter, C. M., Luscher, T. F. (2004) Requirement of JNK2 for scavenger receptor A-mediated foam cell formation in atherogenesis. *Science* **306,** 1558–1561.
89. Ming Cao, W., Murao, K., Imachi, H., Sato, M., Nakano, T., Kodama, T., Sasaguri, Y., Wong, N. C., Takahara, J., Ishida, T. (2001) Phosphatidylinositol 3-OH kinase-Akt/protein kinase B pathway mediates Gas6 induction of scavenger receptor A in immortalized human vascular smooth muscle cell line. *Arterioscler. Thromb. Vasc. Biol.* **21,** 1592–1597.
90. Nagata, K., Ohashi, K., Nakano, T., Arita, H., Zong, C., Hanafusa, H., Mizuno, K. (1996) Identification of the product of growth arrest-specific gene 6 as a common ligand for Axl, Sky, and Mer receptor tyrosine kinases. *J. Biol. Chem.* **271,** 30022–30027.
91. Chen, J., Carey, K., Godowski, P. J. (1997) Identification of Gas6 as a ligand for Mer, a neural cell adhesion molecule related receptor tyrosine kinase implicated in cellular transformation. *Oncogene* **14,** 2033–2039.
92. Anderson, H. A., Maylock, C. A., Williams, J. A., Paweletz, C. P., Shu, H., Shacter, E. (2003) Serum-derived protein S binds to phosphatidylserine and stimulates the phagocytosis of apoptotic cells. *Nat. Immunol.* **4,** 87–91.
93. Todt, J. C., Hu, B., Punturieri, A., Sonstein, J., Polak, T., Curtis, J. L. (2002) Activation of protein kinase C β II by the stereo-specific phosphatidylserine receptor is required for phagocytosis of apoptotic thymocytes by resident murine tissue macrophages. *J. Biol. Chem.* **277,** 35906–35914.
94. Wu, Y., Singh, S., Georgescu, M. M., Birge, R. B. (2005) A role for Mer tyrosine kinase in αvβ5 integrin-mediated phagocytosis of apoptotic cells. *J. Cell Sci.* **118,** 539–553.
95. Haworth, R., Platt, N., Keshav, S., Hughes, D., Darley, E., Suzuki, H., Kurihara, Y., Kodama, T., Gordon, S. (1997) The macrophage scavenger receptor type A is expressed by activated macrophages and protects the host against lethal endotoxic shock. *J. Exp. Med.* **186,** 1431–1439.
96. Jozefowski, S., Arredouani, M., Sulahian, T., Kobzik, L. (2005) Disparate regulation and function of the class A scavenger receptors SR-AI/II and MARCO. *J. Immunol.* **175,** 8032–8041.
97. Camenisch, T. D., Koller, B. H., Earp, H. S., Matsushima, G. K. (1999) A novel receptor tyrosine kinase, Mer, inhibits TNF-α production and lipopolysaccharide-induced endotoxic shock. *J. Immunol.* **162,** 3498–3503.
98. Rothlin, C. V., Ghosh, S., Zuniga, E. I., Oldstone, M. B., Lemke, G. (2007) TAM receptors are pleiotropic inhibitors of the innate immune response. *Cell* **131,** 1124–1136.