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RESEARCH ARTICLE

The class A macrophage scavenger receptor type I (SR-AI) recognizes complement iC3b and mediates NF-κB activation

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ABSTRACT

The macrophage scavenger receptor SR-AI binds to host tissue debris to perform clearance and it binds to bacteria for phagocytosis. In addition, SR-AI modulates macrophage activation through cell signaling. However, investigation of SR-AI signaling on macrophages is complicated due to its promiscuous ligand specificity that overlaps with other macrophage receptors. Therefore, we expressed SR-AI on HEK 293T cells to investigate its ligand binding and signaling. On 293T cells, SR-AI could respond to *E. coli* DH5α, leading to NF-κB activation and IL-8 production. However, this requires *E. coli* DH5α to be sensitized by fresh serum that is treated with heat-inactivation or complement C3 depletion. Anti-C3 antibody inhibits the binding of SR-AI to serum-sensitized DH5α and blocks DH5α stimulation of SR-AI signaling. Further analysis showed that SR-AI can directly bind to purified iC3b but not C3 or C3b. By mutagenesis, The SRCR domain of SR-AI was found to be essential in SR-AI binding to serum-sensitized DH5α. These results revealed a novel property of SR-AI as a complement receptor for iC3b-opsonized bacteria that can elicit cell signaling.

KEYWORDS SR-AI, complement, iC3b, signalling, 293T cells, macrophage

INTRODUCTION

Tissue macrophages play key roles in tissue scavenging, and they modulate tissue inflammation and repair through cytokine production (Gordon and Taylor, 2005). This involves a myriad of macrophage receptors for microbial and endogenous materials (Underhill and Ozinsky, 2002; Taylor et al., 2005). Fc and complement receptors typically recognize opsonized materials (Law, 1988; Daeron, 1997), whereas some receptors like the mannose receptor bind to its ligands directly (Stahl and Ezekowitz, 1998). Macrophages express numerous scavenger receptors with promiscuous polyanionic microbial and host materials as ligands, and there is no report that ligand opsonization is required for SR-AI recognition (Krieger and Stern, 2001; Peiser et al., 2002). SR-A and MARCO are two class A scavenger receptors that have significant structural similarity—both are homotrimeric type II membrane proteins with short cytoplasmic tails and large extracellular portions (Krieger and Stern, 2001; Peiser et al., 2002).

SR-A has two alternative splicing forms, known as SR-AI and SR-AII, which are different in the extracellular portion (Freeman et al., 1990). SR-AI has an extracellular portion consisting of a membrane-proximal triple coiled-coil domain followed by a collagen domain and a C-terminal cysteine-rich (SRCR) domain, but SR-AII lacks the SRCR domain (Freeman et al., 1990; Kodama et al., 1990). MARCO is encoded

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by a separate gene, and it has the collagen and SRCR but not the membrane-proximal coiled-coil domain found in SR-A (van der Laan et al., 1999). The two SR-A receptors recognize polyanionic ligands, such as modified low density lipoprotein (acLDL and oxLDL), lipopolysaccharide (LPS), lipoteichoic acid (LTA) and fucoidan, and earlier studies identified a 22-amino acid segment on the SR-A collagen domain, including 3 lysine residues (K332, K335 and K338 in human SR-A) as the binding site for these ligands (Acton et al., 1993; Doi et al., 1993). SR-A also binds to bacteria for phagocytosis (Peiser et al., 2000; Thomas et al., 2000; Peiser et al., 2002). In line with these SR-A properties, SR-A$^{-/-}$ mice are more susceptible to *Listeria monocytogenes* and type I herpes simplex virus infections than wild type mice (Kurihara et al., 1997; Ishiguro et al., 2001).

Both SR-A and MARCO have short cytoplasmic tails (Freeman et al., 1990; van der Laan et al., 1999; Krieger and Stern, 2001; Peiser et al., 2002b), but they can apparently regulate macrophage cytokine production, which suggests their potentials in initiating cell signaling (Sutterwala et al., 1997; Jozefowski et al., 2005). With respect to SR-A and MARCO signaling, The SR-A ligand fucoidan appeared to elicit multiple signaling pathways in macrophages (Hsu et al., 2001). However, this was later shown to be mediated by CD14 rather than SR-A (Kim et al., 2003). The ligand promiscuity of SR-A results in its overlapping ligand specificity with other macrophage receptors, and this renders it challenging to study SR-A ligands and signaling with macrophages (Krieger and Stern, 2001; Peiser et al., 2002). We therefore expressed SR-AI in the human embryonic kidney 293T cells to investigate the properties of SR-A.

RESULTS

Expression of SR-AI in transfected 293T cells

To investigate the SR-AI signaling with 293T cells, the human SR-AI cDNA was into the pcDNA3.1 vector (pSR-AI), and it was also cloned with C-terminal Myc and His tags (pSR-AI-MH) to facilitate its detection. When 293T cells were transfected with pSR-AI, the 75-kDa protein was detected by Western blotting in the cell lysate (Fig. 1A, insert). As a control, it was not detected in 293T cells transfected with the empty pcDNA3.1 vector (vec-293T). To determine whether SR-AI was surface-expressed, the transfected cells were surface-biotinylated followed by streptavidin-sepharose precipitation. SR-AI was clearly enriched in the biotinylated protein fraction from SR-AI-293T cells and was absent when vec-293T cells were used (Fig. 1A, insert). 293T cells were also transfected with pSR-AI-MH and were then stained using an anti-Myc antibody, and SR-AI was detectable on SR-AI-293T cells by flow cytometry (Fig. 1A, insert), showing that SR-AI is expressed on the surface of transfected 293T cells.

SR-AI is activated by *E. coli* DH5α

We then examined whether SR-AI confers 293T cells the ability to respond to *E. coli* DH5α, a previously identified bacteria ligand for SR-AI (Peiser et al., 2000). In these experiments, a p5xNF-κB-Luc firefly luciferase vector was co-transfected to detect NF-κB activation. A pRL-CMV *Renilla* luciferase vector is also co-trasfected as an internal control. For stimulation, SR-AI-293T and vec-293T cells were first cultured with DH5α for 1 h, and were, after washing, cultured overnight before luciferase determination.

As shown in Fig. 1A, DH5α induced NF-κB activation in SR-AI-293T but not vec-293T cells. Unlike DH5α, The bacteria *Mycobacterium bovis* (BCG) failed to induce NF-κB activation although, as a positive control, it induced NF-κB activation in 293T cells that were transfected to express TLR2 (Fig. 1A). It shows that SR-AI recognizes DH5α but not BCG. The bacteria *Bacillus subtilis*, which was shown to activate TLR2 like DH5α, was similarly examined, and these bacteria also activated SR-AI signaling (Fig. 1B). This is the first demonstration that bacteria induce SR-AI signaling and lead to NF-κB activation. These experiments cannot be performed with macrophages as DH5α are expected to stimulate NF-κB activation through multiple other macrophage receptors.

SR-AI only recognizes fresh serum-sensitized *E. coli* DH5α

An unexpected finding during this study was that DH5α was only able to activate SR-AI signaling when fresh but not heat-inactivated BCS was present (Fig. 2A). It suggests that DH5α requires sensitization by heat-labile serum factor(s), potentially complement proteins, to stimulate SR-AI signaling. When complement receptor 3 (CR3) was expressed in 293T cells (CR3-293T), DH5α induced NF-κB activation and this also requires fresh serum (Fig. 2B). DH5α similarly induced IL-8 production from CR3-293T and SR-AI-293T cells (Fig. 2C).

CR3 mainly recognizes the complement fragment iC3b that deposits on complement-reacted bacteria (Law, 1988). To determine whether SR-AI also recognizes bacteria surface-bound protein(s), we first incubated DH5α with fresh or, as a control, heat-inactivated BCS for 30 min. The bacteria were washed before stimulation of SR-AI-293T cells, which was performed in the presence of heat-inactivated BCS. DH5α pre-incubated with fresh BCS remained potent in NF-κB activation after washing (Fig. 3A), but DH5α pre-incubated with heat-inactivated BCS lost the stimulatory effects (Fig. 3A). However, when DH5α was used to stimulate SR-AI-293T cells with fresh BCS, whether it was pre-sensitized with fresh or heat-inactivated BCS became irrelevant. Collectively, these data show that DH5α acquire surface-bound factors after reaction with fresh BCS, which can stimulate SR-AI signaling.

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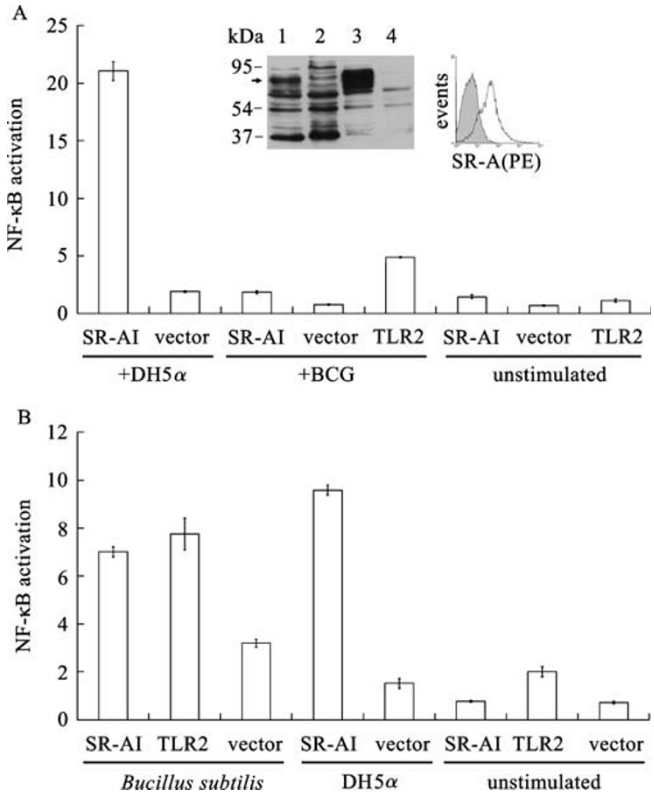

Figure 1. SR-AI signaling is activated by bacteria *E. coli* DH5α and *B. subtilis* but not *M. Bovis* BCG. (A) SR-AI-293T and, as controls, vec-293T cells were first incubated with bacteria *E. coli* DH5α or *M. bovis* BCG for 1 h, and after washing, the cells were cultured overnight. 293T cells were also transfected with pTLR2 for 24 h and then stimulated with *M. bovis* BCG. (Insert) The left panel is a Western blot with a goat anti-human SR-AI antibody. The molecular weight standards (kDa) are labeled and the arrow indicates the 75-kDa SR-AI protein. Total cell lysate (lanes 1 and 2) and biotin-labeled surface proteins (lanes 3 and 4) were separated on 12.5% SDS-PAGE gels under reducing conditions. The cell lysate and biotinylated proteins were derived from SR-AI-293T (lanes 1 and 3) and vec-293T (lanes 2 and 4) cells, respectively. The right panel is a histogram with 293T cells that were transfected with pSR-AI-MH. The cells were stained with an anti-Myc antibody (open histogram) or an isotype control mouse IgG (filled histogram) followed by PE-labeled goat anti-mouse IgG. (B) 293T cells were similarly transfected and stimulated as in (A), except that *B. subtilis* was used in place of BCG. NF-κB-directed firefly luciferase activity in each experiment is normalized to CMV-directed *Renilla* luciferase activity. All experiments were carried out in triplicate and results were presented as means ± SD.

Human serum is different from BCS in DH5α sensitization

We then examined whether human serum also sensitizes DH5α for SR-AI stimulation like BCS. Initial experiments showed that incubation of DH5α with human serum for 30 min caused severe bacteria lysis and the debris only stimulated SR-AI weakly (data not shown). We then incubated DH5α with human serum and, as a control, BCS following a series of time periods up to 30 min. Incubation with BCS steadily increased DH5α stimulation of SR-AI with time, but human serum showed optimal DH5α sensitization at approximately 10 min and then declined rapidly (Fig. 3B). Therefore, DH5α was sensitized for 10 min when human serum was used.

SR-AI is activated by *E. coli* DH5α-bound complement C3

The ability of serum-sensitized DH5α to stimulate CR3 signaling implies the deposition of the CR3 ligand iC3b on the bacteria (Fig. 2B and 2C). To examine whether C3 fragments were involved in SR-AI stimulation, DH5α was sensitized with C3-depleted human serum. As controls, normal or C7-depleted sera were used. DH5α incubated with C3-depleted serum lacked SR-AI stimulation, but DH5α incubated with C7-depleted serum was as potent as DH5α that was sensitized with normal serum (Fig. 3C). Likewise, C3-depleted serum also failed to sensitize DH5α for IL-8 induction from SR-AI-293T cells (Fig. 3D). These data suggest that the complement C3 plays a dominant role in sensitizing DH5α for SR-AI stimulation. In this experiment, BCS-treated DH5α showed little SR-AI stimulation, which was expected because BCS would not sensitize the bacteria effectively in 10 min (Fig. 3B).

The role of C3 in SR-AI stimulation was further verified using an anti-C3 antibody. After sensitization with human

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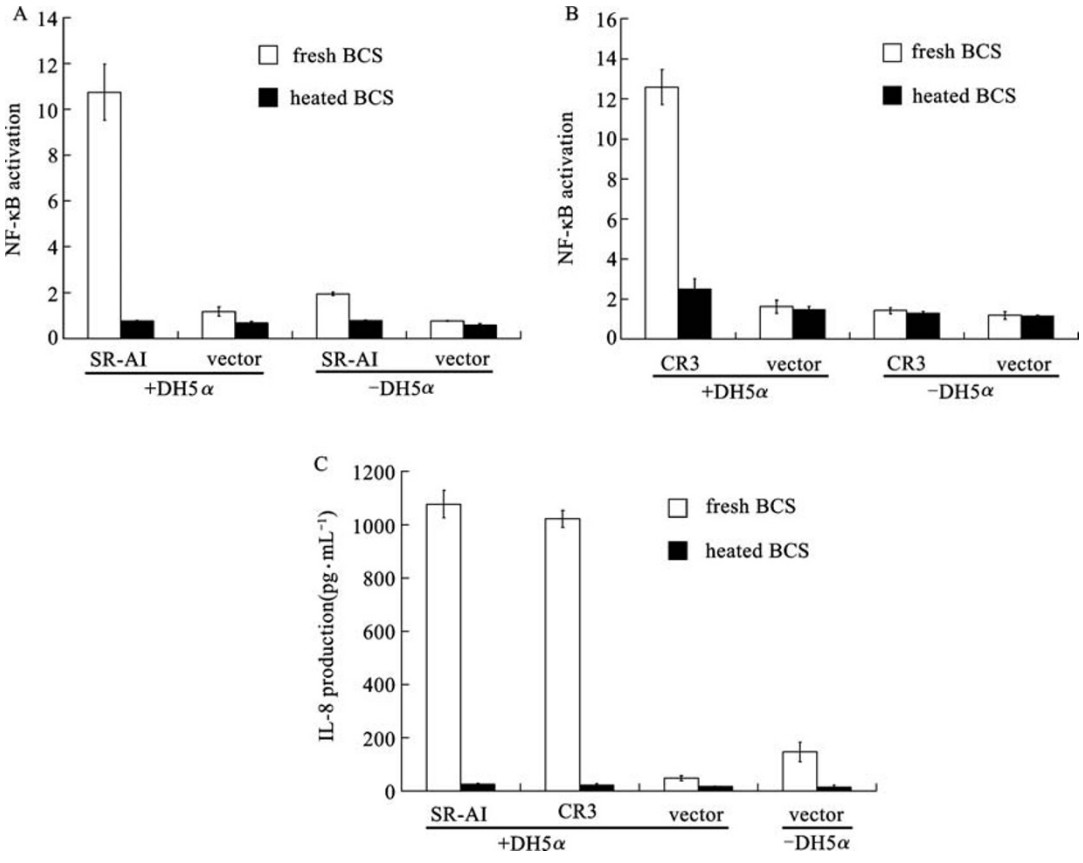

Figure 2. Serum-opsonized *E. coli* DH5α stimulates SR-AI signaling. (A) SR-AI-293T or vec-293T cells were incubated with *E. coli* DH5α for 1 h in the presence of fresh or heat-inactivated BCS. The cells were washed and cultured overnight using the respective BCS. As a control, the cells were similarly cultured without DH5α. (B) CR3-293T and vec-293T cells were similarly treated with *E. coli* DH5α as in (A). NF-κB activation (A and B) or IL-8 production in the media (C) was determined. Results of triplicate experiments were presented as means ± SD.

serum, DH5α was washed and incubated with anti-C3 or, as a control, anti-C1q antibodies before SR-AI stimulation. The anti-C3 antibody nearly completely blocked DH5α stimulation of SR-AI signaling whereas the anti-C1q antibody had no effect (Fig. 3E). This was similarly observed in IL-8 induction (Fig. 3F). Collectively, these results show a dominant role for DH5α-bound C3 or its fragment(s) in SR-AI stimulation. In other words, SR-AI is a receptor for C3-opsonized bacteria.

C3 is deposited on serum-reacted DH5α

To determine whether C3 is actually deposited on the serum-sensitized bacteria, DH5α was transformed to express the green fluorescence protein (GFP). The DH5α-GFP bacteria were pre-treated with human serum and were, after washing, incubated with immobilized anti-C3 antibody. As controls, plates were coated with anti-SR-AI or anti-C1q antibody (Fig. 4). Bound bacteria were viewed under fluorescence microscope. When the bacteria were treated with fresh serum, the bacteria bound prominently to anti-C3 antibody (Fig. 4). However, the bacteria showed little binding to the anti-SR-AI and anti-C1q antibodies. When the bacteria were sensitized with heat-inactivated or C3-depleted serum, there is no significant binding with any of the three antibodies.

SR-AI binds to DH5α-deposited C3 fragments

We then examined whether purified SR-AI binds to serum-reacted DH5α (Fig. 5). For this experiment, the SR-AI extracellular region was expressed as a soluble protein (sSR-AI) and coated on culture plates (Fig. 5). Bovine serum albumin (BSA) was coated as a control. The serum-reacted DH5α bound to sSR-AI but not BSA. When the bacteria were treated with the anti-C3 antibody, the binding to sSR-AI was markedly reduced (Fig. 5). However, treatment of the bacteria with non-immune goat IgG or goat anti-C1q antibody showed no inhibition. Together, these results demonstrate that C3 deposits on serum-reacted DH5α and the deposited C3 or fragment(s) are recognized by SR-AI.

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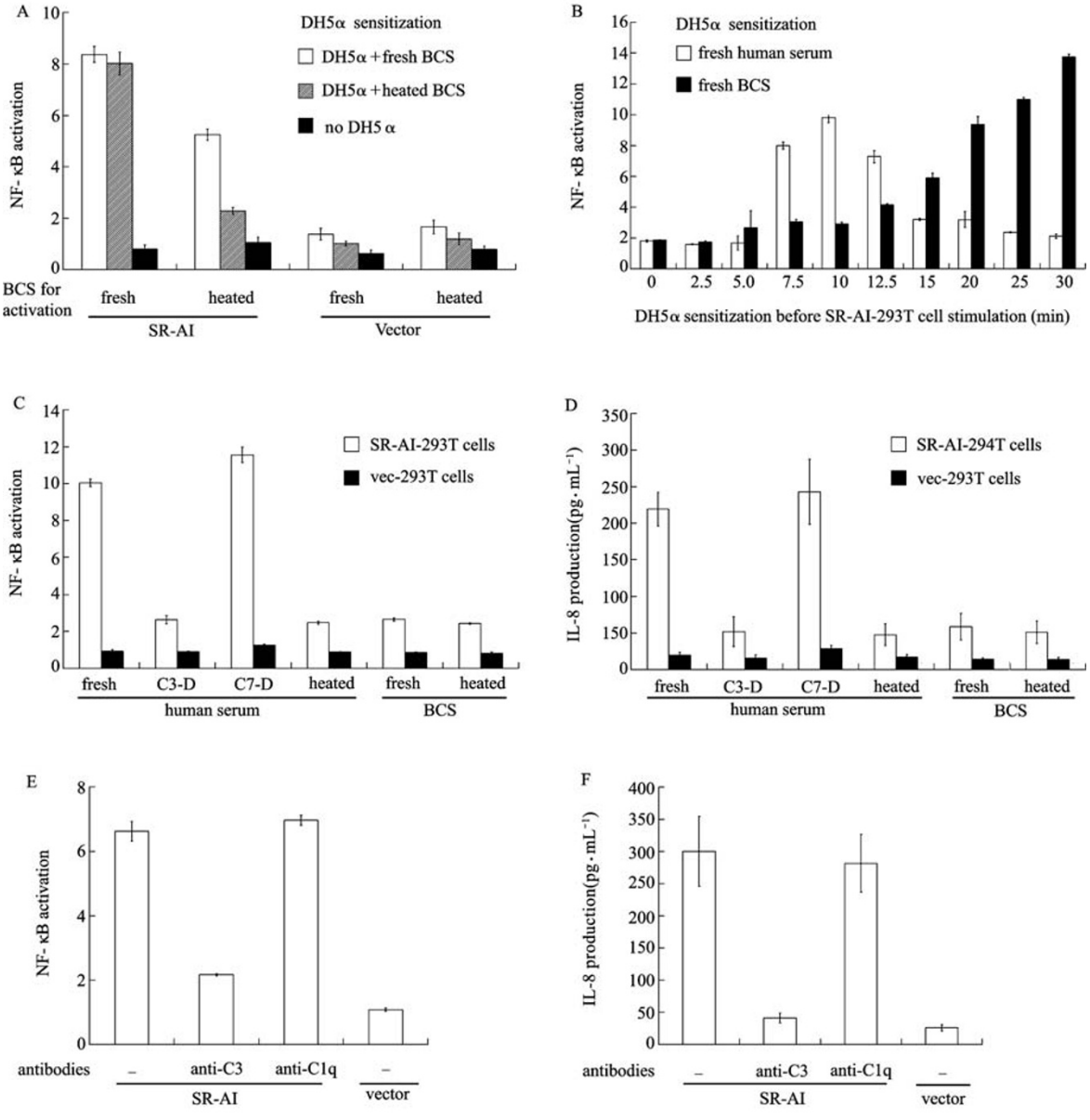

Figure 3. SR-AI signaling is activated by *E. coli* DH5α surface-deposited complement C3. (A) *E. coli* DH5α was pre-incubated with fresh or heat-inactivated BCS (10% in DMEM) for 30 min and washed with serum-free DMEM. SR-AI-293T and vec-293T cells were cultured with or without bacteria for 1 h in the presence of fresh or heated BCS. The cells were washed and cultured overnight before luciferase measurement. (B) *E. coli* DH5α was pre-incubated with 10% (*v/v*) fresh human serum or BCS for 2.5–30 min and was washed for SR-AI-293T cell stimulation. (C and D) *E. coli* DH5α were pre-incubated for 10 min with 10% (*v/v*) fresh, C3-depleted, C7-depleted, or heat-inactivated human serum, and were, after washing, used for SR-AI-293T and vec-293T cell stimulation in the presence of heated BCS. Pre-incubation with fresh or heated BCS for 10 min did not cause significant SR-AI stimulation. NF-κB activation (C) and IL-8 production (D) were measured. (E and F) *E. coli* DH5α was pre-incubated with fresh human serum and was, after washing, further incubated with goat anti-C3 or anti-C1q antibody. The bacteria were then used to stimulate SR-AI-293T and vec-293T cells in the presence of heated BCS. NF-κB activation (E) and IL-8 production (F) were determined. Results of triplicate experiments were expressed as means ± SD.

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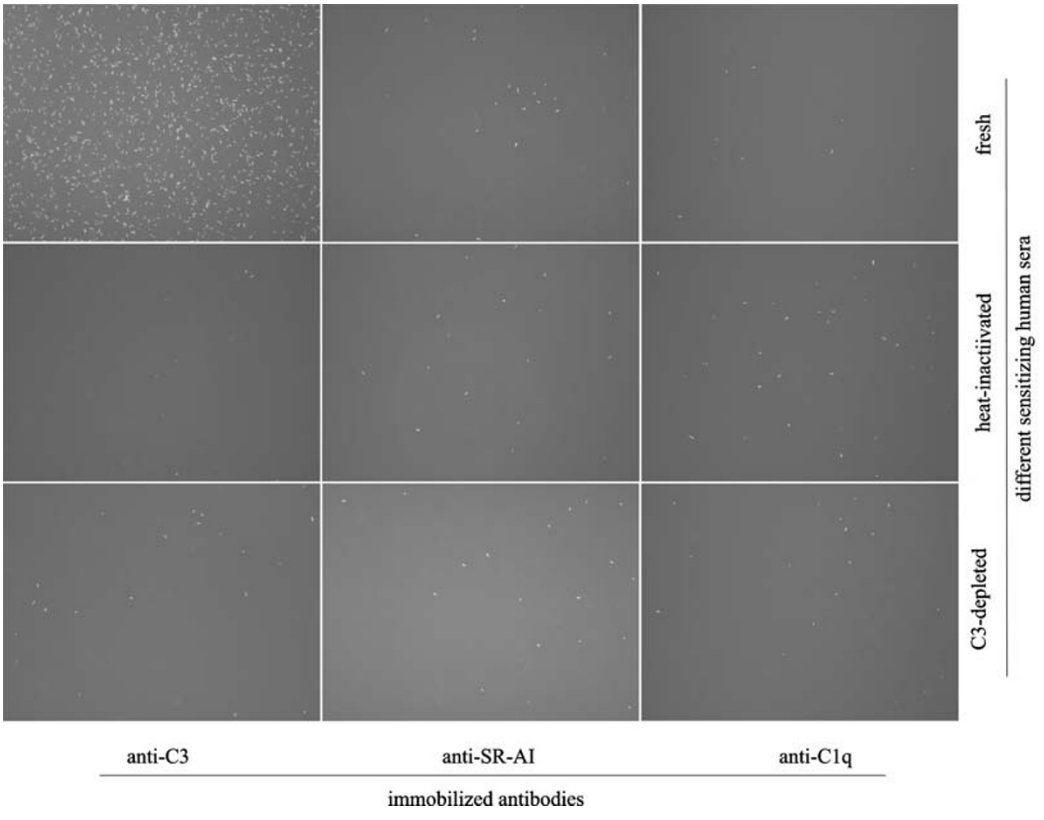

Figure 4. Detection of C3 deposition on serum-reacted DH5α. The DH5α-GFP bacteria were pre-incubated with fresh, heated-inactivated, or C3-depleted human serum for 1 h and were, after washing, incubated with coverslips coated with anti-C3, anti-SR-AI, or anti-C1q antibody. Bound bacteria are fixed with 4% formaldehyde and analyzed using a BX51 Olympus fluorescence microscope (63 × objective) and the Olympus Micro Image software (Olympus, Tokyo, Japan).

SR-AI requires its SRCR domain to respond to DH5α

We then asked which SR-AI domain bound to serum-sensitized DH5α. SR-AI binds to its promiscuous polyanionic ligands with a 22-residue segment in its collagen region, which include a cluster of three lysine residues (K332, K335 and K338 in human SR-A) (Acton et al., 1993; Doi et al., 1993). To determine whether this region also recognizes serum-treated DH5α, these lysine residues were mutated singly or jointly into alanine, which yielded 7 different SR-AI mutants (Table 1). Because these mutants are not recognized by the anti-SR-AI antibody (data not shown), they were expressed with C-terminal Myc and His tags for detection. Where all three lysine residues were mutated, the SR-AI mutant was known as SR-AI-mColl-MH. An 8th SR-AI mutant was constructed in which the entire collagen region was deleted (SR-AI-dColl-MH).

As shown in Fig. 6A, all the 7 SR-AI lysine mutants similarly responded to serum-treated DH5α like wild type SR-AI, but the SR-AI mutant that lacks the entire collagen region showed no response to the bacteria. Therefore, this deletion could have affected the conformation of the SRCR domain. These lysine mutations did not hinder SR-AI surface expression as all 8 SR-AI mutants were accessible to membrane-impermeable biotin and were detected at similar levels (Fig. 6B). How the collagen region contributes to DH5α binding cannot be deduced from this experiment. However, the SR-AI binding site for its poly-anionic ligands is apparently insufficient for DH5α recognition.

We then considered the SRCR domain of SR-AI as a potential binding site for the bacteria. Brannstrom et al. (2002) reported that MARCO uses its SRCR domain to bind bacteria. We first examined SR-AII in DH5α recognition as it naturally lacks the SRCR domain (Freeman et al., 1990; Kodama et al., 1990). As shown in Fig. 6C, unlike SR-AI, expression of SR-AII in 293T cells did not confer response to the bacteria. It suggests that the SRCR domain is required for SR-AI to recognize serum-sensitized DH5α.

It is unclear how the SRCR domain of SR-AI is involved in bacteria-binding, but it has been reported that MARCO uses an 11-residue segment (RGRAEVYYSGT) (Brannstrom et al., 2002). In addition to SR-AI and MARCO, the complement factor I (CFI) contains a closely related SRCR domain that interacts with the complement C3b fragment. We then compared the SRCR sequences of the SR-AI, MARCO and

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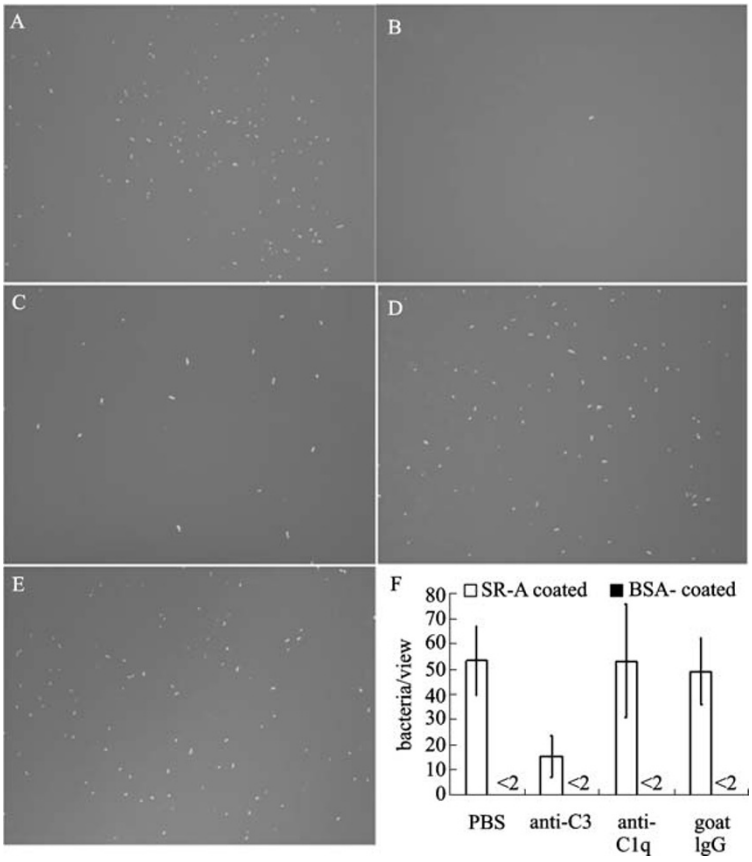

Figure 5. DH5α surface-deposited C3 binds to purified SR-AI. The DH5α-GFP bacteria were pre-incubated with fresh human serum for 1 h and were, after washing, incubated with coverslips coated with purified sSR-AI (A) or BSA (B). The serum-sensitized DH5α was also incubated with anti-C3 antibody (C), anti-C1q antibody (D), or non-immune goat IgG (E) before incubation with coverslip-coated sSR-AI (C–E) or BSA (images not shown). After washing, bound DH5α-GFP was fixed with 4% formaldehyde and analyzed using a BX51 Olympus fluorescence microscope (63 × objective) and the Olympus Micro Image software (Olympus, Tokyo, Japan). (F) DH5α-GFP binding to SR-AI or BSA was also quantified by counting 5 independent views on each coverslip. The numbers of bound bacteria in each view were then presented as means ± SD.

CFI, and two residues G361 or E364 were found completely conserved (Fig. 6D). Two SR-AI mutants were then generated: mutation of the small G361 residue into a rigid proline residue (G361P) and mutation of the acidic E364 residue to a neutral alanine (E364A). Both mutants were detected on the surface of transfected 293T cells (Fig. 6B). However, neither showed response to the sensitized DH5α (Fig. 6C). These results suggest a critical role for the SR-AI SRCR domain in the recognition of C3-opsonized bacteria.

SR-AI requires its SRCR domain to bind serum-sensitized DH5α

The requirement of SRCR domain of SR-AI on binding serum-sensitized DH5α was evaluated using SR-AI, SR-AII and the SR-AI-mColl mutant that were expressed on 293T cells. DH5α-GFP was incubated for 1 h with the transfected cells in the presence of fresh BCS. Bound bacteria were observed by fluorescence microscopy. The bacteria showed prominent binding to the SR-AI-293T cells. As a control, the bacteria exhibited low background binding to vec-293T cells (Fig. 7). Expression of SR-AII in 293T cells did not confer increased DH5α-binding as compared with vec-293T cells. The SR-AI-mColl mutant, which is similar to wild type SR-AI in mediating DH5α-induced signaling (Fig. 6A), was also as potent in DH5α binding (Fig. 7). When heat-inactivated BCS was used, DH5α-GFP was unable to bind to SR-AI or the SR-AI-mColl mutant. These data show that the SRCR domain is required for SR-AI to bind to C3-sensitized DH5α.

SR-AI selectively binds to iC3b

C3 can deposit on bacteria as C3b but it can be further cleaved to become iC3b and C3d (Law, 1988). Since serum-sensitized DH5α was found to activate CR3 signaling, the CR3 ligand iC3b is likely to be abundant on these bacteria

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Table 1 Mutation, deletion and cloning strategies for the generation of SR-A expression vectors

original codon	new codon	restriction sites	mutant expression vectors
TAA (Stop)	TCA (Ser)	-	pSR-AI-MH
AAA	GCA	-	pSR-AI-K332A-MH
AAA	GCA	-	pSR-AI-K335A-MH
AAG	GCG	-	pSR-AI-K338A-MH
AAA…AAA	GCA…GCA	-	pSR-AI-K332/5A-MH
AAA…AAG	GCA…GCG	-	pSR-AI-K332/8A-MH
AAA…AAG	GCA…GCG	-	pSR-AI-K335/8A-MH
AAA…AAA…AAG	GCA…GCA…GCG	-	pSR-AI-mColl-MH
GGTCCT…GGAAAC	GGATCC (BamHI)	BamHI/BamHI	pSR-AI-dColl-MH
CAACTC…GGGCCC	GGATCC…GGGCCC	BamHI/ApaI	psSR-AI-MH
GGAAAC…GGGCCC	GGTACC…GGGCCC	KpnI/ApaI	pSRCR-MH
GGG	CCG	-	pSR-AI-G361P-MH
GAG	GCG	-	pSR-AI-E364A-MH
TAA (stop)	GCA (Ala)	-	pSR-AII-MH

Note: The pSR-AI and pSR-AII vectors were constructed as described in 'Materials and Methods'. The stop codon in pSR-AI (TAA) was mutated into a serine codon (TCA) so that SR-AI is fused to C-terminal Myc/His tags (pSR-AI-MH). Likewise, the stop codon in pSR-AII (TAA) was mutated into an alanine codon (GCA) (pSR-AII-MH). A series of point mutations were introduced to pSR-AI-MH to generate K332A, K335A, K338A, E364A and G361P mutants. In pSR-AI-mColl-MH, K332A, K335A and K338A are jointly introduced. Mutations were also introduced into pSR-AI-MH to allow the deletions of the collagen domain (pSR-AI-dColl-MH) or the expression of sSR-AI (psSR-AI-MH), or SRCR (pSRCR-MH). All vectors were sequence-verified.

(Fig. 2B and 2C). Here we purified C3 from human plasma and generated the C3b and iC3b fragments (Fig. 8A). SR-AI interaction with C3, C3b and iC3b was assessed in two different experiments. Firstly, sSR-AI was coated on the plates to incubate with these C3 molecules, and bound C3 or C3 fragments were detected using an anti-C3 antibody. As shown in Fig. 8B and 8C, SR-AI showed prominent binding to iC3b but not C3 or C3b. As a control, immobilized BSA showed little binding to these C3 molecules. The SR-AI SRCR domain was also expressed and purified (Supplemental Fig. 1), but the immobilized sSRCR domain showed little binding to soluble iC3b (Fig. 8C).

In another experiment, iC3b was immobilized and incubated with sSR-AI or the SRCR domain. When SR-AI was used at 10 μg/mL, binding was not detectable (data not shown). At 100 μg/mL, binding of SR-AI to immobilized iC3b was detected (Fig. 8C). At both concentrations, the binding of SRCR to immobilized iC3b was not detectable (Fig. 8C). SRCR is ~20 kDa in size, which is expected to cluster as a trimer on SR-AI. Without the other SR-AI regions, the SRCR domain has apparently lost the trimeric conformation because it can pass 30-kDa cutoff filters (data not shown).

DISCUSSION

Macrophages are major tissue scavengers and express many phagocytic receptors for endogenous and microbial materials (Underhill and Ozinsky, 2002). These are mainly low affinity receptors that have promiscuous and overlapping ligand specificity and achieve ligand-binding through multivalent interactions. The signaling properties of these receptors are generally less understood partly due to this complexity. We therefore expressed one such receptor, SR-AI, in 293T cells to study its ligand recognition and cell signaling. In 293T cells, SR-AI-mediated NF-κB activation was clearly detected in the absence of other macrophage receptors and it also led to IL-8 production from the 293T cells. The intracellular mechanisms that lead to NF-κB activation, which are currently under investigation, are independent of MyD88 or the TLR (data not shown). In this report, we describe an unexpected finding that, while SR-AI recognizes DH5α as previously reported (Peiser et al., 2000), it requires the bacteria to be pre-sensitized with complement C3.

Results from a series of experiments showed that SR-AI was stimulated by complement C3-opsonized DH5α with iC3b directly binding to SR-AI and was the most likely ligand on serum-reacted DH5α. Firstly, in 293T cells, SR-AI only confers DH5α binding when these bacteria were sensitized with fresh serum. Secondly, C3 or its fragment(s) was detected on serum-reacted DH5α and the ability of these bacteria to stimulate CR3 suggests the abundance of iC3b. Thirdly, these bacteria could bind to purified SR-AI and the binding was inhibited by anti-C3 antibody. Finally, direct binding between purified SR-AI and iC3b has been demonstrated. While our conclusion is in discordant with the previous report that SR-AI directly binds to DH5α (Peiser et

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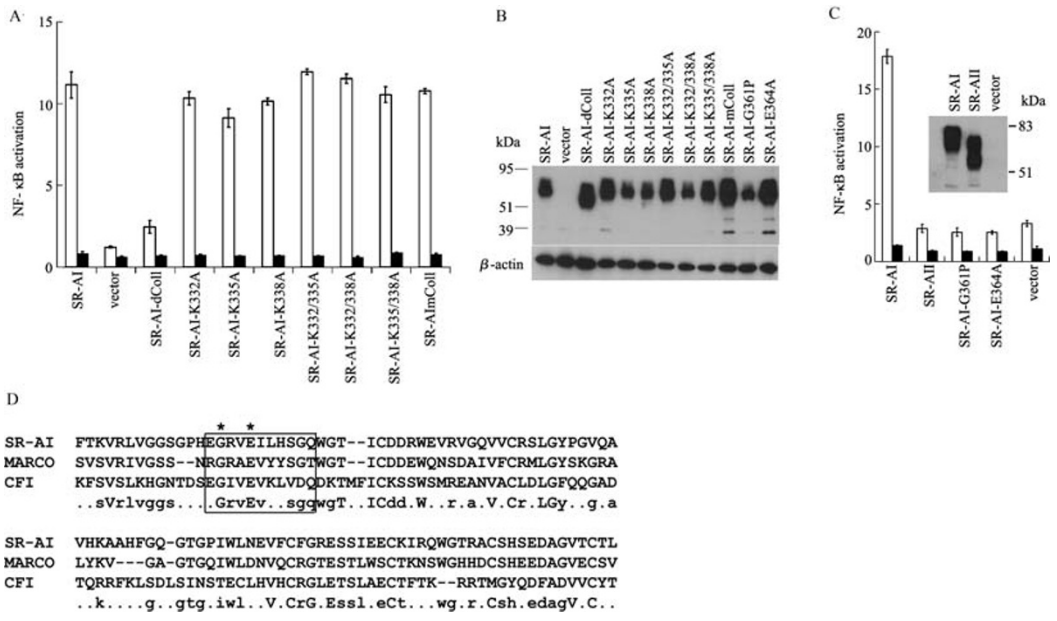

Figure 6. The integrity of the SRCR domain is essential for SR-AI response to serum-opsonized *E. coli* DH5α. (A) pSR-AI-MH and the different mutant vectors for the K332A, K335A, K338A, G361P and E364A mutation or the deletion of the collagen domain were expressed in 293T cells, which were stimulated with *E. coli* DH5α sensitized with fresh human serum (open bars). As controls, the transfected cells were not stimulated with the bacteria (solid bars). NF-κB activation was determined. Results of triplicate experiments were expressed as means ± SD. (B) Samples were prepared as described in (A) except that the transfected 293T cells were surface-biotinylated. Biotin-labeled proteins were precipitated with streptavidin-agarose and detected by Western blotting using a mouse anti-Myc antibody. (C) 293T cells were transfected with pSR-AI-MH, pSR-AII-MH or vectors containing the G361P or E364A mutations. The cells were stimulated with DH5α in the presence of fresh BCS (open bars) or, as controls, unstimulated (solid bars). NF-κB activation was measured. (D) Alignment of the SRCR sequences of SR-AI, MARCO and CFI. The 11-residue bacteria-binding region in MARCO and equivalent sequences in SR-AI and CFI are indicated by a rectangle. Residues G361 and E364 were indicated by `*'.

al., 2000), it is consistent with a more recent study showing that the bacteria *Francisella tularensis*, which infects macrophages through SR-AI, only infects macrophages when the bacteria were sensitized by heat-labile serum factor(s), implying the involvement of complement (Pierini, 2006).

Another novel finding is the identification of the SR-AI SRCR domain in SR-AI binding to C3-sensitized DH5α. SR-AII lacks the SRCR domain and it also lacks binding to the bacteria. For the polyanionic ligands, the binding site on SR-AI has been mapped to a 22-residue region of the collagen domain including three clustered lysine residues (Acton et al., 1993; Doi et al., 1993; Krieger and Stern, 2001; Peiser et al., 2002b). However, mutation of the lysine residues showed no effect on SR-AI binding and response to serum-sensitized DH5α, but mutations in the SRCR domain abolished SR-AI response to the bacteria (Fig. 6). It shows that SR-AI recognizes C3-sensitized bacteria through its SRCR domain rather than its collagen domain. We also expressed the SRCR domain, but the purified SRCR domain showed no binding to iC3b while purified SR-AI did. A reasonable explanation is that the SRCR domain is trimeric on SR-AI and, without the other SR-AI domains, it loses this conformation and also loses the ability to bind to iC3b. The purified SRCR domain behaved like a monomer (data not shown). When isolated, the MARCO SRCR domain also dissociates into dimers and monomers and also lacks bacteria binding (Ojala et al., 2007).

The fact that complement factor I (CFI) contains a SRCR domain that is closely related to the SRCR domain in SR-AI and MARCO supports the relevance of the demonstrated SR-AI and iC3b interaction in this study. CFI is a serine protease that binds to C3b so as to cleave C3b into iC3b. In MARCO, the bacteria-binding site was mapped to an 11-residue segment in the SRCR domain. Alignment of the SR-AI, MARCO and CFI SRCR sequences identified two residues in this 11-residue segment (G361 and E364), which are

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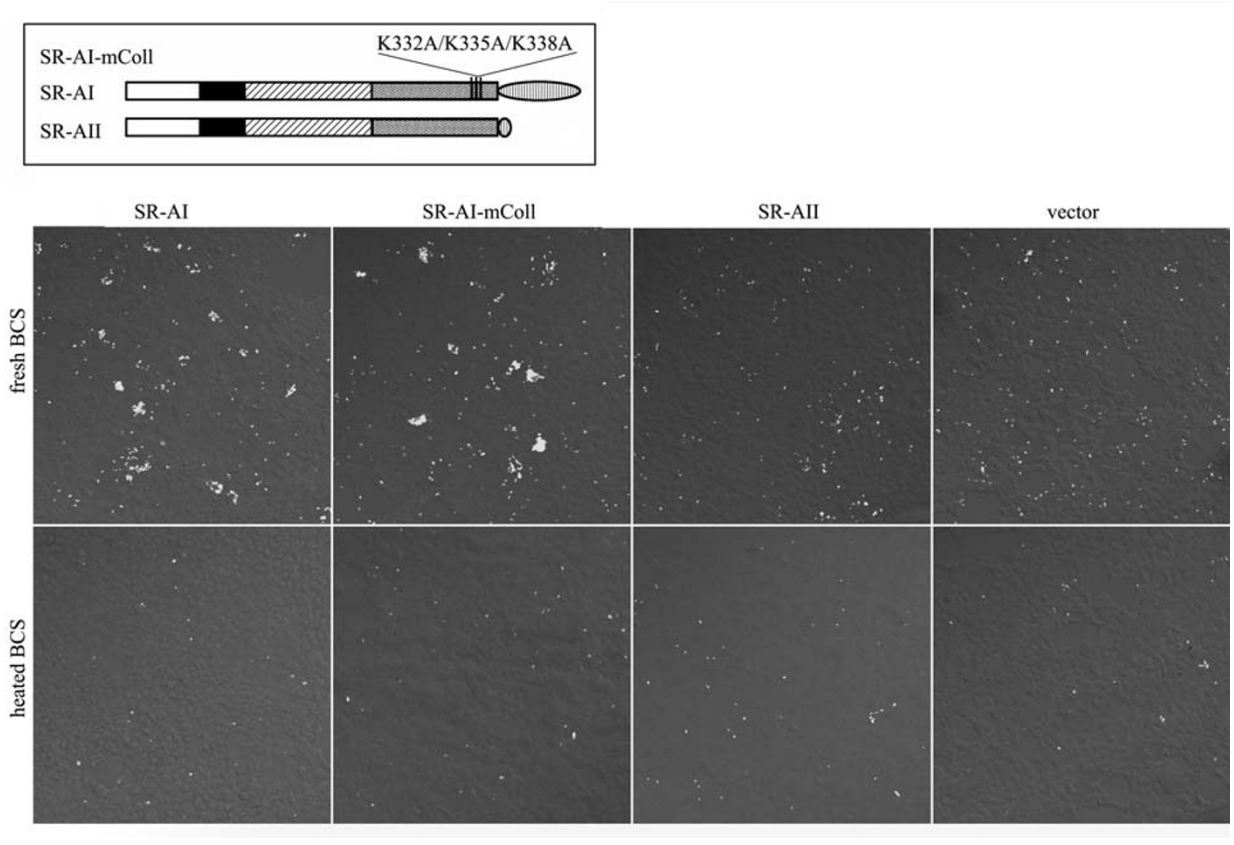

Figure 7. ***E. coli*** **DH5α binds to surface-expressed SR-AI but not SR-AII.** 293T cells were transfected with pSR-AI-MH, pSR-AII-MH, pSR-AI-mColl-MH, or pcDNA3.1 on coverslips and then incubated for 1 h with DH5α-GFP in the presence of fresh or heat-inactivated BCS. After washing, the cells were fixed in 4% (*w/v*) formaldehyde and analyzed using a LSM510 laser-scanning microscope. The diagram inside the rectangle illustrates the domain organizations of SR-AI, SR-AII and SR-AI-mColl-MH.

completely conserved. We show that mutation of either residue in SR-AI abolishes its response to serum-sensitized bacteria. One remaining puzzle is that, while MARCO binds to bacteria with its SRCR domain, it binds to bacteria directly without C3 sensitization. In this regard, it is interesting to note that MARCO has been reported to interact with the complement C4b and C4d fragments although its relevance was not further elaborated (Chen et al., 2006).

The ability of SR-AI to recognize complement C3-sensitized DH5α offers a mechanism by which SR-AI is able to recognize a broader range of targets. For example, we showed, for the first time, SR-AI recognition of the bacteria *B. subtilis*. We also showed that it was not activated by the bacteria *M. bovis* BCG, although it is unclear why BCG is unable to activate SR-AI. An earlier study showed that the related bacteria *Mycobacterium avium* are, after complement reaction, surface-deposited with predominantly C3b rather than iC3b (Schorey et al., 1997). Further work is required to evaluate how the SRCR domain might distinguish iC3b from C3b and to dissect the signalling events from SR-AI that leads to NF-κB activation and IL-8 production.

MATERIALS AND METHODS

Cell culture and reagent

Human embryonic kidney cells, 293T (ATCC), were cultured in DMEM supplemented with BCS (HyClone, Logan, UT), 100 units/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine at 37°C and 5% CO_2. *Bacillus subtilis* (ATCC) and *E. coli* DH5α (Invitrogen, Carlsbad, CA) were cultured in L-broth and DH5α-GFP (kindly provided by Dr. Yunn Hwen Gan, National University of Singapore) was cultured in L-broth supplemented with ampicillin (100 μg/mL). *M. bovis* BCG (Connaught strain, Aventis Pasteur) was cultured at 37°C in 7H9 Middlebrook medium (Difco) supplemented with 10% ADC supplement (0.85% (*w/v*) NaCl, 5% (*w/v*) BSA, 2% (*w/v*) dextrose and 0.003% (*w/v*) catalase), 0.05% (*v/v*) Tween-80 and 0.2% (*v/v*) glycerol. All bacteria were cultured with shaking at 130 rpm and harvested at $OD_{600\ nm}$ readings of 0.7 to 0.8. LPS (*E. coli* O5 : 55), C3- and C7-depleted human sera and goat anti-human C3 and C1q antibodies were obtained from Sigma-Aldrich (St Louis, IL). The mouse anti-Myc monoclonal antibody was purchased from Roche Diagnostics Co. (Indianapolis, IN). The goat anti-human SR-A antiserum was obtained from Serotec (MprphoSys UK Ltd, Oxford, UK).

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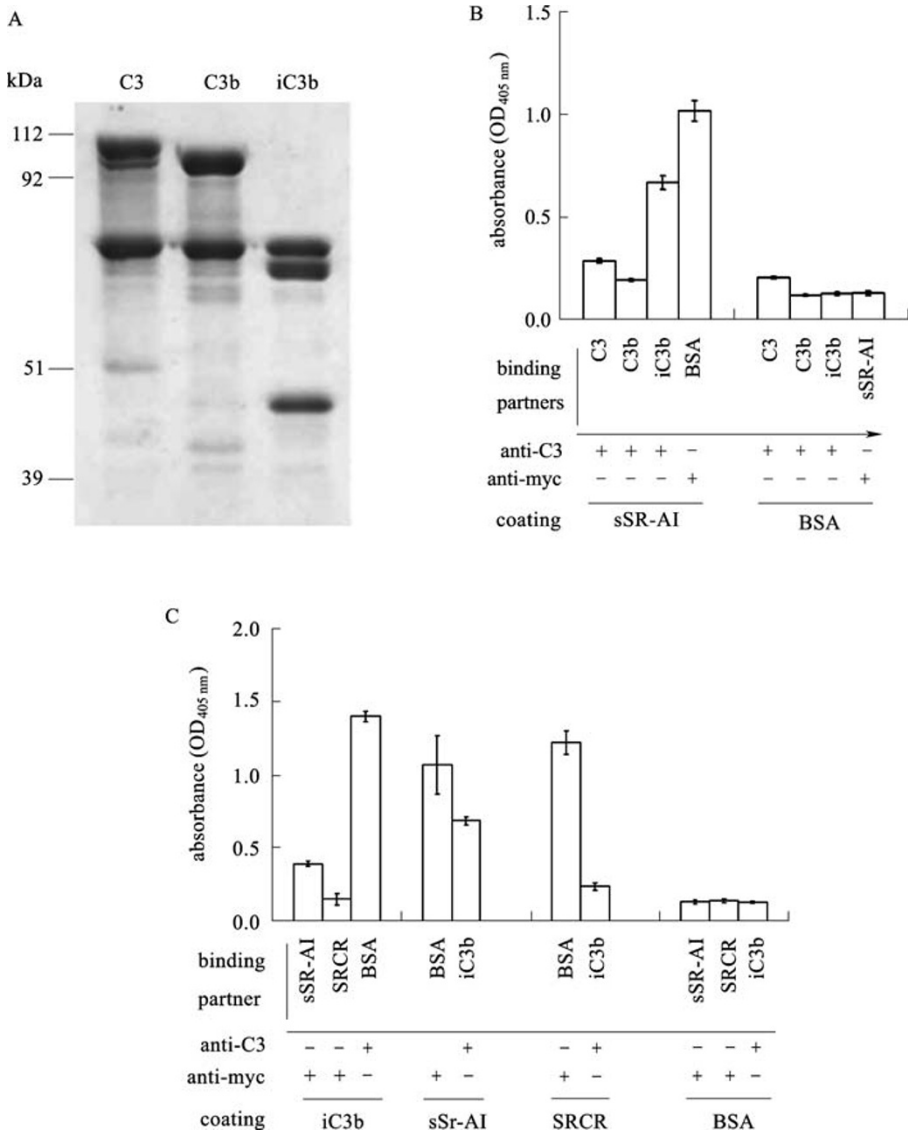

Figure 8. SR-AI selectively binds to iC3b. (A) Purified C3 and its C3b and iC3b fragments were subjected to SDS-PAGE on 10% (*w/v*) gels and stained with Coomassie blue. (B) 96-well plates were coated with sSR-AI or BSA (10 μg/mL) and then incubated with C3, C3b, iC3b or BSA (10 μg/mL). Bound C3 or its fragments were detected using a goat anti-human C3 antibody. As controls, coated sSR-AI was directly detected using an anti-Myc antibody. (C) C3, C3b and iC3b were immobilized (10 μg/mL) in 96-well plates to incubate with sSR-AI or SRCR (100 μg/mL). At 10 μg/mL, neither sSR-AI nor SRCR showed binding to immobilized iC3b, C3b or C3 (data not shown). Results of triplicate experiments were expressed as means ± SD.

Expression vectors

The pTLR2 vector was constructed as previously described (Zhong et al., 2005). The CR3 expression vectors for the αM and β2 subunits were kindly provided by Dr. Alex Law, University of Oxford, UK (Al-Shamkhani et al., 1998). The SR-AI cDNA was amplified by RT-PCR from human macrophage RNA using a pair of SR-AI-specific primers (5'-3', cgaggtaccgccaccatggagcagtgggatcac/cgcatcgggcccttataaagtgcaagtgactcc, NM_138715) and cloned into the KpnI/ApaI sites of the pcDNA3.1 plasmid (Invitrogen) to yield the pSR-AI vector. The cDNA for SR-AII was separately amplified with the same forward primer but an SR-AII-specific reverse primer (5'-3', cgcatcttcgaattaagagggccctgccctaa, NM_002445) and cloned into the KpnI/SfuI sites of pcDNA3.1 (pSR-AII). The stop codons in the pSR-AI and pSR-AII vectors were mutated into serine and alanine codons respectively to express both receptors with C-terminal Myc and His tags (pSR-AI-MH and pSR-AII-MH) (Table 1). Further expression vectors were constructed using pSR-AI-MH by site-directed mutagenesis combined with deletion to express SR-AI mutants and to generate the psSR-AI-MH and pSRCR-MH vectors for the expression of soluble sSR-AI and the SRCR domain. These are described in Table 1.

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NF-κB luciferase Assay

NF-κB luciferase Assay was performed as previously described (Zhong et al., 2005). 293T cells were cultured overnight in 24-well plates at $1x10^5$/well and transfected using the GenePorter 2 reagents (Gene Therapy Systems, La Jolla, CA). Each expression vector was used at 300 ng/well unless otherwise stated and the p5xNFκB-Luc (Stratagene, La Jolla, CA) and pRL-CMV (Promega, Madison, WI) luciferase reporter plasmids were co-transfected each at 100 ng/well. After 24 h, the cells were incubated with bacteria at a bacteria:cell ratio of 100 : 1 ($2×10^7$ bacteria/well) for 1 h at 37°C. The cells are then washed 3 times with warm medium and cultured overnight before NF-κB activation was measured using the Dual Luciferase Assay kit (Promega). All bacteria were washed 3 times with serum-free DMEM before incubation with 293T cells. In some experiments, *E. coli* DH5α was pre-incubated at 37°C with 10% (*v/v*) fresh or heat-inactivated BCS or human serum in DMEM and washed 3 times in serum-free DMEM prior to 293T cell stimulation. Serum was heated for 45 min at 56°C to inactivate complement activities. Where normal or complement C3- and C7-depleted human sera (Sigma-Aldrich) were used to sensitize *E. coli* DH5α, this was carried out for 8 min at 37°C unless otherwise specified. In some experiments, *E. coli* DH5α that had been pre-incubated for 10 min with human serum, were subsequently incubated with goat anti-C3 or goat anti-C1q IgG antibodies (Sigma-Aldrich) (20 μg/mL) prior to 293T cell stimulation.

293T cells were also transfected with pTLR2 (10 ng/mL) and then similarly stimulated with *M. bovis* BCG or *B. sublitis*. NF-κB activation was determined and expressed as NF-κB-directed firefly luciferase expression normalized to CMV-directed *Renilla* luciferase expression taking the latter as 100. All experiments were carried out in triplicate and results were expressed as means ± SD. The supernatants were collected in some experiments to measure IL-8 by ELISA (BD Biosciences, San Diego, CA).

Western blotting

Transfected 293T cells were washed in PBS and lysed for 1 h at 4°C in a lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (*v/v*) Nonidet P-40 and the complete protease inhibitor cocktail (Roche)). After centrifugation, the lysates were subjected to SDS-PAGE on 12.5% (*w/v*) gels and Western blotting using a goat anti-human SR-AI antibody (Serotec). Transfected 293T cells were also washed in ice-cold PBS (pH 8.0) and re-suspended at $2×10^7$/mL. The cells (0.1 mL) were incubated for 30 min at room temperature with 50 μg of EZ-Link™Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL) and then quenched with 0.1 M glycine (pH 8.0). After washing, the cells were lysed in 0.1 mL of lysis buffer and the cleared lysate was incubated with 40 μL streptavidin-agarose (Pierce). The beads were washed and bound proteins were analyzed by SDS-PAGE on 12.5% (*w/v*) gels. SR-AI was detected by Western blotting using the goat anti-human SR-AI or a mouse anti-Myc antibody (Roche). Signals were visualized using the Immune-Star substrate pack (Bio-Rad, Hercules, CA).

Flow cytometry

293T cells were transfected with pSR-AI-MH (300 ng/well) and were, after 24 h, harvested and washed in FACSwash (PBS containing 2% (*v/v*) BCS and 0.05% (*w/v*) NaN_3). The cells were then incubated for 45 min with a mouse anti-Myc antibody or isotype control IgG and, after washing, incubated with a PE-labeled goat anti-mouse IgG for 30 min. The cells were washed and analyzed on a FACSCalibur using the CellQuest software (Becton Dickinson Immunochemistry Systems, San Jose, CA).

Expression and purification of sSR-AI and SRCR

293T cells were transfected with the psSR-AI or pSRCR vector (Table 1) at 50 μg/T75 flask using calcium phosphate (Cao et al., 2006). After 6 h, the cells were washed with serum-free DMEM containing BSA (100 μg/mL) and cultured for 2 days in the same media. The conditioned media (60 mL from 3 flasks) were passed through 0.22 μm filters and incubated overnight at 4°C with Ni-NTA-agarose (0.5 mL) (Qiagen). After washing, the beads were eluted with imidazole (Cao et al., 2006). The proteins were dialyzed and concentration was measured using the Bio-Rad Protein Assay kit.

***E. coli* DH5α binding assay**

293T cells were cultured on glass coverslips in 24-well plates and transfected with the pSR-AI-MH, pSR-AII-MH, pSR-AI-mColl-MH, or pcDNA3.1 vector. After 24 h, the cells were incubated for 1 h with DH5α-GFP ($2×10^7$/well) using fresh BCS. The cells were washed 3 times with culture medium, fixed in 4% (*w/v*) formaldehyde, and analyzed using a LSM510 laser-scanning microscope and the Zeiss LSM Image Browser software.

In another binding assay, 96-well plates were coated with sSR-AI or BSA (50 μg/mL in PBS) overnight at 4°C. After washing with a TBS buffer (20 mM Tris, pH 7.4, 150 mM NaCl , 5 mM $CaCl_2$ and 5 mM $MgCl_2$), the plates were blocked for 1 h with the TBS buffer containing 0.1% (*w/v*) BSA. DH5α-GFP was pre-incubated in 10% (*v/v*) fresh human serum (in DMEM) for 10 min ($4×10^8$/mL) and was, after washing, re-suspended in serum-free DMEM to the same density. The bacteria (1 mL) were incubated with the anti-C3 antibody (100 μg) for 15 min on ice. As controls, the bacteria were incubated with the anti-C1q antibody, non-immune goat IgG, or PBS. The bacteria were then incubated for 30 min at 4°C with sSR-AI-coated plates (0.1 mL/well). The plates were washed with DMEM and analyzed using an Olympus 1X81 Inverted fluorescence microscope and the Image-Pro MC 5.1 software (Olympus America Inc, Center Valley, PA). Bound DH5α-GFP in each well was counted using five independent views and the number of bound bacteria in each well was presented as mean ± SD.

Similarly, 96-well plates were coated with anti-C3, anti-SR-AI and anti-C1q antibodies (50 μg/mL). The plates were washed and blocked with BSA. DH5α-GFP was incubated with 10% fresh, heat-inactivated or C3-depleted human serum for 10 min. After washing, the bacteria were re-suspended at $2×10^8$/mL in serum-free DMEM and then incubated with the plates for 30 min at 4°C. The plates were washed with DMEM and analysed using the Olympus 1X81 fluorescence microscope and the Image-Pro MC 5.1 software.

Preparation of C3 and its fragments

Complement C3 was isolated from human plasma and C3b was generated from C3 following a published method (Dodds, 1993). iC3b was generated by incubation of human plasma with thiol-Sepharose and eluted with L-cysteine as previously reported (Cai and Wright, 1995). The eluted iC3b was further purified by FPLC using a Mono Q

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column (HR5/5). The purified C3, C3b and iC3b were examined by SDS-PAGE on 10% (*w/v*) gels with Coomassie blue staining.

Solid phase protein binding assay

Ninety-six-well plates were coated with sSR-AI, SRCR, C3, C3b, iC3b or BSA (10 μg/mL in PBS) overnight at 4°C. The plates were washed in PBS containing 0.1% (*v/v*) Tween-20 (PBS-T) and blocked using the same buffer. The plates were then incubated with the indicated soluble protein partners (10 or 100 μg/mL) overnight at 4°C. Immobilized or bound proteins on the plates were detected, using a goat anti-human C3 antibody (20 μg/mL) (Sigma-Aldrich) or a mouse anti-Myc monoclonal antibody (2 μg/mL; Roche) as specified, by incubation for 2 h at 4°C followed by washing with PBS-T, and then by a 2 h incubation with alkaline phosphatase-labeled secondary antibodies in PBS-T. After washing, the plates were developed using the substrate p-nitro phenyl phosphate (pNPP) (Sigma) and read at 405 nm using a Bio-Rad Model 680 microplate reader.

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ABBREVIATIONS

BSA, bovine serum albumin; CFI, complement factor I; CR3, complement receptor 3; GFP, green fluorescence protein; LDL, low density lipoprotein; LPS, lipopolysaccharide; LTA, lipoteichoic acid; MARCO, macrophage receptor with collagenous structure; pNPP, p-nitro phenyl phosphate; SR-AI, scavenger receptor class A type I; SRCR, scavenger receptor cycteine-rich; TLR, Toll-like receptor

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