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Drosophila Scavenger Receptor CI Is a Pattern Recognition Receptor for Bacteria

Mika Rämet,[1,4] Alan Pearson,[1,2] Pascal Manfruelli,[1] Xiohung Li,[3] Henry Koziel,[3] Verena Göbel,[1] Ed Chung,[2] Monty Krieger,[2] and R. Alan B. Ezekowitz[1,4]

[1] Laboratory of Developmental Immunology
MassGeneral Hospital for Children
Department of Pediatrics
Harvard Medical School
Boston, Massachusetts 02114

[2] Department of Biology
Massachusetts Institute of Technology
Cambridge, Massachusetts 02138

[3] Department of Medicine
Division of Pulmonary and Critical Care Medicine
Beth Israel Deaconess Medical Center and
Harvard Medical School
Boston, Massachusetts 02115

Summary

One hallmark of innate immunity apparently conserved from primitive life forms through to humans is the ability of the host to recognize pathogen-associated molecular patterns (PAMPs). Since macrophage pattern recognition receptors are not well defined in *Drosophila,* we set out to identify such receptors. Our findings reveal that *Drosophila* macrophages express multiple pattern recognition receptors and that the *Drosophila* scavenger receptor, dSR-CI, is one such receptor capable of recognizing both gram-negative and gram-positive bacteria, but not yeast. Our data indicate that scavenger receptor bacterial recognition is conserved from insects to humans and may represent one of the most primitive forms of microbial recognition.

Introduction

A consensus is emerging that the templates of innate immunity in mammals have ancient origins (Hoffmann et al., 1999) and hence that genetically tractable model systems like *Drosophila melanogaster* can be informative in identifying genes whose products play a role in the innate immunity of many species. The characterization of the Toll family of proteins in *Drosophila* and mammals and their respective involvement in host defense illustrates this idea (Anderson et al., 1985; Lemaitre et al., 1997; Medzhitov et al., 1997). Progress in innate immunity is fueled by an interchange of experimental findings in mammalian systems with more primitive lifeforms, which indicate important similarities and differences (Ezekowitz and Hoffmann, 2001).

Receptor-mediated phagocytosis of pathogens is a highly conserved cellular process. Macrophages are able to recognize disparate surface structures on particles and microorganisms they ingest. One well-defined mechanism is Fc receptor-mediated recognition of microbes that have been opsonized by epitope-specific antibodies (Aderem and Underhill, 1999). It has been proposed that pathogen-associated molecular patterns (PAMPs), conserved structures that adorn the surfaces of microorganisms, are recognized by pattern recognition molecules and receptors (Janeway, 1989). Some of these receptors are expected to signal the presence of pathogens for integrated responses of the organism to pathogen, while others may primarily serve as endocytosis/phagocytosis receptors that primarily clear, and subsequently destroy, the pathogen. Ligands can be opsonized with soluble pattern recognition molecules such as complement, mannose binding proteins and lipopolysaccharide (LPS) binding protein for subsequent ingestion mediated by complement receptors, collectin receptors, and CD14, respectively (Aderem and Underhill, 1999). In addition, macrophages can directly recognize microorganisms via pattern recognition receptors (PRRs) like the mannose receptor and other lectin-like receptors (Stahl and Ezekowitz, 1998) and the scavenger receptors (SRs) (Krieger, 1997; Gough and Gordon, 2000). There are at least six structurally distinct classes of SRs, defined by their ability to bind acetylated and or oxidized low-density lipoprotein (LDL) (Gough and Gordon, 2000). Most classes of SRs are additionally able to recognize a broad range of both common and receptor-specific ligands, including polyanionic compounds, apoptotic cells, bacteria, and LPS (Hampton et al., 1991; Ashkenas et al., 1993; Dunne et al., 1994; Krieger, 1997).

In contrast to mammalian systems, no transmembrane macrophage PRR for bacteria has been unambiguously described in insects. Several soluble proteins that recognize peptidoglycan, LPS, and β-1-3-glucan, and several complement-like proteins have been proposed as pattern-recognition molecules in various invertebrate species (Lee et al., 1996; Richman et al., 1997; Kang et al., 1998; Beschin et al., 1998, Ochiai and Ashida, 1999, 2000; Ma and Kanost, 2000; Werner, et al. 2000, Levashina et al., 2001). The *Drosophila* gram-negative binding proteins appear to participate in the induction of antimicrobial peptide synthesis in vitro, but their association with the membrane remains uncertain (Kim et al., 2000). There are two well-characterized macrophage receptors in *Drosophila*: croquemort (Franc et al., 1996) and the *Drosophila* SR, dSR-CI (Pearson et al., 1995). Croquemort belongs to the CD36 superfamily of proteins, and it is expressed specifically in macrophages. In vitro and in vivo experiments demonstrated a key role for croquemort in the recognition of apoptotic cells (Franc et al., 1996, 1999). However, macrophages lacking croquemort expression, while defective in apoptotic cell engulfment, were still able to ingest *E. coli* and *S. aureus* (Franc et al., 1999). Thus, *Drosophila* macrophages must possess other PRRs for phagocytosis of bacteria. Like croquemort, dSR-CI appears to be macrophage specific. In contrast to croquemort, dSR-CI recognizes a broad range of polyanionic ligands, much like the mammalian class A SR (Pearson et al., 1995). Comparison of the ligand binding properties of the *Drosophila* S2 cell line (Abrams et al., 1992), from which

[4] Correspondence: ezekowitz.alan@mgh.harvard.edu (R.A.B.E.), mramet@partners.org (M.R.)

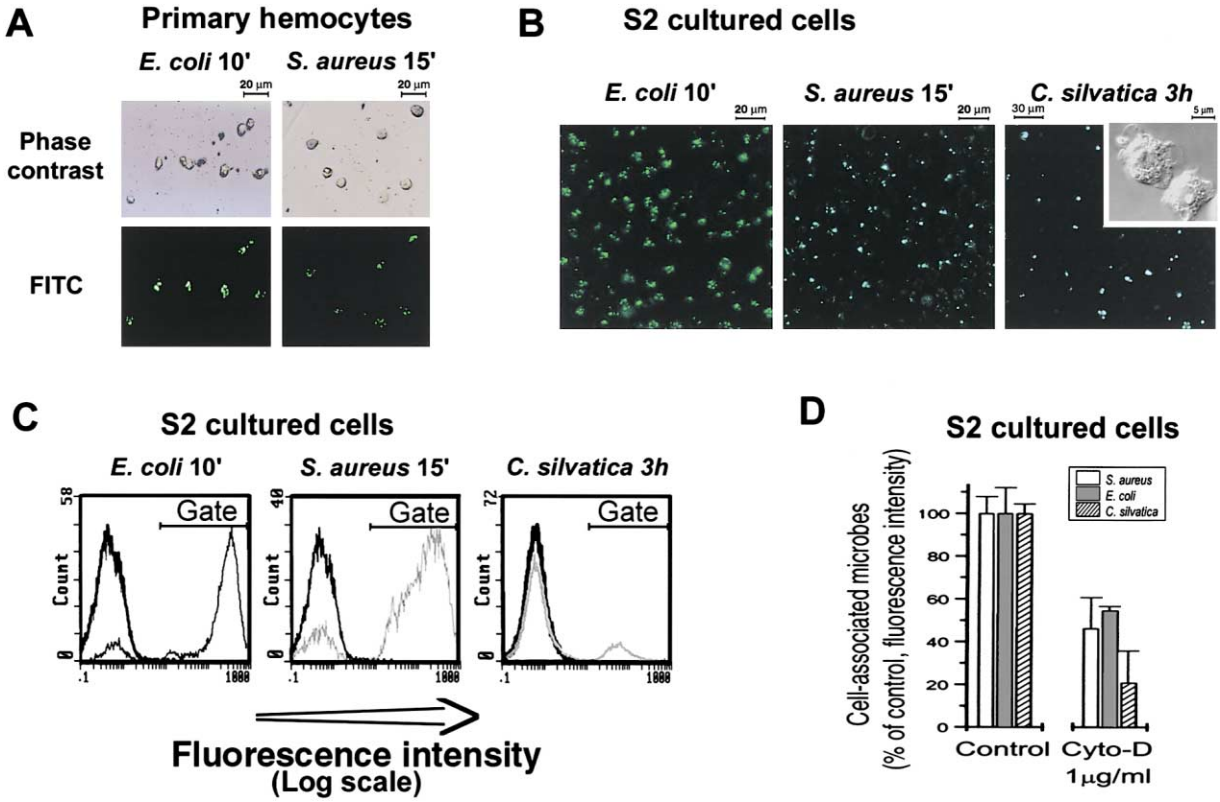

Figure 1. Phagocytosis of Microbes by S2 Cells and Primary *Drosophila* Hemocytes

(A) Phagocytosis of heat-killed, FITC-labeled bacteria by primary hemocytes from third instar larvae. Ten bacteria per cell were added to primary hemocytes for 10 min at 26°C, and the fluorescence of extracellular bacteria was quenched by the addition of Trypan blue.

(B) Phagocytosis of heat-killed, FITC-labeled *E. coli*, *S. aureus*, and *C. silvatica* by S2 cells after 10, 15, or 180 min of incubation, respectively. Fluorescence of extracellular organisms was quenched by Trypan blue. Inset shows a high-magnification phase-contrast confocal microscope image of phagocytosis of *C. silvatica*.

(C) Quantitation of cell-associated microbes by FACS. FITC-labeled microbes were added to S2 cells as in (B). Cell-associated microbes were assayed by FACS (gray lines). Black line represents background autofluorescence of S2 cells.

(D) Approximately 20%–60% of the cell-associated fluorescence is due to binding since it is not cytochalasin D inhibitable. S2 cells were pretreated for 20 min with cytochalasin D (1 μg/ml) and were then incubated in the continued presence of cytochalasin D at 26°C with labeled *E. coli*, *S. aureus*, or *C. silvatica* for 10, 15, or 180 min, respectively. The cells were fixed and analyzed by FACS. Data shown as mean ± standard error (SE).

dSR-CI was cloned, with dSR-CI expressing stably transfected CHO cells suggests that *Drosophila* is likely to have additional SRs (Pearson et al., 1995).

In this study we found that dSR-CI is a bacterial PRR that accounts for 20%–30% of the total bacterial binding activity in S2 cells. Our results suggest that SRs are primordial PRRs and that, as in mammalian systems, insects have additional as yet to be defined PRRs.

Results

S2 Cell as a Model for Studying Phagocytosis

Drosophila macrophages are capable of binding and ingesting both *E. coli* and *S. aureus* in vivo (Rizki and Rizki 1980; Franc et al., 1999). To develop an in vitro cell culture system for studying phagocytosis in *Drosophila*, we examined whether S2 cells were phagocytosis competent. The S2 cells are derived from a primary culture of late stage *Drosophila* embryos (Schneider, 1972) and express macrophage-specific markers like dSR-CI and croquemort. As a first step toward evaluating whether S2 cells have macrophage-like phagocytic properties, we compared S2 cell and primary larval hemocyte (macrophage) phagocytosis. Both primary larval hemocytes (Figure 1A) and S2 cells (Figure 1B) exhibited phagocytosis of fluorescently labeled *E. coli* and *S. aureus* within 15 min of particle addition. Similar results (data not shown) were obtained using the macrophage-like mbn-2 cell line derived from third instar larval hemocytes. The phagocytosis of the yeast *Candida silvatica* was less efficient in both S2 cells and primary larval hemocytes. After 15 min, less than 5% of the cells had internalized any *C. silvatica*. By 3 hr, about 20% of the S2 cells had internalized one to several *C. silvatica* (Figure 1B). Zymosan was internalized poorly by S2 cells.

In order to quantitatively measure the association of bacteria with S2 cells, we incubated the cells for 10 or 15 min at 26°C with FITC-labeled, heat-killed bacteria and measured the fluorescence in the cells using FACS. In this assay, greater than 90% of the S2 cells had cell-associated bacteria (Figure 1C). While this assay does not distinguish between binding and internalization of the bacteria, we found that 30%–50% of the cell-associated fluorescence appears to be the result of particle

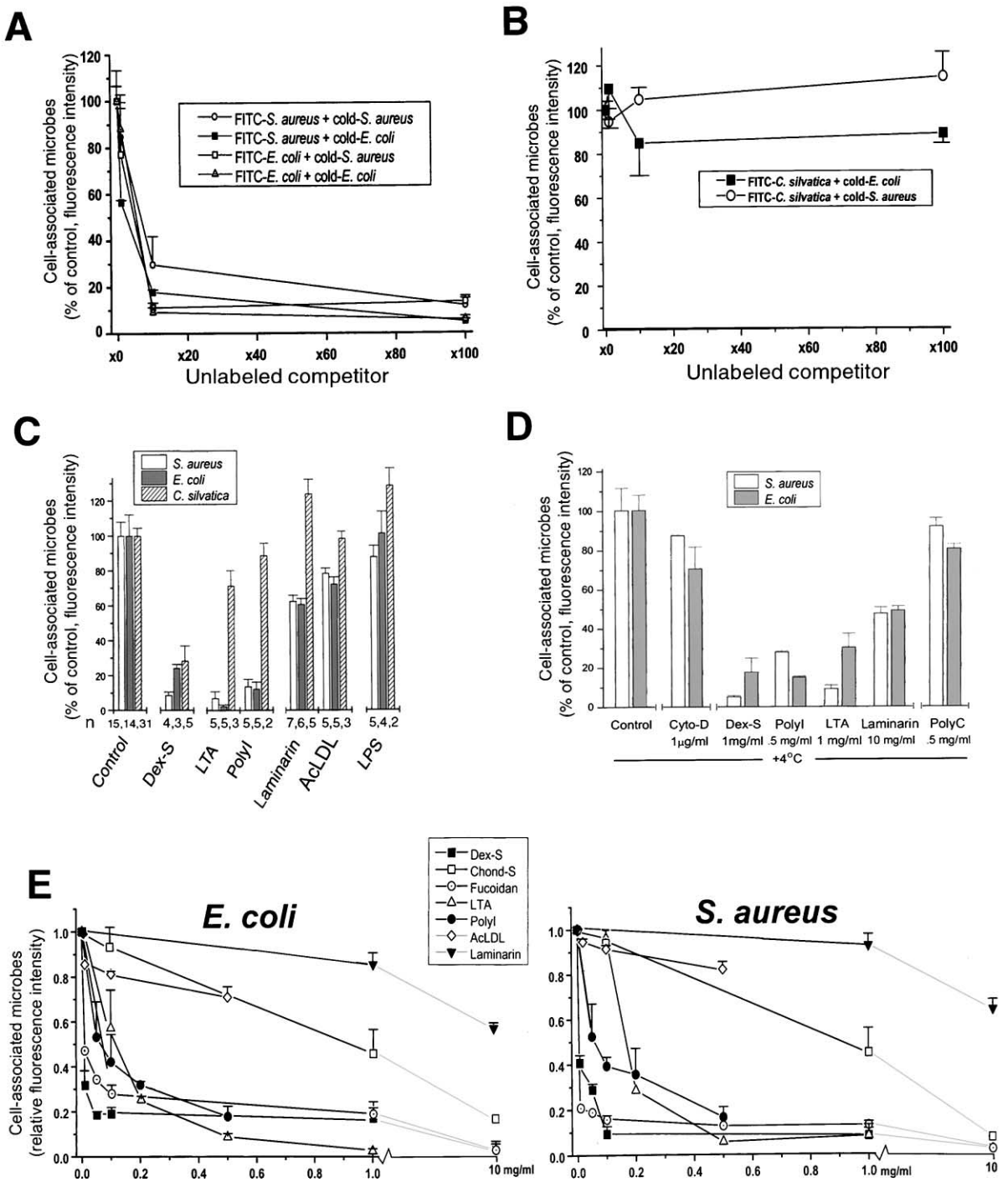

Figure 2. Evidence for Common Receptors for Gram-Positive and Gram-Negative Bacteria

(A and B) Cross-competition between *E. coli* and *S. aureus* (A) and between *C. silvatica*, *E. coli*, and *S. aureus* (B). Ten FITC-labeled microbes per S2 cell were incubated for 5 (A) or 180 (B) minutes in the presence or absence of unlabeled competitor. Cell-associated FITC-labeled microbes were quantitated by FACS. Each point represents the mean ± SE of two to four experiments.

(C) Inhibition profiles of *E. coli*, *S. aureus*, and *C. silvatica* association with S2 cells. S2 cells were incubated at 26°C with labeled *E. coli*, *S. aureus*, or *C. silvatica* in the presence or absence of the indicated inhibitors for 10, 15, or 180 min, respectively, and analyzed by FACS. The number of experiments is indicated under each column. Data shown as mean ± standard error. Dex-S 1 mg/ml; LTA 1 mg/ml; Polyl 500 μg/ml; laminarin 10 mg/ml; AcLDL 500 μg/ml; LPS 1 mg/ml.

(D) Effect of selected inhibitors on cell association of *E. coli* and *S. aureus* at 4°C. S2 cells were incubated at 4°C with labeled *E. coli* or *S. aureus* in the presence of indicated inhibitors for 10 or 15 min, respectively. At least three experiments were performed with each inhibitor.

(E) Dose response curves of the effective inhibitors. S2 cells were incubated at 26°C with FITC-*E. coli* or *S. aureus* in the presence of the indicated inhibitors for 10 or 15 min, respectively. Cell-associated microbes were quantitated using FACS. Data are shown as mean ± SE. Two to five individual experiments were done for each data point.

Table 1. Effect of Various Treatments on Cell Association of FITC-Labeled Microbes

Compound	Concentration	Yeast	Bacteria
No effect			
Asialofetuin	10 mg/ml	–	ND
Chitin	0.5 mg/ml	–	ND
Dextran	10 mg/ml	–	–
EDTA	10 mM	–	–
EGTA	20 mM	–	ND
Fructose	100 mM	–	ND
D-Fucose	100 mM	–	–
L-Fucose	100 mM	–	–
Galactose	100 mM	–	–
N-Acetyl-Galactosamine	100 mM	–	ND
β-Glucan	1.0 mg/ml	–	ND
Glucose	100 mM	–	–
N-Acetyl-Glucosamine	100 mM	–	–
HDL	0.5 mg/ml	–	–
Heparinase I	5 U/10^5 S2 cells	–	–
Lactose	100 mM	–	ND
LDL	0.5 mg/ml	–	–
LPS	1.0 mg/ml	–	–
Maltose	100 mM	–	ND
Mannose	100 mM	–	–
N-Acetyl-Mannosamine	100 mM	–	–
Polycytidylic acid (PolyC)	0.5 mg/ml	–	–
Nonselective inhibition			
Chondroitin sulphate	25 mg/ml	↓	⇊
Cytochalasin-D	10 μg/ml	⇊	⇊
Dextran sulphate	10 mg/ml	⇊	⇊
Fetuin	10 mg/ml	↓	↓
Fucoidan	10 mg/ml	⇊	⇊
Heparin	100 U/ml	↓	↓
Neuraminic acid	25 mM	⇊	↓
Selective inhibition			
Acetylated LDL	0.5 mg/ml	–	↓
Laminarin	10 mg/ml	–	↓
Lipoteichoic acid	1.0 mg/ml	–	⇊
Polyinosinic acid (Polyl)	0.5 mg/ml	–	⇊

–, cell association 75%–125% of control; ↓, 50%–75% of control; ⇊, <50% of control; ND, not determined. Bacteria represent *E. coli* and/or *S. aureus*. No significant differences were observed between gram-negative and gram-positive bacteria.

internalization as it is inhibitable by prior treatment of S2 cells with cytochalasin D (Figure 1D), an inhibitor of actin polymerization. Taken together, these results indicate that phagocytosis by S2 cells is similar to phagocytosis by primary larval hemocytes of *Drosophila* and that, as in mammalian systems, both cell types use actin polymerization-dependent phagocytic mechanisms.

Inhibition Profiles of *Drosophila* PRRs for Microbes—Evidence for Common Receptor(s) for Gram-Positive and Gram-Negative Bacteria

In order to determine whether *E. coli* and *S. aureus* were recognized by the same or distinct S2 cell PRRs, we performed cross-competition experiments. As shown in Figure 2A, unlabeled *S. aureus* competed for the cell association of FITC-labeled *S. aureus* in a dose-dependent manner. A similar result was observed for *E. coli*. Coincubation of 10 labeled bacteria with 10 unlabeled bacteria/S2 cell resulted in 20%–40% inhibition (i.e., 1× in Figure 2A); 100 unlabeled to 10 labeled bacteria results in 70%–90% inhibition (i.e., 10× in Figure 2A). *Drosophila* hemocytes and S2 cells have a lower phagocytic index compared to mammalian macrophages and hence fewer unlabeled bacteria should be required for competing out binding and ingestion of labeled bacteria. Interestingly, unlabeled *E. coli* was also able to compete for the cell association of labeled *S. aureus*, and unlabeled *S. aureus* was able to compete for labeled *E. coli*. Importantly, neither *E. coli* nor *S. aureus* were able to inhibit the cell association of FITC-*C. silvatica* (Figure 2B). These results suggest that there may be a common receptor(s) for *E. coli* and *S. aureus* and a distinct receptor(s) for *C. silvatica*.

In order to further test this hypothesis, a variety of molecules were tested for their ability to inhibit binding and phagocytosis of *E. coli*, *S. aureus*, and *C. silvatica*. Of the many potential inhibitors tested (Table 1), only a few appeared to have an effect. Dextran sulfate (Dex-S) and fucoidan inhibited the cell association of all three microorganisms (Figure 2C). In contrast, several other inhibitors were more selective. Polyinosinic acid (polyI), acetylated LDL (AcLDL), laminarin, and lipoteichoic acid (LTA) were all inhibitors of bacterial, but not yeast, association with S2 cells (Figure 2C). The effects of polyI, LTA, and laminarin are probably due primarily to competition with the bacteria for receptor binding since these compounds reduced the binding of bacteria to S2 cells at 4°C (Figure 2D). For those compounds that did not inhibit binding, we indicate the highest concentrations used (Table 1). Inhibition of bacterial binding was dose dependent for all effective inhibitors (Figure 2E). Interestingly, we observed a difference in efficacy among the bacteria-specific inhibitors of S2 cell association: whereas polyI inhibited cell association of bacteria almost completely, both laminarin and AcLDL were partial inhibitors (Figures 2C–2E). These results suggest that there is more than one bacteria-specific phagocytic receptor in *Drosophila* in addition to distinct receptor(s) for *C. silvatica*.

Although LTA is a cell wall component of gram-positive bacteria, it was also a very effective inhibitor of binding of gram-negative bacteria. This effect was specific since, in addition to having no effect on the association of yeast with S2 cells, LTA had no effect on latex bead phagocytosis or on endocytosis of FITC-BSA (data not shown). Similar results were obtained with primary larval hemocytes (on average 3.8 FITC- *E. coli* per cell were phagocytosed by untreated cells compared to 0.5 by cells treated with 1 mg/ml LTA), indicating that the inhibition was not an artifact due to the use of a cultured cell line. The ability of *S. aureus*-derived LTA to inhibit gram-positive and gram-negative bacterial binding is contrasted with the failure of *E. coli*-derived LPS to act as an inhibitor of either *S. aureus* or *E. coli* association (Figure 2C). As unlabeled whole *E. coli* were effective inhibitors, this suggests that the failure of soluble LPS to act as an inhibitor is either an in vitro artifact or that other components of the gram-negative bacterial membrane, with or without LPS, are required.

dSR-CI Is a Candidate PRR for Bacteria

The inhibitor profile of S2 cell binding and phagocytosis of bacteria is similar to the known ligand specificities of both the mammalian class A SRs and the macrophage-

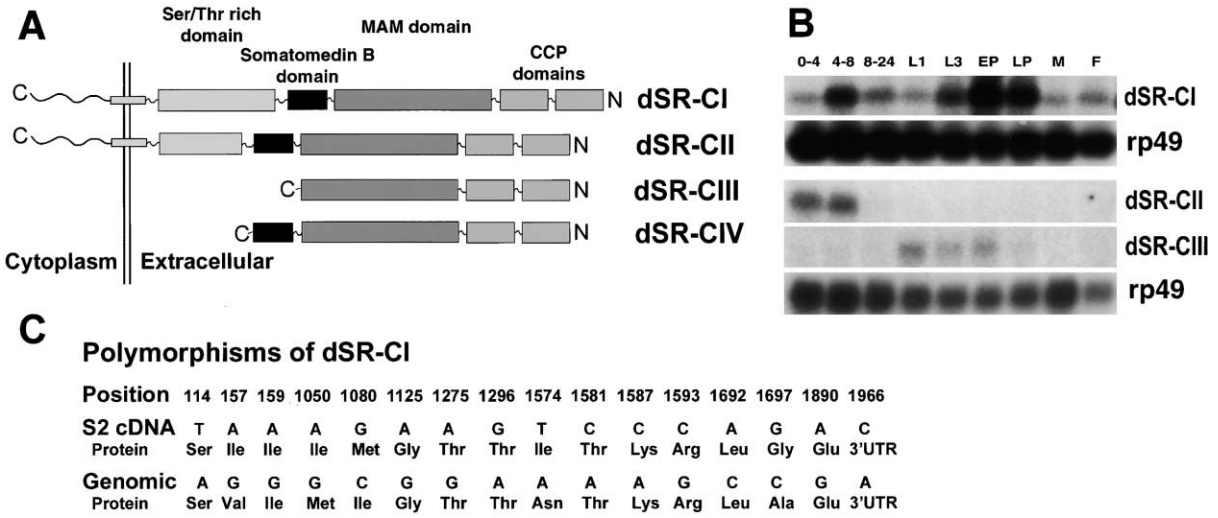

Figure 3. dSR-CI Is a Potential Candidate Receptor for Bacteria

(A) Schematic representation of SR-Cs indicating different domains. MAM, a domain found in Meprin, A5 antigen, and RPTP Mu. CCP, complement control protein domain.

(B) Developmental expression analysis of the SR-C family by Northern blot. Each dSR-C has a unique expression profile, only dSR-CI being expressed throughout development. L, larvae; EP, early pupae; LP, late pupae; M, adult male; F, adult female.

(C) Polymorphic nucleotides of dSR-CI.

specific *Drosophila* SR, dSR-CI (Pearson et al., 1995; Krieger, 1997). dSR-CI is the defining member of a small family of proteins in *Drosophila*. We identified two additional dSR-C family members by genomic and cDNA cloning, while a homology search against all known *Drosophila* genes and ESTs identified a fourth member. All four molecules (shown schematically in Figure 3A) have two N-terminal complement control protein (CCP) domains followed by a MAM domain. dSR-CI, dSR-CII, and dSR-CIV then have a somatomedin B domain and dSR-CI and dSR-CII further have Ser/Thr-rich, transmembrane, and cytoplasmic domains. dSR-CIII and dSR-CIV are predicted to encode secreted proteins.

Several experiments were performed in order to gain insight into which of the dSR-Cs might be candidate PRR for bacteria. First, conditioned media from S2 cells did not affect binding or ingestion of bacteria, suggesting that secreted proteins like dSR-CIII or dSR-CIV are unlikely to play a role in this process under our assay conditions (data not shown). Second, developmental analysis of dSR-C gene expression (Figure 3B) showed that dSR-CI is expressed throughout the life cycle of the fly from early embryogenesis to adults. Furthermore, dSR-CI exhibits hemocyte-specific expression in the embryo (Pearson et al., 1995), is expressed in the larval hemocyte-derived mbn-2 cells (Dimarcq et al., 1997), and its expression is induced at the onset of pupariation, perhaps to aid in the phagocytic clearance of cell corpses and debris generated during metamorphosis (Lanot et al., 2001). In contrast, dSR-CII is expressed only in the embryo from 2 to 8 hr after egg laying and with no apparent hemocyte expression (Figure 3B and data not shown). Interestingly, sequencing of the Oregon R genomic DNA encoding dSR-CI revealed 16 single nucleotide polymorphisms (SNPs), 5 of which are nonconservative, compared to the sequence of the cDNA derived from S2 cells (Figure 3C). These SNPs indicate a level of diversity that is sometimes associated with host defense molecules.

dSR-CI Binds Both Gram-Positive and Gram-Negative Bacteria and Is Necessary for Optimal Phagocytosis of Bacteria by S2 Cells

In order to determine if dSR-CI expression is sufficient for cells to bind *E. coli* and/or *S. aureus*, we tested the bacterial binding capability of a stably transfected CHO cell line expressing dSR-CI (CHO[dSR-CI]) (Pearson et al., 1995). CHO[dSR-CI] cells were able to bind both *E. coli* (Figure 4A) and *S. aureus* (Figure 4B) but not *C. silvatica* or latex beads (data not shown). While there was avid bacterial binding to transfected cells, there was little ingestion of the bound bacteria (data not shown). Untransfected CHO cells bound neither *E. coli* (data not shown) or *S. aureus* (Figure 4C). The uptake of labeled AcLDL is an accepted measure of SR activity in S2 cells (Pearson et al., 1995). For this reason we determined that dSR-CI CHO transfectants (Figure 4A), but not mock transfectants, internalized DiI-AcLDL, and therefore we conclude that these transfectants express functional dSR-CI. There was a positive correlation between the level of DiI-AcLDL uptake and the amount of bacterial binding in this population of CHO[dSR-CI] cells that exhibited a somewhat heterogeneous distribution of dSR-CI expression. Those cells that efficiently endocytosed AcLDL also bound bacteria (white arrows in Figures 4A and 4B), while those cells that were unable to take up AcLDL had, like the untransfected CHO cells, few or no bound bacteria (gray arrows in Figure 4A).

We also performed similar experiments on COS cells transiently transfected with the dSR-CI cDNA. Although there is significant background binding of FITC-labeled bacteria to untransfected COS cells (data not shown),

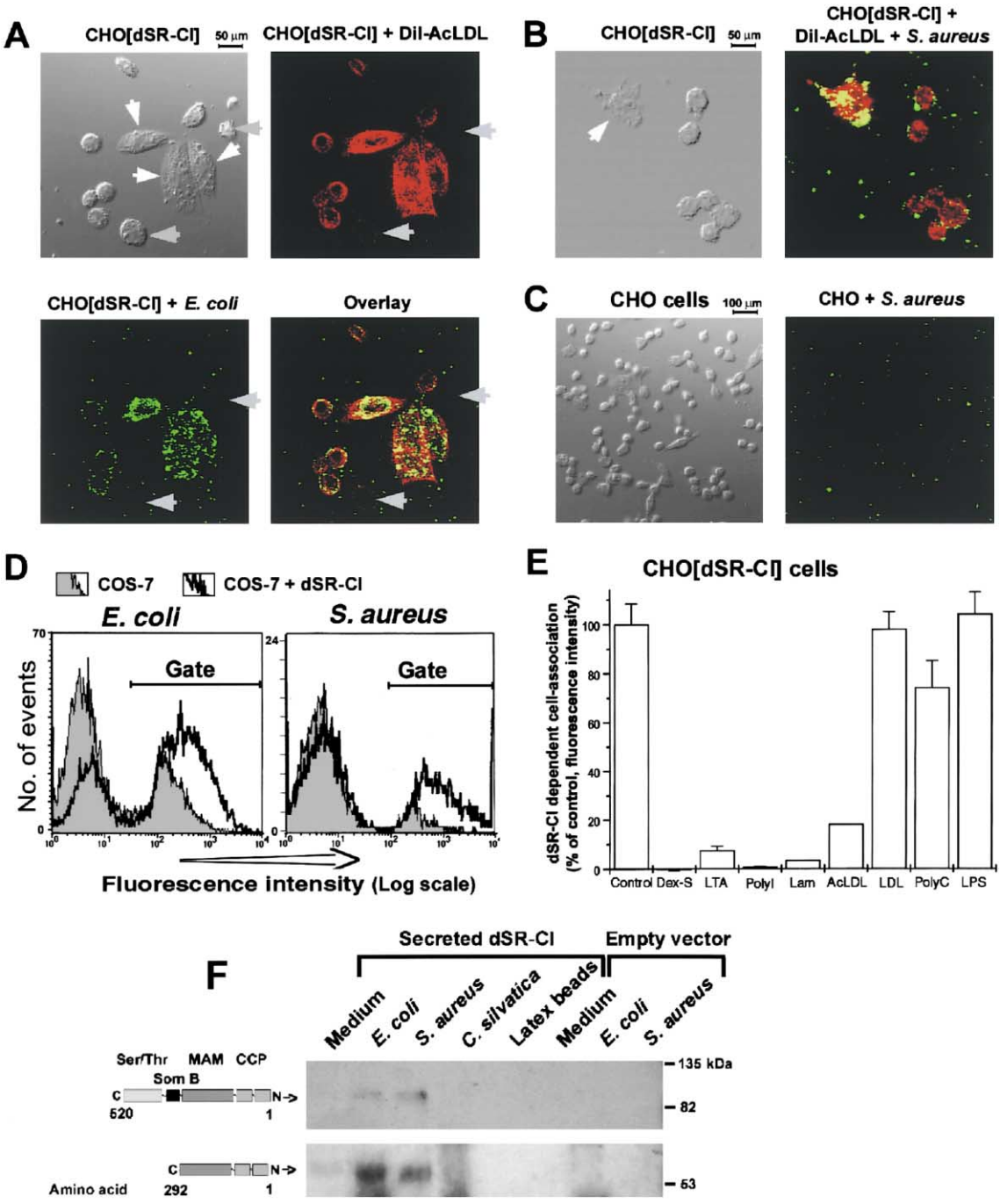

Figure 4. CHO and COS-7 Cells Transfected with dSR-CI Bind Both *S. aureus* and *E. coli*

(A and B) Confocal images of dSR-CI-expressing CHO cells (CHO[dSR-CI]) incubated with FITC-labeled *E. coli* (A) or *S. aureus* (B) for 20 min and thereafter 20 min with DiI-AcLDL (5 μg/ml). Prior to fixing, cells were washed five times with 1xPBS. (A) Top left panel, Nomarski optics. Rhodamine channel shows DiI-AcLDL in red (top right). FITC channel shows FITC-*E. coli* in green (bottom left). Overlay of Rhodamine and FITC channels shown in bottom right panel. (B) Left panel, Nomarski optics. Right panel, overlay of rhodamine channel showing DiI-AcLDL in red and FITC channel showing FITC-*S. aureus* in green.

(C) Confocal images of untransfected CHO cells incubated for 20 min with FITC-labeled *S. aureus* followed by washes as in (A). Left panel, Nomarski optics. Right panel, FITC channel showing FITC-*S. aureus* in green.

(D) FACS analysis of FITC-*E. coli* and FITC-*S. aureus* binding to mock-transfected (gray diagram) and to dSR-CI transfected (transparent diagram) COS-7 cells after 20 min incubation with FITC-labeled bacteria. Prior to analysis, cells were washed as in (A).

(E) Inhibition profile of FITC-*E. coli* binding to dSR-CI. CHO[dSR-CI] and untransfected CHO cells were incubated with FITC-labeled *E. coli* for 20 min in the presence of the indicated competitor. Cells were washed five times with 1xPBS and fixed with 1% paraformaldehyde. Cell-associated microbes were quantitated by FACS. The amount of dSR-CI-dependent cell association of FITC-*E. coli* was calculated by subtracting the background binding of parent CHO cells from total binding by CHO[dSR-CI] cells. Each column represents mean ± SE of two to four experiments. Dex-S 1 mg/ml; LTA 1 mg/ml; Polyl 500 μg/ml; Laminarin 10 mg/ml; AcLDL 500 μg/ml; LDL 500 μg/ml; Polycytidylic acid 500 μg/ml; LPS 1 mg/ml.

the results shown in Figure 4D demonstrate that dSR-CI transfectants clearly exhibited enhanced *E. coli* and *S. aureus* binding (solid line) compared to mock-transfected COS cells (shaded lines) or COS cells transfected with the croquemort cDNA (data not shown).

The inhibition profile for dSR-CI-dependent *E. coli* binding to CHO[dSR-CI] cells (Figure 4E) was compared to that observed with S2 cells. As with S2 cells, Dex-S, polyI, and LTA were effective inhibitors of *E. coli* cell association with dSR-CI, while LPS was a poor inhibitor (compare Figures 2B and 4E). Of note, laminarin (10 mg/ml) and AcLDL (500 μg/ml), which are avid ligands of dSR-CI, almost totally inhibited dSR-CI-dependent *E. coli* binding to CHO[dSR-CI] cells, whereas these compounds were only partial inhibitors of *E. coli* binding to S2 cells (compare Figures 2B and 4I). This result suggests again that additional receptors beyond dSR-CI play a role in bacterial binding to S2 cells.

To demonstrate direct binding of bacteria, and to define the binding site in more detail, we created three recombinant soluble forms of the receptor (Figure 4F). All of these soluble receptors bound *E. coli* and *S. aureus,* but not *C. silvatica* or latex beads (Figure 4F shows results of full-length secreted ectodomain and a construct encoding the MAM and CCP domains). Based on these results, we conclude that the CCP domains together with the MAM domain are sufficient to bind bacteria in vitro, whereas the Ser/Thr-rich domain and somatomedin B domain are not essential. Further domain analysis was hampered by the fact that removal of the first CCP domain yielded a protein that was trapped inside the cell. Therefore, a more directed mutagenesis is required to define the minimal effective binding domains.

To more directly explore the role of dSR-CI in S2 cell binding and phagocytosis of *E. coli* and *S. aureus*, we utilized dsRNAi-mediated gene silencing, an effective method to diminish protein expression in *Drosophila* and in S2 cells (Kennerdell and Carthew, 1998; Hammond et al., 2000). dMoesin, an abundant membrane-associated protein, was used as a control in all of the dsRNAi experiments. Using semiquantitative RT-PCR (Figure 5A), we found that dSR-CI and dMoesin transcript levels were strongly and specifically decreased 96 hr after treatment with dSR-CI or dMoesin dsRNA, respectively (Figure 5B). The decreases in mRNA expression were accompanied by corresponding decreases in protein levels as determined on Western blots (Figure 5C). When tested for their ability to bind and engulf bacteria, S2 cells treated with dSR-CI dsRNA exhibited a 20%–30% reduction in the level of *E. coli* and *S. aureus* cell association as compared to untreated cells (Figure 5D). In contrast, treatment of S2 cells with dMoesin dsRNA or sham RNA had no significant effect on bacterial cell association (Figure 5D). None of the dsRNAi treatments affected *C. silvatica* association to S2 cells (Figure 5D).

In order to determine if the dsRNA treatment eliminated all dSR-CI activity in S2 cells, we examined whether a specific dSR-CI inhibitor would enhance the effect of dsRNA treatment in S2 cells. The addition of AcLDL to dSR-CI dsRNA-treated S2 cells did not enhance the effect of dsRNA treatment alone (Figure 5E), indicating that the dsRNA treatment effectively blocked most of the dSR-CI-dependent bacterial cell association. This result lends further support to the hypothesis that the residual binding activity observed when treating cells with AcLDL is likely due to one or more PRRs distinct from dSR-CI.

dSR-CI Does Not Appear to Be Required for the Antibacterial Peptide Response Triggered by Bacteria in S2 Cells

Secretion of antimicrobial peptides as a response to microbial challenge is one of the cornerstones of innate immunity (Steiner et al., 1981; Lehrer and Ganz, 1999). While the Toll antifungal pathway is now well defined in *Drosophila* (Lemaitre et al., 1997; Imler and Hoffmann, 2000), it is not yet clear how bacteria trigger antibacterial responses. At least one genetically defined pathway, termed the immune deficiency (IMD) pathway, appears to account for most antibacterial peptide (e.g., attacin) gene induction (Imler and Hoffmann, 2000). However, none of the *Drosophila* Toll-related receptors appear to be involved in the regulation of the antibacterial genes (Tauszig et al., 2000). To determine if dSR-CI triggers the induction of antimicrobial genes in S2 cells, we used an attacin promotor-driven luciferase reporter gene (Tauszig et al., 2000), since attacin expression is controlled entirely by the IMD pathway (Imler and Hoffmann, 2000). As shown in Figure 6, a 12 hr incubation of S2 cells with *E. coli* resulted in an approximately 10-fold increase in attacin-reporter activity. Incubation with *S. aureus* had a similar effect (data not shown). dsRNA-mediated silencing of dSR-CI expression did not reduce luciferase induction, suggesting that dSR-CI is not necessary for the attacin response in S2 cells. Furthermore, transfection of S2 cells with the dSR-CI cDNA did not induce luciferase activity in the absence of bacteria, nor did it superinduce luciferase activity in response to *E. coli* (Figure 6) or *S. aureus* (data not shown). These results indicate that dSR-CI is not necessary for attacin gene induction in S2 cells.

Discussion

The use of genetically tractable model systems like *Drosophila* has proven invaluable in defining necessary genes and pathways that play a role in innate host defense. However, unlike mammalian systems, there is a paucity of well-characterized *Drosophila* cell lines. Of the *Drosophil*a cell lines, the embryo-derived S2 cell line

(F) Secreted dSR-CI constructs bind *E. coli* and *S. aureus* but not *C. silvatica* or latex beads. Two different secreted forms of dSR-CI (as shown schematically in the figure) were expressed in COS-7 cells for 36 hr, and cell culture medium (containing 0.1% serum) was collected for in vitro binding assay; 100 μg of *E. coli*, *S. aureus*, *C. silvatica*, or latex beads were added in 400 μl of the medium and incubated 1 hr at 4°C with mild agitation. Thereafter the mixture was centrifuged and the pellet was washed five times with 1 ml of 1xPBS. dSR-CI protein bound to the pellet was detached by adding 50 μl of SDS-PAGE loading buffer, boiled for 10 min, and visualized by Western blot using dSR-CI antibody against the MAM domain. First lane of each construct, 20 μl of the medium. Following lanes, 400 μl of medium incubated for 1 hr with 100 μg of indicated ligand.

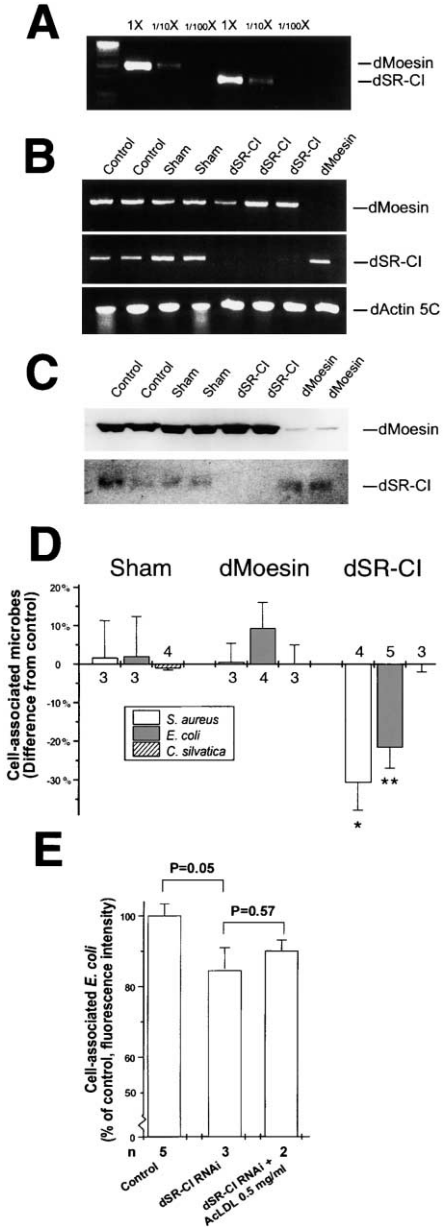

Figure 5. dSR-CI Is Necessary for Optimal Binding/Phagocytosis of Bacteria

(A) Semiquantitative RT-PCR to estimate mRNA levels of dMoesin and dSR-CI. Lane 1, size standards. Lanes 2–4, dMoesin RT-PCR using 1× (lane 2), $/_{10}$× (lane 3), or $/_{100}$× (lane 4) dilutions of S2 cell lysate as a template. S2 cell lysate was diluted to cell lysate (CHO) that did not contain dMoesin or dSR-CI mRNA. Lanes 5–7, corresponding experiment for dSR-CI. Lane 8, dSR-CI and dMoesin PCR, no template.

(B and C) dsRNAi-mediated gene silencing specifically decreases both RNA (B) and protein (C) levels of the target protein. Representative dMoesin, dSR-CI, and dActin RT-PCR shown in (B) and dSR-CI and dMoesin Western blot in (C). 10 μg of dsRNA was added to 1.0×10^6 cells, and the cells cultured for 96 hr. Equivalent amounts of total protein were loaded to each lane based on OD_{545}. Immunodetection of dMoesin and dSR-CI was performed on the same blot.

(D) FACS quantitation of microbe S2 cell association in the presence or absence of dsRNA treatment. 1.0×10^6 cells were incubated for 96 hr with 10 μg of dsRNA and subsequently for 5 min with FITC-labeled *E. coli,* for 10 min with FITC-*S. aureus*, or for 2 hr with FITC-*C. silvatica* and analyzed by FACS. Twenty-five bacteria or ten *C. silvatica* were used per cell. Number of experiments is shown below each column. Each of these experiments consists of two to four independent assays. Error bars represent SE. Sham, RNA of non-*Drosophila* origin. *, $p < 0.05$; **, $p < 0.01$.

(E) The effect of AcLDL (500 μg/ml) and dSR-CI RNAi on association of FITC-labeled *E. coli* with S2 cells. Each column represents the mean of two to four experiment ± SE.

is the most widely used. Several markers that define *Drosophil*a macrophages in vivo, including peroxidasin, croquemort, and dSR-CI, are expressed in S2 cells. We have found that S2 cells are similar to primary larval macrophages in their ability to phagocytose *C. silvatica*, *E. coli*, and *S. aureus*. S2 cell phagocytosis of bacteria is rapid and, like mammalian macrophage phagocytosis, is cytochalasin sensitive. These results provide strong support that S2 cells should be considered macrophage-like and that they represent a valid in vitro model system to study phagocytosis in *Drosophila*. This will likely complement and inform more traditional in vivo genetic studies.

Using S2 cells we have identified the *Drosophila* SR, dSR-CI, as a PRR for *E. coli* and *S. aureus*. This conclusion is based on several findings. First, cross-competition studies indicate that S2 cells are likely to have a common receptor(s) for gram-positive and gram-negative bacteria. Second, inhibition profiles of cell association of both *E. coli* and *S. aureus* were similar to each other and to the known ligand specificities of both the mammalian type A SRs (Hampton et al., 1991; Ashkenas et al., 1993; Fraser et al., 1993; Dunne et al., 1994) and dSR-CI (Pearson et al., 1995). Third, mammalian CHO and COS cells transfected with dSR-CI as well as secreted dSR-CI constructs selectively bind *E. coli* and *S. aureus*. Finally, association of both *E. coli* and *S. aureus* with S2 cells was reduced by 25% when expression of dSR-CI was abolished by RNAi treatment. Therefore, dSR-CI appears necessary for optimal phagocytosis/binding of both gram-negative and gram-positive bacteria by S2 cells.

Comparisons between CHO[dSR-CI] and S2 cells in our initial study (Pearson et al., 1995) suggested that S2 cells might have multiple SRs. Our current results are reminiscent of these findings in that almost all binding of *E. coli* to CHO[dSR-CI] was inhibited by AcLDL and laminarin, whereas these compounds had only partial inhibitory effect on association of bacteria to S2 cells. Furthermore, dSR-CI RNAi-treated S2 cells had a 25% reduction in cell association of bacteria, and this could not be further inhibited by addition of AcLDL. This argues that there are other bacterial PRRs in S2 cells. In addition, *C. silvatica* appears to be recognized differently from bacteria based on the distinct inhibition profile and cross-competition results.

A common feature of the expanding family of SRs is their ability to bind a broad range of polyanionic ligands. Mammalian SR-AI and SR-AII recognize *E. coli*-derived bacterial LPS and its bioactive precursor, lipid IV_A, as well as *S. aureus* and its outer cell wall component lipoteichoic acid (Hampton et al., 1991; Ashkenas et al., 1993; Dunne et al., 1994). It is of interest that different bacterial strains of *E. coli* are recognized differently by

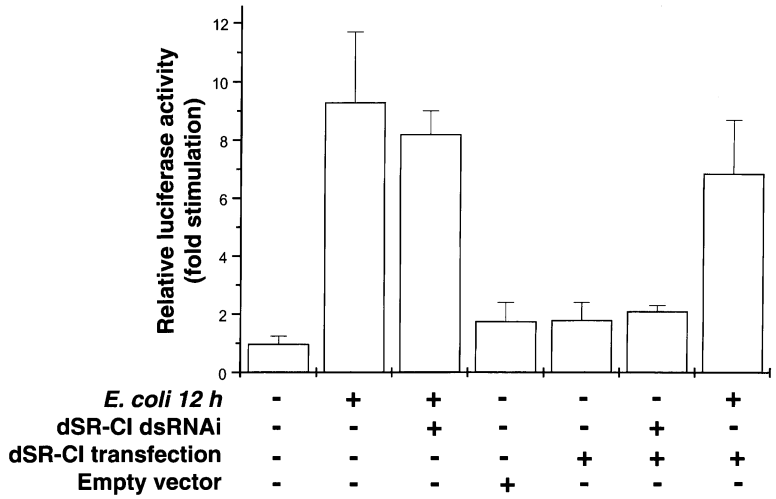

Figure 6. dSR-CI Is Not Required for *E. coli*-Induced Attacin Expression in S2 Cells

S2 cells were incubated for 12 hr with 1.0×10^7 *E. coli*. Attacin reporter activity was quantitated using a luciferase assay as described in Experimental Procedures. dSR-CI transfection was performed 36 hr prior to addition of *E. coli*. dSR-CI dsRNAi treatment was performed as described in Figure 5. Each experiment was repeated twice using both *E. coli* and *S. aureus* as elicitors with similar results. In each experiment, two to four independent assays were done for each condition. A representative experiment is shown. Error bars represent SE.

SR-A and that gram-negative bacteria express other—yet incompletely characterized—SR ligands (Peiser et al., 2000). Microbial binding activity may indeed reflect physiologically relevant roles of SR-AI and SR-AII in innate host defense as SR-$A^{-/-}$ mice are more susceptible to infection with *Listeria monocytogenes, S. aureus*, and *Herpes simplex virus type1* compared to their wild-type littermates (Suzuki et al., 1997; Thomas et al., 2000). The precise role of SR-AI and SR-AII in the control of inflammatory response is currently unclear. (Haworth et al., 1997; Kobayashi et al., 2000). A third class A SR, macrophage receptor with collagenous structure (MARCO), has also been found to bind *E. coli* and *S. aureus* (Elomaa et al., 1995). The other class of mammalian bacteria binding SRs is represented by LOX-1, which is expressed on vascular endothelial cells and was recently shown to support adhesion of *E. coli* and *S. aureus* (Shimaoka et al., 2001). In spite of similar ligand binding properties, the SR-As and LOX-1 are structurally distinct. The class A SRs are trimeric type II membrane proteins that bind their ligands via their collagen-like domains (Kodoma et al., 1990; Rohrer et al., 1990; Acton et al., 1993; Doi et al., 1993; Elomaa et al., 1995), while LOX-1 has a C-type lectin-like structure (Shimaoka et al., 2001). Based on the results shown in this study, dSR-CI can be now added to SRs with bacteria binding properties. Again, dSR-CI bears no structural homology to mammalian SRs. Our data show that dSR-CI is not required for antibacterial peptide (attacin) response triggered by *E. coli* or *S. aureus*. Additional studies will be required to determine if dSR-CI is required for the induction of host defense responses initiated by other elicitors.

In this study we demonstrated that there are three additional members in the *Drosophila* class C SR family. dSR-CII is predicted to be a transmembrane protein, whereas dSR-CIII and dSR-CIV appear to be secreted. dSR-CI is expressed throughout the life cycle of the fly, while dSR-CII expression was detected only in very early embryos, making it unlikely that dSR-CII would have any significant role in innate immunity at later stages of *Drosophila* development. In the course of this study, we sequenced genomic DNA encoding dSR-CI and identified 16 SNPs compared to the sequence of the cDNA derived from S2 cells. Even more extensive polymorphisms have been observed in dSR-CI in wild populations of *Drosophila melanogaster* and *Drosophila simulans* (T. Schlenke and D. Begun, personal communication). These variations are consistent with dSR-CI playing a role in pattern recognition in host defense because high-frequency polymorphism would be expected to confer some individual variation in capacity to recognize pathogens, a property well established with major histocompatibility loci in the adaptive immune system. Interestingly, significant variations in the coding sequences in the SR-AI/II gene have also been reported in mice (Fortin et al., 2000; Daugherty et al., 2000). Although the functional significance of these changes is not yet clear, it is interesting to speculate that, like variants in other host defense molecules such as the mannose binding lectin, the surfactant apoproteins, and human Toll receptor, these may all generate subtle differences in the host response to infection (Turner and Hamvas, 2000; Rämet et al., 2001; Arbour et al., 2000).

Our thesis is that pattern recognition is a cornerstone of innate immunity and that this concept has been conserved from the most primitive multicellular organisms to humans. Accordingly, it appears that polyanionic ligands represent one basic pattern and hence that SRs having a broad range of polyanionic ligand binding specificity have been maintained throughout evolution. Our in vitro results indicate that SRs appear to be involved in recognition of bacteria in invertebrates, providing a further example of functional conservation of innate immunity from insects to mammals. We therefore postulate that SRs represent primordial pattern recognition molecules and that they may mediate evolutionarily conserved innate immunity linked functions.

Experimental Procedures

Cell Culture and Isolation of Primary Hemocytes

Schneider S2 cells (S2 cells) and mammalian CHO and CHO[dSR-CI] (clone 2.6a) cells were maintained as described earlier (Pearson et al., 1995). COS-7 cells were maintained in Dulbecco's modified Eagle's medium with 4 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, and 1.0 mM sodium pyruvate and 10%

FCS. For isolation of primary hemocytes, third instar larvae were washed briefly in 5% sodium hypochlorite and thereafter twice in water. Hemocytes were obtained from groups of four larvae opened with forceps into 300 μl Shields and Sang M3 insect medium (Sigma, St Louis, MO) in 96-well ultralow-adhesion plates (Costar).

Fluorescence Microscopy and Determining Phagocytic Index

Approximately 10 heat-killed, FITC-labeled microbes (Molecular Probes, Eugene, OR) per cell (unless stated otherwise) were added to cells in serum-free medium in tissue culture plates, which were then centrifuged 2 min at 100 × g and incubated for the indicated time at 26°C. The fluorescence of extracellular particles was quenched by replacing the medium with 0.2% Trypan blue in 1xPBS (pH 5.5), and the cells were analyzed by fluorescence microscopy. The phagocytic index is determined as the number of particles giving bright fluorescence per cell.

Confocal Microscopy

A Leica confocal laser scanning microscope consisting of a Leica TCS 4D scanner attached to a Leitz DMBR/E microscope was operated using the TCS-NT software.

FACS Analysis to Quantitate Cell-Associated Microbes

S2 cells were incubated for the indicated time with FITC-labeled, heat-killed *E. coli*, *S. aureus*, or *C. silvatica* in Schneider's medium without serum or antibiotics, then fixed with 1% paraformaldehyde in 1xPBS, and subsequently analyzed with FACS using the CELLQuest program (Becton Dickinson) or System II (Coulter). S2 cells showing increased fluorescence were gated as shown in Figure 1C. The level of microbe cell association was quantitated as the percentage of fluorescence positive cells multiplied by the mean fluorescence of these cells, and 3,000–10,000 cells were counted from each sample. All inhibitors were added 20 min prior to the addition of FITC-labeled ligand. All compounds tested for an inhibitory effect on microbe cell association were purchased from Sigma unless stated otherwise. LDL and HDL were isolated as previously described (Hannuksela et al., 1996). Fluorescent 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate-labeled AcLDL (DiI-AcLDL) was produced as previously described (Gu et al., 2000).

Northern Blotting and RT-PCR Analysis

Total RNA was isolated with Trizol reagent (GIBCO BRL, Gaithersburg, MD) and analyzed by Northern blot following standard procedures. First-strand cDNA was synthesized from 1.0 μg of total RNA using Omniscript Reverse Transcriptase (Qiagen, Stanford, CA). The primers used for RT-PCR analysis correspond to nucleotides 211–240 and 1176–1204 of the dSR-CI cDNA (GenBank accession number U17693), nucleotides 137–166 and 1865–1892 of the dMoesin cDNA (GenBank accession number L38909), and nucleotides 161,755–161,774 and 162,874–162,893 of genomic clone AE003452 containing Actin 5C. To distinguish mRNA-derived PCR products from products generated from any residual genomic DNA contamination of the RNA preparations, all primers were chosen to span introns.

Transfections

Transfections of COS cells were performed using Superfect transfection reagent (Qiagen) according to the manufacturer's instructions. The DES expression vector (Invitrogen, Carlsbad, CA) was used to overexpress dSR-CI in S2 cells. Deletion mutants of the dSR-CI gene containing C-terminal *myc*-tag were cloned into pcDNA1Amp expression vector (Invitrogen) using standard methodology.

RNAi

First-strand cDNA was synthesized from 1.0 μg of total RNAas described above. The templates for dsRNA synthesis were generated from cDNA by a two-step PCR reaction. The first primers consisted of nucleotides 211–240 and 1176–1204 of the dSR-CI cDNA or nucleotides 137–166 and 1865–1892 of the dMoesin cDNA. A second PCR reaction was performed using nested primers containing a T7 promoter sequence on its 5′-end (GAATTAATACGACTCACTATAG GGAGA) attached to gene-specific sequences: nucleotides 350–379 and 1060–1089 for dSR-CI and nucleotides 418–447 and 1094–1119 for dMoesin. Both sense and antisense RNAs were synthesized simultaneously from a single PCR product using the T7 MegaScript RNA polymerase (Ambion, Austin, TX). DNAase-treated dsRNA was analyzed by agarose gel electrophoresis. The concentration of dsRNA was 10 μg per 10^6 S2 cells in each experiment. Cells were incubated with dsRNA under serum-free conditions for 30 min before adding FCS.

Antibody Production and Western Blotting

A synthetic peptide containing amino acids RRPWKRVSTVSDIHSLR TGPRHC of the MAM domain of dSR-CI was coupled to KLH as previously described (Kozarsky et al., 1986) and used to generate a rabbit polyclonal antiserum that specifically recognizes the dSR-CI protein (data not shown). A mouse polyclonal dMoesin antibody was a generous gift from Dr R. Fehon (Department of Botany and Zoology, Duke University, Durham, NC), and was used at 1:10,000 concentration. Western blot detection was conducted using the Renaissance chemiluminescence reagent (Life Science, Boston, MA).

Luciferase Reporter Assay

Attacin-reporter activity was measured essentially as described earlier (Tauszig et al. 2000). In brief, 1.0 × 10^6 S2 cells were transfected with 1.0 μg of attacin-reporter plasmid and β-galactosidase expression vector (Tauszig et al., 2000) and with 1.0 μg of the experimental expression plasmid using Superfect (Qiagen). When dsRNAi was used, 15 μg of dsRNA was added 6 hr after the initial transfection. After 48 hr, cells were treated as indicated, washed with 1xPBS, and lyzed in reporter lysis buffer (Promega). Luciferase and β-galactosidase activities were measured using Promega Luciferase assay system and Tropix Galacton-Plus substrate, respectively. β-galactosidase activity was used to normalize transfection efficiency.

Statistical Analysis

Data were analyzed using one-way ANOVA. $p < 0.05$ was considered to be significant.

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