

# Deletion of scavenger receptor A gene in mice resulted in protection from septic shock and modulation of TLR4 signaling in isolated peritoneal macrophages

Robert Drummond<sup>1</sup>, David M Cauvi<sup>2</sup>, Dennis Hawisher<sup>2</sup>, Donghuan Song<sup>2</sup>, Diego F Niño<sup>3</sup>, Raul Coimbra<sup>2</sup>, Stephen Bickler<sup>2,4</sup> and Antonio De Maio<sup>2,4</sup>

Innate Immunity  
19(1) 30–41  
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DOI: 10.1177/1753425912449548  
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## Abstract

Scavenger receptor A (Sra), also known as macrophage scavenger receptor I (MsrI), is a surface glycoprotein preferentially present in macrophages that plays a primary role in innate immunity. Previous studies have shown that Sra is a modifier gene for the response to bacterial LPS in mice at the level of IL-10 production, in particular. In the present study, we found that *Sra*( $-/-$ ) mice are more resistant to septic shock induced by cecal ligation and puncture than wild-type C57BL/6J (B6) mice. In addition, *Sra*( $-/-$ ) mice displayed initial elevated high density lipoprotein (HDL) circulating levels. Naïve peritoneal macrophages (PM $\phi$ s) were isolated from *Sra*( $-/-$ ) mice to understand the possible protective mechanism. Incubation of these cells with LPS was found to modulate TLR4 signaling, leading to a reduction in IL-10 and IL-6 mRNA levels, but not TNF- $\alpha$  expression, at low concentrations of LPS in comparison with PM $\phi$ s isolated from B6 mice. No differences were found in LPS binding between PM $\phi$ s derived from *Sra*( $-/-$ ) or B6 mice. The lack of Sra binding to LPS was confirmed after transfection of Chinese hamster ovary (CHO) cells with the *Sra* gene. The contribution of Sra to the outcome of sepsis may be a combination of changes in TLR4 signaling pathway and elevated levels of HDL in circulation, but also LPS toxicity.

## Keywords

Cholesterol, cytokines, HDL, inflammation, lipopolysaccharide, macrophage, sepsis, toll-like receptor

Date received: 13 January 2012; revised: 7 March 2012; accepted: 16 April 2012

## Introduction

The innate immune system is the first line of defense against invading pathogens, which is orchestrated by a network of various cell-types, including macrophages (M $\phi$ s), dendritic cells (DCs), polymorphic nuclear leukocytes (PMNL), and monocytes. These cells express an array of molecules involved in the recognition of pathogens or their products that distinguish specific molecular features common to these microorganisms, broadening their scope and reaction capacity. These molecules have been coined pattern recognition receptors (PRRs) and their ligands coined pathogen associated molecule patterns (PAMPs). The recognition of PAMPs by PRRs triggers a variety of responses directed at destroying, neutralizing, and clearing pathogens in order to resolve the infection.<sup>1</sup>

It has become clear that PRRs are also involved in the response to injury, and the clearance of apoptotic cells and cellular debris secondary to tissue necrosis.

<sup>1</sup>MSTP program Johns Hopkins University, School of Medicine, Baltimore, MD, USA

<sup>2</sup>Department of Surgery, University of California San Diego, La Jolla, CA, USA

<sup>3</sup>Biomedical Sciences Graduate Program, University of California San Diego, La Jolla, CA, USA

<sup>4</sup>Center for Investigations of Health and Educational Disparities, University of California San Diego, La Jolla, CA, USA

## Corresponding author:

Antonio De Maio, University of California San Diego, School of Medicine, 9500 Gilman Drive, #0739, La Jolla, CA 92093-0739, USA.  
Email: ademai@ucsd.edu

Therefore, PRRs seem to play a wide role in many pathological conditions. PRRs can be localized in the extracellular environment as soluble molecules (e.g. mannan binding lectin), associated with the plasma membrane (e.g. scavenger receptors, C-type lectins) or combined between the cell surface or subcellular compartment, as is the case with the TLR family.<sup>1</sup>

Scavenger receptors, as parts of PRRs, comprise a large family of cell surface molecules with the common characteristic of recognizing complex macromolecules. They are classified into two subgroups: one that binds modified lipoproteins and polyanionic ligands (class A) and another that recognizes Ig-like domains (class B). They have also been grouped according to their molecular structure, such as by the presence of collagenous domains that characterize scavenger receptor A (Sra) and macrophage receptor with collagenous domain (MARCO). Sra, also known as macrophage scavenger receptor 1 (*Msr1*) or CD204, is a cell surface glycoprotein primarily expressed on myeloid cells.<sup>2–4</sup> Sra is present in three isoform products of alternative splicing, all containing exons 1–8, but differing in the presence of exons 10 and 11 (SraI), exons 9 (SraII), and 10 (SraIII). SraI and II have been detected in cells, whereas SraIII is of questionable expression.<sup>5</sup> The typical collagenous domain, which is considered the binding site for multiple ligands, is present in all isoforms, as well as the coiled-coil region that is responsible for trimerization, whereas a cystein-rich domain is only present in SraI.<sup>2,4</sup> These receptors are type II glycoproteins with a very short cytosolic tail at the N-terminus of the molecule. Sra is expressed in Mφs, but not in PMNL or monocytes.<sup>3,4</sup>

Sra was initially discovered as a binding protein for modified lipoproteins [e.g. acetylated low density lipoprotein (LDL), oxidized LDL]. It is particularly present in foam cells and its role in arteriosclerosis has been well recognized.<sup>3,4,6,7</sup> Sra has also been implicated in the development of Mφ apoptosis under endoplasmic reticulum stress.<sup>8,9</sup> Sra is also up-regulated during Mφ differentiation.<sup>10</sup> It plays a significant role in phagocytosis of bacteria<sup>11–14</sup> and apoptotic cells.<sup>15</sup> Likewise, Sra is involved in cell adhesion.<sup>16,17</sup> Inbred mouse strains lacking *Sra* (*Msr1*) showed increased susceptibility to *Listeria monocytogenes* and herpes simplex virus,<sup>18</sup> malaria,<sup>19</sup> and *Neisseria meningitidis*.<sup>13,20</sup> Similarly, *Sra*(–/–) mice were reported to be more susceptible to LPS if they were primed by injection of bacillus Calmette-Guerin<sup>21</sup> or in absence of priming.<sup>22–24</sup> In contrast, other studies have shown that *Sra*(–/–) mice are protected from a LPS challenge.<sup>25</sup>

We identified *Sra* (*Msr1*) as a candidate gene in genetic screening for phenotypic differences in the inflammatory response between mouse strains, which could be used as a potential predictor of the incidence of sepsis.<sup>26</sup> Sepsis is a serious health problem with an incidence of 750,000 cases per year in the USA, a mortality rate of

30–50%, and an annual healthcare cost of more than US\$17 billion.<sup>27</sup> *Sra* (*Msr1*) was recognized based on differences in LPS-induced plasma levels of IL-10 between C57BL/6J (B6) and A/J mice.<sup>28</sup> Polymorphisms in this gene, which have been previously described,<sup>29</sup> were correlated with differences in LPS-induced IL-10 expression levels in various mouse strains.<sup>28</sup> We found that LPS-induced IL-10 levels were depressed in *Sra*(–/–) mice as opposed to wild-type B6 mice.<sup>28</sup> We extended our prior studies to further investigate the role of Sra in the inflammatory process during sepsis. In this study, we observed that Sra is a modifier of the TLR4 signal transduction pathway, which was independent of direct LPS binding to the scavenger receptor. Moreover, we found that *Sra*(–/–) mice were more resistant to septic shock, which is correlated to initial elevated high density lipoprotein (HDL) levels in the transgenic mouse.

## Materials and methods

### Mice

*Sra*(–/–) mice, which were backcrossed over seven generations to B6 mice,<sup>30</sup> were originally obtained as a gift from Dr Mason Freeman. These animals were bred in our colony at University of California San Diego and were certified to be *Helicobacter*-free. In addition, a colony of B6 mice was maintained in the same environment as *Sra*(–/–) mice and used as a wild-type control. Both strains of mice were fed with the same regular chow diet. All procedures were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health, and were approved by the Institutional Animal Care and Use Committee of the University of California, San Diego School of Medicine.

### Experimental animal models

Male mice (8–9-wks-old) were fasted for 16 h before any intervention, which was always performed during the morning between 10:00 and 11:30. Male mice were anesthetized with isoflurane via a vaporizer at 1.5–2.5 minimum alveolar concentrations (MAC). Under sterile conditions, a 2-cm incision was made in the lower abdominal region and the cecum was exposed. The distal portion of the cecum was ligated 1.5 cm from the end with a 4-0 silk suture and punctured once with either an 18- or 16-gauge needle. The cecum was replaced in the peritoneal cavity and squeezed to place a small portion of its content (bacteria and feces) into the peritoneum. Then, the peritoneal wall and skin were closed with double sutures. Mice were resuscitated with a 1-ml subcutaneous injection of sterile saline (0.9%). After the procedure, mice had access to water and food *ad libitum*.<sup>31,32</sup>

### Isolation of peritoneal M $\phi$

Resident naïve peritoneal M $\phi$ s (PM $\phi$ s) were isolated by peritoneal cavity lavage as described previously.<sup>33</sup> Male mice (8–9-wks-old) were fasted for 16 h before the procedure and euthanized under anesthesia with isoflurane via a vaporizer at 1.5–2.5 MAC during the morning. Under sterile conditions, a 16-gauge catheter was inserted into the peritoneum. Cold RPMI 1640 medium supplemented with 1% heparin (20 U/ml), 1% penicillin (100 U/ml), and 1% streptomycin (100 U/ml) was introduced (5 ml) via the catheter into the peritoneum. The abdomen was gently massaged and the lavage was withdrawn through the catheter. The peritoneal lavage was centrifuged at 800 rpm (115  $\times$  g) for 10 min. The pellet containing the cells was suspended in 1 ml of RPMI 1640 medium supplemented with 10% FBS, 1% penicillin, and 1% streptomycin (approximately  $1 \times 10^6$  PM $\phi$ /mouse). Cell suspension was seeded on a 12-well plate at a concentration of  $5 \times 10^5$  cells per well. Cells were allowed to attach for 1 h at 37°C in a CO<sub>2</sub> incubator. Non-adherent cells were removed and fresh medium was added. Cells were incubated for an additional 16 h and then stimulated (or not) with LPS (1 or 100 ng/ml *Escherichia coli* 026:B6; Sigma-Aldrich, St Louis, MO, USA) in RPMI 1640 medium supplemented with 10% FBS for 3 h at 37°C. Cellular viability was determined by the trypan-blue exclusion test (90% viability was considered acceptable). Purity of the cell population was determined by staining with F4/80 Ab. A 90–95% population of M $\phi$  was considered acceptable. PM $\phi$ s isolated by this procedure did not appear to be activated by the lack of cytokine gene expression in absence of LPS stimulation.

### Cloning of *SraI* and *II* and cell transfection

*SraI* and *SraII* were cloned from a cDNA library prepared using PolyA selected RNA obtained from PM $\phi$  isolated from A/J or B6 mice. *Sra* genes were cloned by PCR using platinum pfx polymerase (Invitrogen, Carlsbad, CA, USA) and the following primers: 5' UTR (CGAGGA TCCGTAAAGAGAGGGAAGTGGA) common to both *SraI* and *II*; and specific 3' UTR of *SraI* (GTAGCTCGAGCATTATGAAGTACAAGTGACC), and 3' *SraII* (CATCTCGAGGTTATACTGATCTTG ATCC) corresponding to exons 11 and 9, respectively. Restriction sites for BamHI and XhoI were added to the primers for subsequent cloning. *Sra-I* and *II* genes were finally cloned into pCMVscript (Stratagene, Santa Clara, CA) and their identity confirmed by sequencing. Chinese hamster ovary (CHO)-K1 cells were maintained in Ham's F12K media supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C in the presence of 5% CO<sub>2</sub>. CHO-K1 cells ( $5 \times 10^5$  cells) were transfected with *Sra* gene in pCMVscript vector (2  $\mu$ g of DNA) using fugegene reagent (2:1 reagent/DNA ratio; Promega, Madison, WI).

### Immunostaining

Transfected cells were analyzed for *Sra* expression by fluorescent microscopy using a rabbit polyclonal Ab against all *Sra* isoforms.<sup>28</sup> CHO-K1 cells were incubated with 10% fetal sheep serum (FSS) in PBS for 30 min at 25°C to block non-specific binding. Cells were incubated with rabbit anti-*Sra* Ab (1/200) in PBS-5% FSS for 1 h at 25°C. Cells were washed three times with PBS and incubated with AlexaFluor 488-conjugated anti-Rabbit Ab in PBS-5% FSS for 40 min at 25°C. Cells were then washed  $3 \times$  w/PBS, fixed with 4% paraformaldehyde for 10 min, DAPI stained, then washed again  $3 \times$  w/PBS. Alternatively, cells were incubated with AlexaFluor488-conjugated-Ac-LDL (1/50) or LPS-Biotin (visualized by AlexaFluor 594-conjugated streptavidin) following the same protocol as described above.

### Measurement of mRNA levels by quantitative real time PCR

PM $\phi$ s were washed twice with PBS and homogenized in TRIzol reagent (Invitrogen) and total RNA was isolated, as recommended by the manufacturer. Total RNA was treated with DNase I (DNA-free kit, Ambion, Austin, TX, USA) and reverse transcribed to cDNA using the High Capacity Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Newly synthesized cDNA was stored at –20°C. Samples of cDNA were amplified by real time (RT) PCR in a 7500 Fast Real-Time PCR System (Applied Biosystems) using QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA, USA) with the following QuantiTect validated primer sets (Qiagen): TNF- $\alpha$  (QT00104006), IL-6 (QT00098875), IL-10 (QT00106169), CD14 (QT00246190), and TLR4 (QT00259042). Standards corresponding to each target gene were added in each PCR reaction. The results for each sample were normalized by copy number of GAPDH (QT01658692) used as a marker of cDNA inputs. All results were expressed as copy numbers of target gene per copy numbers of GAPDH.

### Flow cytometry analysis

PM $\phi$ s were gently scraped, centrifuged, and re-suspended in FACS staining buffer (FSB, DPBS without Ca<sup>2+</sup>/Mg<sup>2+</sup> supplemented with 0.5% BSA). PM $\phi$ s were then incubated with rat anti-mouse CD16/CD32 (mouse Fc block, BD Pharmingen, San Diego, CA, USA) for 15 min. In some experiments, PM $\phi$ s were incubated with APC-conjugated anti-F4/80 Abs (eBioscience, San Diego, CA, USA) and biotinylated LPS for 30 min followed by AlexaFluor488-conjugated streptavidin (BD Pharmingen). In other experiments, PM $\phi$ s were stained for 30 min with a combination of FITC-conjugated anti-CD14 (eBioscience) and

Allophycocyanin (APC)-conjugated anti-TLR4/MD2 Abs (eBioscience). PM $\phi$ s were also incubated with biotin-conjugated LPS (10  $\mu$ g/ml for 30 min) followed by AlexaFluor488-conjugated streptavidin. PM $\phi$ s were then washed and re-suspended in FSB. Fluorescence was acquired using a BD FACSCanto II and analyzed by FlowJo software (Tree Star, Ashland, OR, USA).

### Cholesterol analysis

Male mice (8–9-wks-old) were nil per os (NPO) for 16 h before sample collection. Mice were euthanized under isoflurane anesthesia, and approximately 500  $\mu$ l of blood was drawn from mice via cardiac puncture using a 16-gauge needle and placed in EDTA-coated tubes on ice. Tubes were spun down at 5000g for 5 min and plasma was collected from the supernatant, placed into fresh tubes, and stored at  $-20^{\circ}\text{C}$  until further analysis. Cholesterol levels were performed by the hematology core facility at the University of California San Diego.

### Statistical analysis

All data were analyzed using GraphPad Prism software (GraphPad Prism Software, San Diego, CA, USA). Significance was analyzed using a Student's *t*-test or one-way ANOVA followed by Newman-Keuls Multiple Comparison Test. A *P* value of  $<0.05$  was considered statistically significant. Statistical significance was analyzed by log-rank test for comparison of mortality rates.

## Results

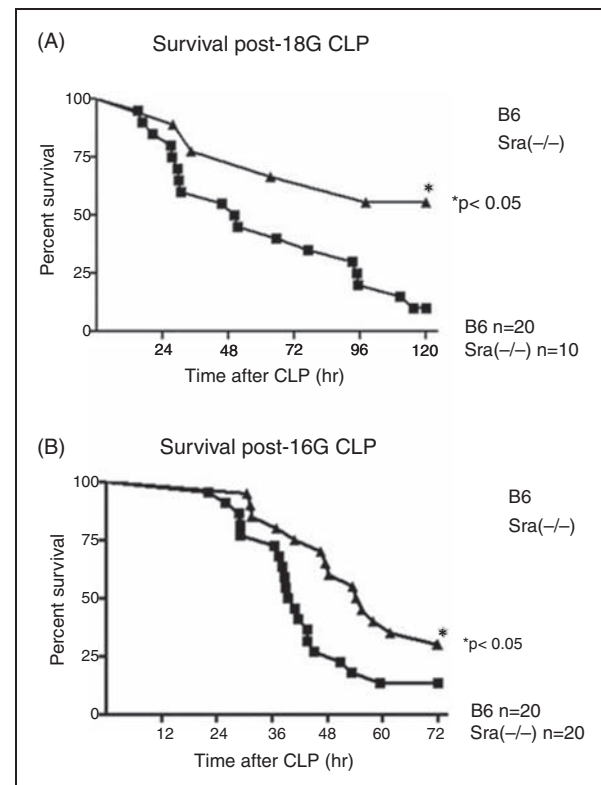
### *Sra*( $-/-$ ) mice are more resistant to septic shock induced by cecal ligation and puncture

*Sra1* (*Msr1*) was mapped as a modifier gene of the inflammatory response induced by LPS.<sup>28</sup> In the present study, we investigated the response of *Sra*( $-/-$ ) mice to septic shock. We used cecal ligation and puncture (CLP), which is the gold standard model to study sepsis in rodents, because it mimics many features of the condition in humans. Male *Sra*( $-/-$ ) or B6 mice were subjected to CLP using a single perforation with an 18- or 16-gauge needle that resulted in higher mortality rates depending on the increase in puncture size. *Sra*( $-/-$ ) mice displayed better survival rates (56%) than B6 mice (10%) after CLP with 18-gauge perforation, which was statistically significant ( $P < 0.05$ ) (Figure 1A). These results were corroborated with a more severe injury (CLP with 16-gauge perforation), displaying 30% survival for *Sra*( $-/-$ ) mice and 14% for B6 mice ( $P < 0.05$ ) (Figure 1B). B6 mice reached 50% mortality within 38 h and 48 h after 16- or 18-gauge perforation, respectively. In contrast,

*Sra*( $-/-$ ) mice displayed 50% mortality within 54 h after CLP with 16-gauge perforation and did not reach 50% mortality after CLP with 18-gauge puncture (Figure 1).

### Cytokine expression is altered in PM $\phi$ s derived from *Sra* ( $-/-$ ) mice

The potential role of *Sra1* in the response to LPS was investigated in PM $\phi$ s isolated from naïve *Sra*( $-/-$ ) or wild-type (B6) mice. These primary cells were incubated in culture conditions with two concentrations of LPS (1 ng/ml or 100 ng/ml). The lower LPS concentration activates M $\phi$ s exclusively via CD14, which is the major LPS binding site on M $\phi$ s, whereas the higher concentration activates M $\phi$ s independently of the presence of CD14.<sup>34</sup> LPS-induced cytokine expression (mRNA levels) was measured by quantitative RT-PCR (qRT-PCR), which provided a rapid and accurate assessment of gene expression. LPS-induced TNF- $\alpha$  mRNA levels were no different between PM $\phi$ s



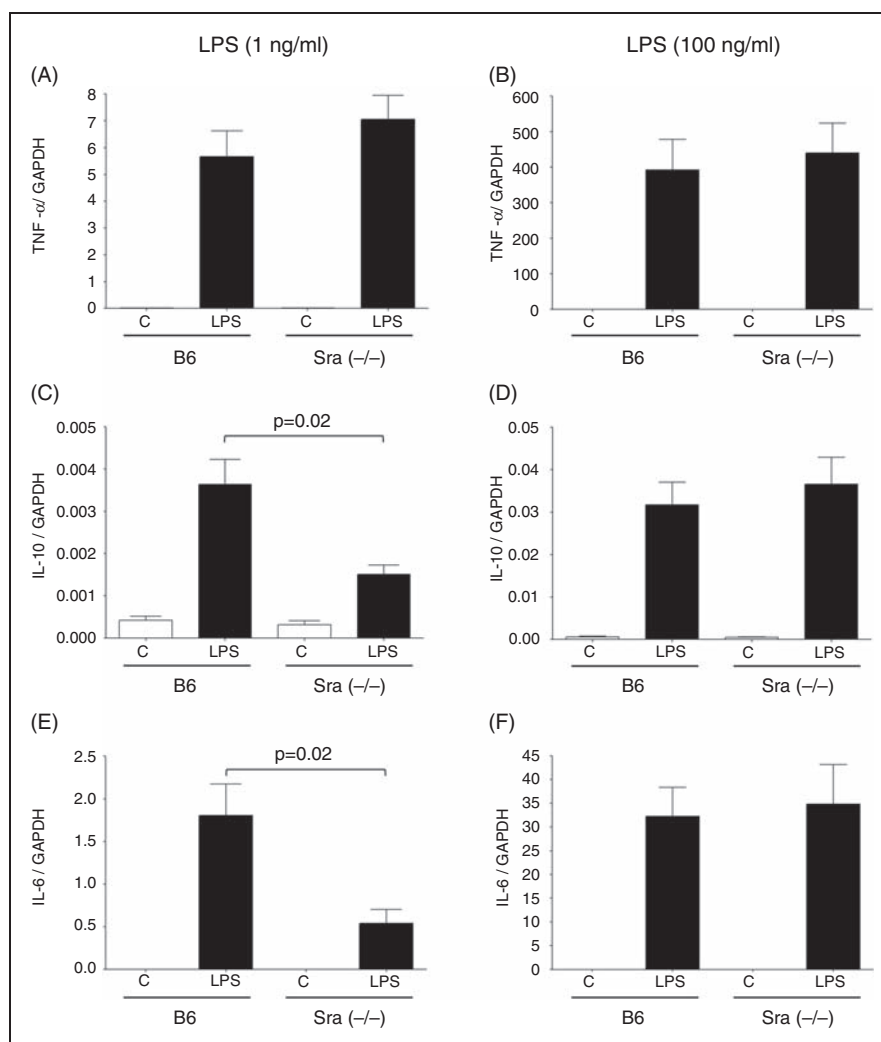
**Figure 1.** Survival of *Sra*( $-/-$ ) and B6 mice after CLP. Male *Sra*( $-/-$ ) or B6 mice were NPO for 16 h before the procedure. Anesthetized mice were subjected to CLP (1.5-cm ligation) and puncture with 18- or 16-gauge needle. Survival was monitored continuously for 120 h (18-gauge) or 72 h (16-gauge) after CLP. Statistical significance was analyzed by log-rank test. (A) CLP (18-gauge), *Sra*( $-/-$ ) *n* = 10, B6 *n* = 20, \**P* < 0.05 *Sra*( $-/-$ ) vs. B6. (B) CLP (16-gauge), *Sra*( $-/-$ ) *n* = 20, B6 *n* = 20, \**P* < 0.05 *Sra*( $-/-$ ) vs. B6.



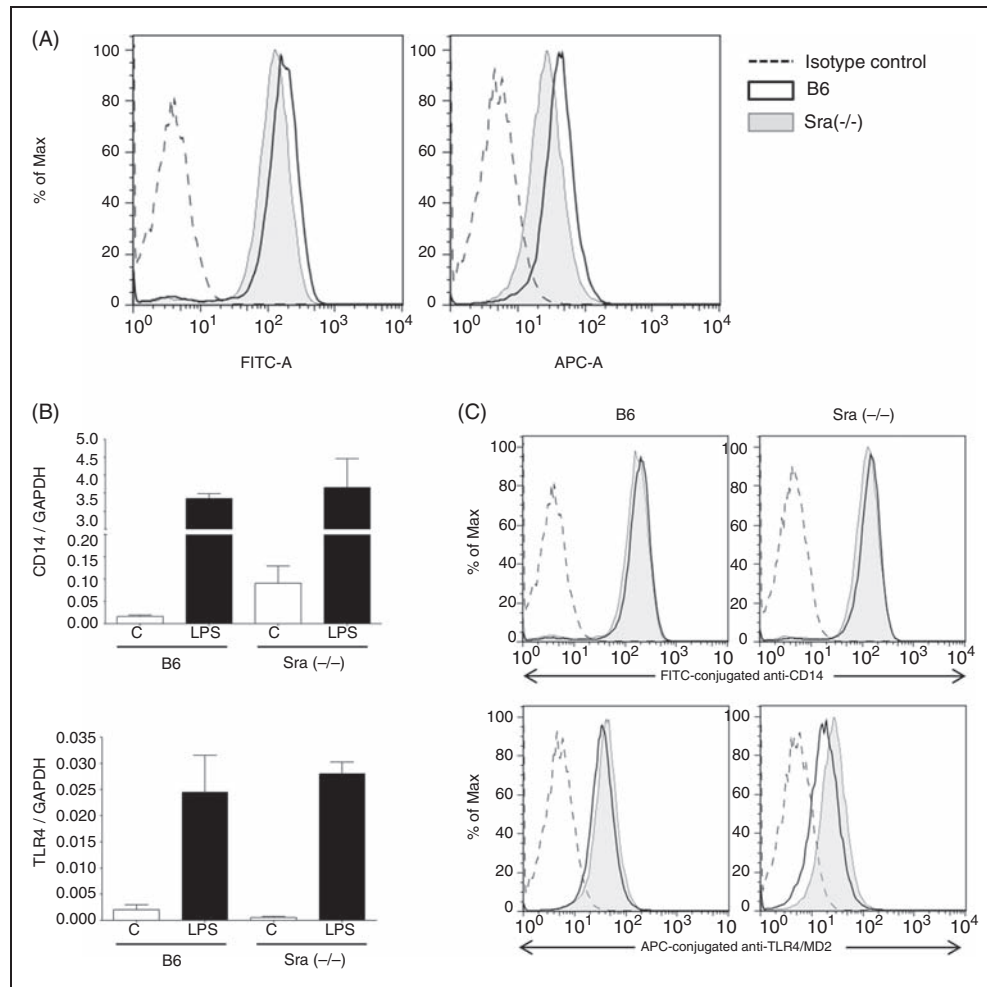
isolated from *Sra*( $-/-$ ) and B6 mice at both LPS concentrations (Figure 2A, B). In contrast, IL-10 expression was significantly reduced in PM $\phi$ s derived from *Sra*( $-/-$ ) mice in comparison with cells from B6 mice at the low LPS concentration (Figure 2C), but not at high LPS concentrations (Figure 2D). Similar observations to IL-10 were made for IL-6 expression (Figure 2E, F). These observations suggest that *Sra* modulates TLR4 signaling, depending on the concentration of LPS. We also investigated the response of PM $\phi$ s isolated from *Sra*( $-/-$ ) or B6 mice to a different ligand, zymosan, which activates M $\phi$ s via TLR2 or dectin. Expression of both TNF- $\alpha$  and IL-10 was equally elevated in PM $\phi$ s isolated from *Sra*( $-/-$ ) and B6 mice (data not shown).

### TLR4 and CD14 expression is similar between PM $\phi$ s derived from *Sra* ( $-/-$ ) and B6 mice

As the preceding observations suggest that the TLR4 signaling pathway is altered in the absence of *Sra* at low concentrations of LPS, we evaluated the expression of TLR4 and CD14 in PM $\phi$ s isolated from *Sra*( $-/-$ ) mice or B6 mice after incubation with LPS (1 ng/ml) in culture. Surface levels for both TLR4 and CD14 in resting cells were slightly reduced in PM $\phi$ s isolated from *Sra*( $-/-$ ) as opposed to B6 mice (Figure 3A). The expression of both receptors (mRNA levels) was equally increased upon LPS stimulation in PM $\phi$ s isolated from *Sra*( $-/-$ ) and B6 mice (Figure 3B). Similarly, cell surface levels of CD14 were similar



**Figure 2.** LPS-induced cytokine profile in PM $\phi$ s isolated from *Sra*( $-/-$ ) or B6 mice. Naïve PM $\phi$ s were isolated from *Sra*( $-/-$ ) or B6 mice by peritoneal lavage in the absence of recruiting agents, as described in the 'Materials and methods'. Cells were incubated (or not) with LPS (1 ng/ml or 100 ng/ml as indicated) for 3 h at 37°C in RPMI 1640 medium containing FBS (10%). At the end of the incubation time, RNA was then isolated, DNase treated, and reverse-transcribed to cDNA. TNF- $\alpha$ , IL-10, and IL-6 mRNA levels were determined by qRT-PCR using the standard curve method. The housekeeping gene GAPDH was used to normalize the data. Results are expressed as average  $\pm$  SE ( $n = 4-5$ ). Significance was analyzed by student's *t*-test between cells under each condition isolated from *Sra*( $-/-$ ) or B6 mice.  $P < 0.02$  for IL-10 *Sra*( $-/-$ ) vs. B6.  $P < 0.02$  for IL-6 *Sra*( $-/-$ ) vs. B6.



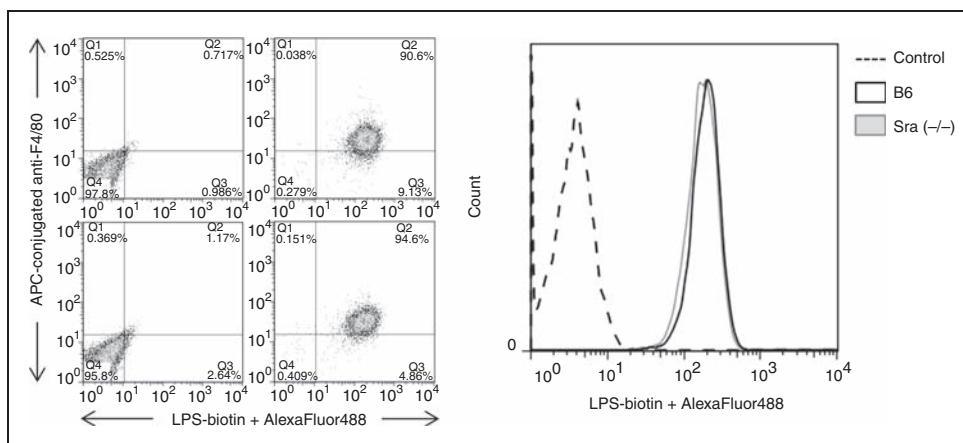
**Figure 3.** Comparison of CD14 and TLR4 mRNA and surface levels on *Sra*<sup>-/-</sup> and B6-derived PMφs before and after incubation with LPS. Naïve PMφs were isolated from *Sra*<sup>-/-</sup> or B6 mice by peritoneal lavage in the absence of recruiting agents, as described in the 'Materials and methods'. (A) PMφs were stained with a combination of FITC-conjugated anti-CD14 and APC-conjugated anti-TLR4/MD2 Abs. Fluorescence was acquired using a BD FACSCanto II flow cytometer and analyzed by FlowJo software. (B) Cells were incubated (or not) with LPS (1 ng/ml) for 3 h at 37°C in RPMI 1640 medium containing FBS (10%). RNA was then isolated, DNase treated, and reverse-transcribed to cDNA. CD14 and TLR4 mRNA levels were determined by qRT-PCR using the standard curve method. The housekeeping gene GAPDH was used to normalize the data. Results are expressed as average  $\pm$  SE ( $n = 4-5$ ). Significance was analyzed by student's *t*-test between cells under each condition isolated from *Sra*<sup>-/-</sup> or B6 mice. (C) LPS-treated PMφs were stained with a combination of FITC-conjugated anti-CD14 and APC-conjugated anti-TLR4/MD2 Abs. Fluorescence was acquired using a BD FACSCanto II flow cytometer and analyzed by FlowJo software. Representative histogram plots are shown with filled grey histograms for control PMφs (untreated) and open black histograms for LPS-treated PMφs. Dashed histograms represent CD14 or TLR4 corresponding isotype control Abs.

between PMφs isolated from *Sra*<sup>-/-</sup> and B6 mice, even after LPS (1 ng/ml) stimulation in culture. A small reduction in TLR4/MD2 surface levels between PMφs isolated from B6 or *Sra*<sup>-/-</sup> mice after incubation with LPS was observed (Figure 3C).

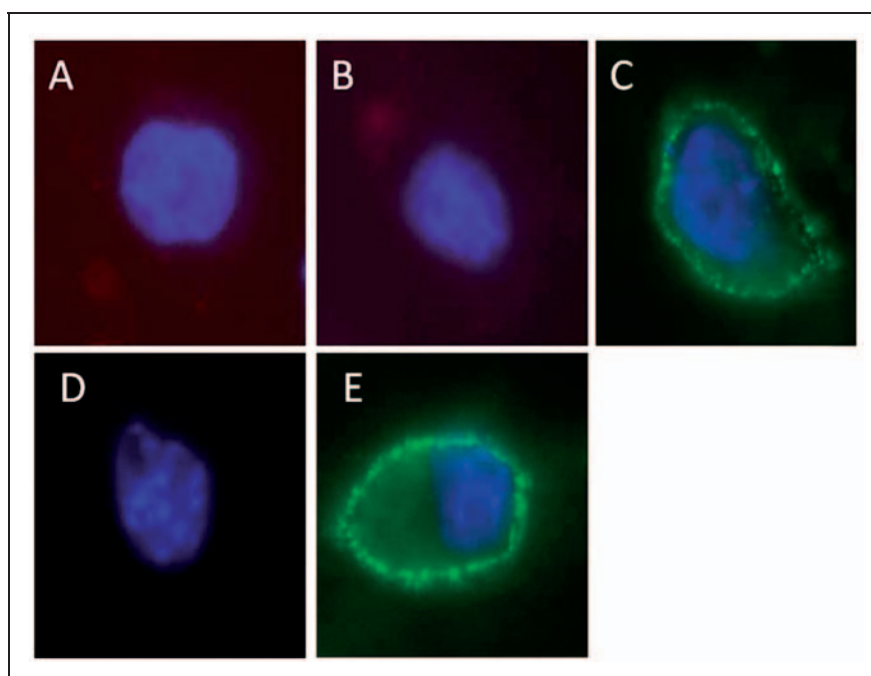
### *Sra* (*Msr1*) does not contribute to LPS binding

The above observations suggest that the TLR4 pathway, which is the major signal transducer for LPS, is altered in absence of the *Sra* gene at low concentrations of LPS, but not at higher amounts of the endotoxin. As the interaction of LPS with *Sra* has been proposed

previously,<sup>2,35</sup> we investigated whether or not LPS binds directly to *Sra*. Binding of biotin-conjugated LPS to PMφs isolated from B6 or *Sra*<sup>-/-</sup> mice was measured by flow cytometry. We did not observe any difference in LPS binding between the knockout- and wild type-derived PMφs (Figure 4), suggesting that if, indeed, LPS binds to *Sra*, it may be just a small contribution to the total binding of endotoxin. To further corroborate the preceding observations, CHO cells that did not present any LPS binding proteins were transfected with mouse *Sra* gene and binding of biotin-conjugated LPS was visualized by fluorescent microscopy. Mock-transfected CHO cells did not



**Figure 4.** PMφs isolated from *Sra*( $-/-$ ) or B6 mice showed an identical LPS binding profile. Naïve PMφs were isolated from *Sra*( $-/-$ ) or B6 mice by peritoneal lavage in the absence of recruiting agents, as described in the 'Materials and methods'. (A) Isolated PMφs were characterized by staining with APC-conjugated anti-F4/80 Abs and biotin-conjugated LPS followed by AlexaFluor488-conjugated streptavidin. An isotype control was also included. Notice that there is no difference in LPS binding between PMφs isolated from *Sra*( $-/-$ ) or B6 mice. Fluorescence was acquired using a BD FACSCanto II flow cytometer and analyzed by FlowJo software.



**Figure 5.** CHO cells transfected with *Sra* did not display any LPS binding. CHO cells were transfected with murine *Sra* (plasmid) using Eugene HD. After 24 h of the transfection, cells were analyzed using various reagents. (A) Mock-transfected CHO cell; (B–E) *Sra* transfected CHO cells. Cells were stained with biotin-conjugated LPS followed by AlexaFluor594-conjugated streptavidin (A, B), anti-*Sra* Ab and AlexaFluor488-conjugated anti-rabbit secondary Ab (C), AlexaFluor488-conjugated anti-rabbit secondary Ab in absence of primary Ab (D), and AlexaFluor488-conjugated-Ac-LDL (E). Notice the lack of LPS binding to *Sra* transfected cells.

display any detectable LPS binding (Figure 5A). Similarly, CHO cells transfected with mouse *Sra* did not present any association with LPS (Figure 5B). The presence of *Sra* on the cell surface of transfected cells was demonstrated by using an Ab specific for mouse *Sra* (Figure 5C), but not by the secondary Ab alone (Figure 5D). Moreover, we demonstrated by

positive binding of Alexafluor 488-labeled AC-LDL that *Sra* on the surface of transfected cells was functional (Figure 5E). The two isoforms of the *Sra* gene (*Sra-I* or *Sra-II*), which are present on Mφs, could differ in LPS binding. Thus, CHO cells were transfected with either *SraI* or *SraII*, or both, and surface expression of these receptors was detected by various

reagents. Indeed, both *SraI* and *SraII* were recognized by anti-*Sra* Ab (Figure 6A, E, I), and both isoforms bound acetylated (Ac)-LDL (Figure 6C, G, K). However, no LPS binding was observed in cells transfected with each of them nor a combination of both (Figure 6D, H, L). The preceding observations suggest that *Sra* (*Msr1*) does not bind to LPS.

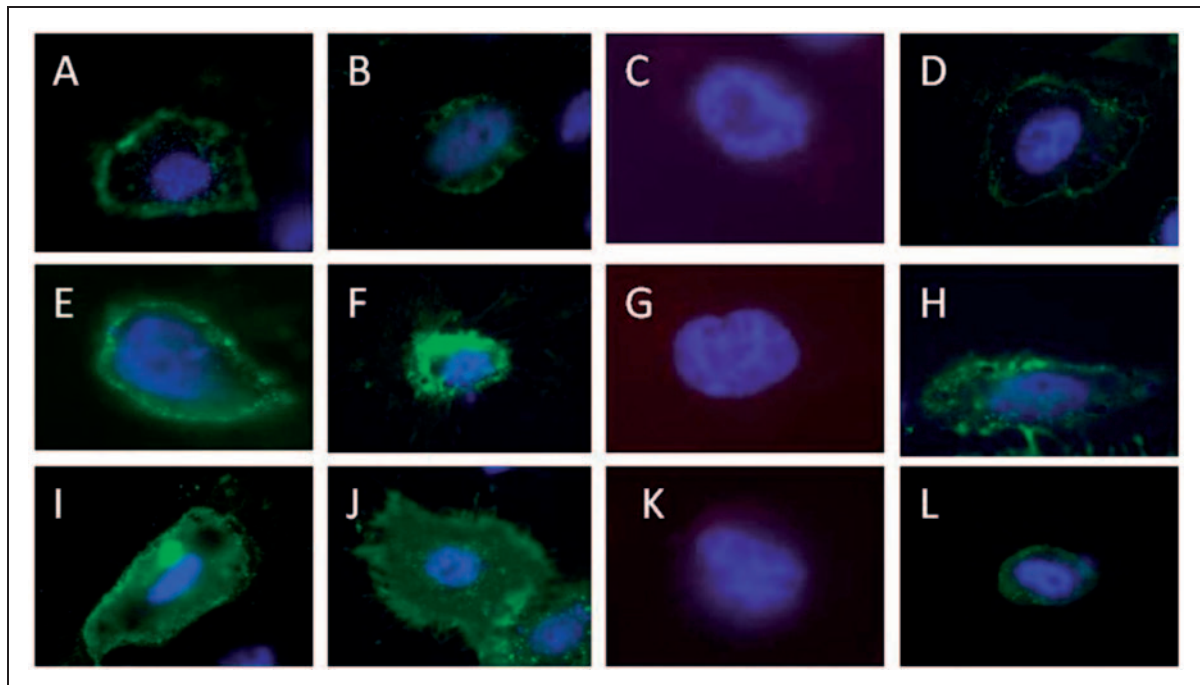
#### *Sra*( $-/-$ ) mice displayed an alteration of lipid profile in comparison with B6 mice

As protection in *Sra*( $-/-$ ) mice from endotoxic and septic shock is unlikely to be related to the presence of LPS, we searched for other factors that could explain the improved survival of *Sra*( $-/-$ ) mice from LPS and CLP. We measured the lipid profile of naïve *Sra*( $-/-$ ) or B6 mice in non-operated conditions or after CLP. Total cholesterol levels were elevated in *Sra*( $-/-$ ) mice as compared with B6 mice, and no change in these levels were observed after CLP in both strains (Figure 7A), as reported previously.<sup>36</sup> The elevated total cholesterol levels in *Sra*( $-/-$ ) mice were mainly due to high HDL levels in comparison with B6 mice (Figure 7B). No alterations in HDL levels were observed after CLP (Figure 7B). Similarly, LDL levels (calculated) were higher in *Sra*( $-/-$ ) than in B6 mice (Figure 7C). An increase in LDL levels was observed in B6 mice after CLP, but not in *Sra*( $-/-$ ) mice

(Figure 7C). There was no difference in triglycerides between *Sra*( $-/-$ ) and B6 mice, and a decrease after CLP was detected in both cases (Figure 7D). Very low density lipoprotein (VLDL) and lipase levels were also not different between *Sra*( $-/-$ ) and B6 mice (Figure 7E, D). Based on these observations, it could be proposed that high levels of HDL may be responsible for the improved survival of *Sra*( $-/-$ ) mice during sepsis. Indeed, several reports have shown that HDL is capable of reducing the inflammatory response induced by LPS.<sup>37,38</sup>

#### Discussion

The innate immune system is the initial and more rapid response to infections and injury. Pathogens, dead cells, and cellular debris are recognized by a group of cell surface receptors named PRRs, which direct clearance and activation of necessary pathways to resolve the insult. Scavenger receptors, which are part of PRRs, have gained increasing attention because of the versatility of the ligands that they recognize and the cellular processes in which they are involved.<sup>2,4</sup> We have previously identified a member of the scavenger receptor family, *SraI* or *Msr1*, as a modifier gene of the inflammatory response induced by LPS during a genetic screening.<sup>28</sup> Moreover, we observed that *Sra*( $-/-$ ) mice presented reduced LPS-induced IL-10 plasma

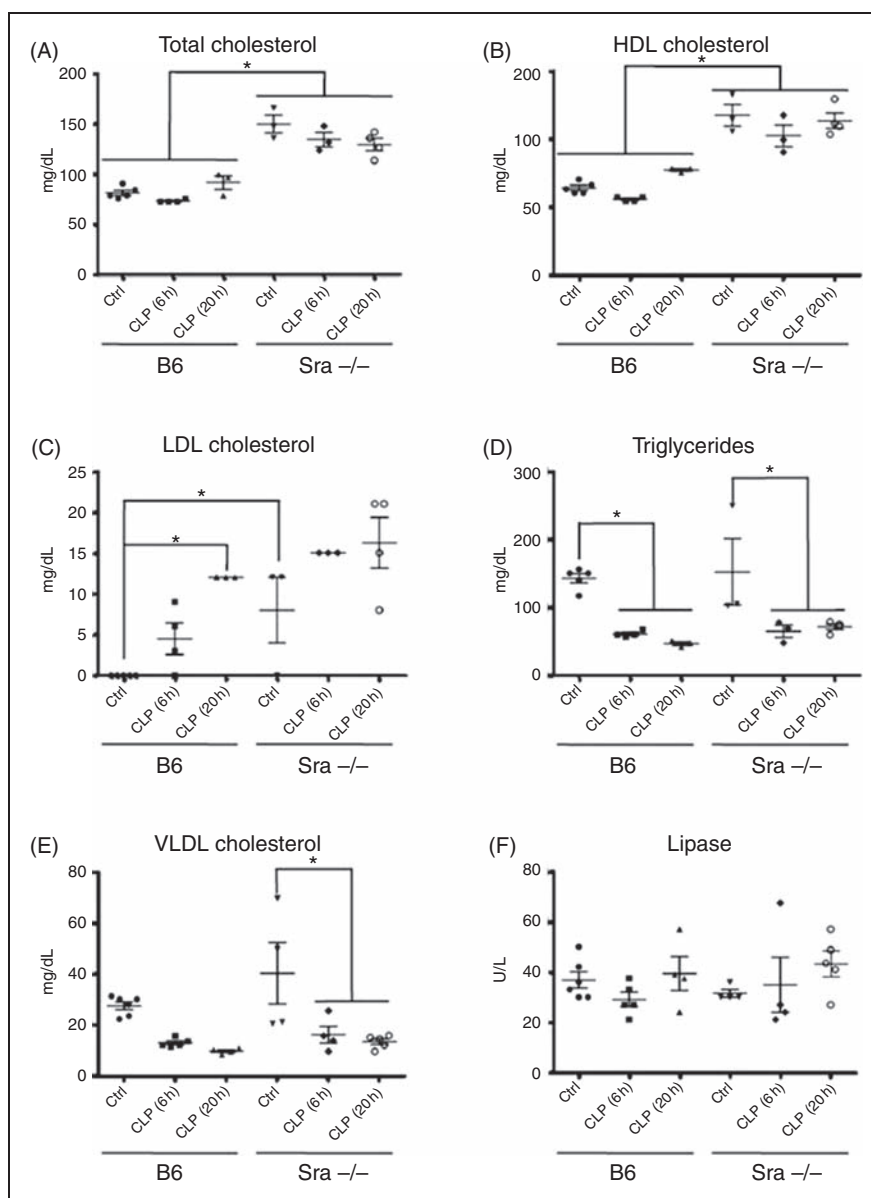


**Figure 6.** CHO cells transfected with *SraI*, *SraII*, or both did not bind LPS. CHO cells were transfected with murine *SraI*, *SraII*, or both (plasmid) using Fugene HD. After 24 h of the transfection, cells were analyzed using various reagents. *SraI*-transfected CHO cells (A–D), *SraII* (E–H), *SraI* and *SraII* (I–L). Cells were stained with anti-*Sra* Ab and AlexaFluor488-conjugated anti-rabbit secondary Ab (A, C, E, I); AlexaFluor488-conjugated anti-rabbit secondary Ab in absence of primary Ab (B, F, J); AlexaFluor488-conjugated-Ac-LDL (C, G, K); biotin-conjugated LPS followed by AlexaFluor594-conjugated streptavidin (D, H, L).



levels in comparison with wild type B6 mice.<sup>28</sup> In the present study, we evaluated whether or not the presence of *Sra* altered the response to a clinically relevant septic insult induced by CLP, which is the gold standard animal model for the study of sepsis. We observed that *Sra*( $-/-$ ) mice displayed a better survival rate than wild-type mice during sepsis induced by two variants of the CLP model that differ in the severity of the insult. Therefore, these observations clearly displayed an advantage in the outcome by the absence of *Sra*. Prior studies have shown increased mortality of *Sra*( $-/-$ ) mice after injection with LPS,<sup>22–24</sup> or after

a combination of LPS and priming with bacillus Calmette-Guerin.<sup>21</sup> In contrast, other studies have reported increased survival of *Sra*( $-/-$ ) mice after injection of LPS.<sup>25</sup> As the CLP model is composed of an area of necrosis and leakage of the gut polymicrobial flora into the peritoneum, it is likely that it does not depend on LPS toxicity alone. Thus, other factors in this insult are likely to play a role in the outcome, in which *Sra* may play a critical part. Indeed, we did not find any direct binding of biotin-conjugated LPS to naïve PM $\phi$ s isolated from *Sra*( $-/-$ ) in contrast to B6 (wild-type)-derived cells or after transfection of CHO



**Figure 7.** Lipid profile of *Sra*( $-/-$ ) and B6 mice. Male *Sra*( $-/-$ ) ( $n = 10$ ) or B6 ( $n = 12$ ) mice were NPO for 16 h before the procedure. Anesthetized mice were subjected to CLP (1.5-cm ligation) and puncture with a 16-gauge needle or were non-operated [NO; B6  $n = 5$ ; *Sra*( $-/-$ )  $n = 3$ ]. The lipid profile was determined in plasma samples from NO mice, 6 or 20 h after CLP. Total cholesterol (A), HDL (B) calculated LDL (C), triglyceride levels (D), calculated VLDL (E), and lipase (F). Significance was analyzed using a one-way ANOVA followed by Newman-Keuls multiple comparison test for each time point ( $P < 0.0001$ ).

cells with *SraI* or *SraII* genes. Previous studies have proposed that SraI interacts with LPS, as assessed by competition binding of lipid A, which is a component of LPS, and typical Sra ligands.<sup>35</sup> However, no direct LPS binding to Sra was demonstrated. Peiser et al.<sup>13</sup> showed that Sra recognized *N. meningitidis* independently of the presence of LPS.

In spite of the lack of direct binding of LPS to Sra, we observed that the response to LPS via the TLR4 signal transduction pathway was altered in PM $\phi$ s isolated from *Sra*( $-/-$ ) mice. The absence of Sra did not result in altered expression of TLR4 or CD14, which could have been a compensatory mechanism to increase LPS binding. We observed that deletion of the *Sra* gene resulted in reduced IL-10 and IL-6 mRNA levels after challenging naïve PM $\phi$ s with low concentrations of LPS (1 ng/ml). In contrast, expression of TNF- $\alpha$  was not affected by the presence (or not) of Sra, suggesting that the TLR4-NF- $\kappa$ B pathway is independent of the presence of the scavenger receptor. The reduced expression of IL-10 and IL-6 in PM $\phi$ s derived from *Sra*( $-/-$ ) mice was not observed at high concentrations of LPS (100 ng/ml). There is an important distinction between these two doses of LPS. The activation of M $\phi$ s at low concentrations (1 ng/ml) is exclusively dependent on the presence of CD14, as demonstrated in *CD14*( $-/-$ ) mice.<sup>34</sup> At higher concentrations of LPS (e.g. 100 ng/ml), LPS is recognized by reduced affinity receptors, such as TLR4.<sup>39</sup> Based on this assumption, we propose that Sra forms a complex with CD14 and TLR4 that is involved in the expression of IL-6 and IL-10 at low LPS concentrations, which are likely similar to the concentrations of LPS in circulation during infection. Previous studies have also proposed an interaction of Sra with TLR4<sup>9,39,40</sup> and a modulation of the TLR4 signal transduction pathway.<sup>23,24,41,42</sup> Recently, it has been reported that TLR4 can differentially activate the TIRAP-MyD88 and the TRAM-TRIF pathways depending on the subcellular localization of this receptor. Thus, the TIRAP-MyD88 pathway is activated when TLR4 is on the plasma membrane, whereas the TRAM-TRIF pathway is triggered by the internalization of TLR4, which is localized within early endosomes.<sup>43</sup> As the TRAM-TRIF pathway is directly involved in IL-10 expression and indirectly in IL-6 production, Sra may modulate the translocation of TLR4 from the plasma membrane to early endosomes, resulting in alteration of cytokine expression. In this regard, we observed small differences in the down-regulation of TLR4 from the cell surface in presence or absence of Sra after stimulation with LPS. Other studies have found that internalization of Sra is required for M $\phi$  activation via various ligands for this receptor.<sup>44</sup> Elegant studies by Seimon et al.<sup>9</sup> have shown that ligands for Sra, such as fucoidan, triggered apoptosis in ER-stressed M $\phi$ s via association with TLR4 and blocked the pro-survival IRF-3-IFN- $\beta$  pathway. In

contrast, LPS triggered an alternative survival pathway via the activation of TRIF-TRAM and, subsequently, IRF3 and the production of IFN- $\beta$ . These observations suggest the activation of two alternative pathways in M $\phi$ s by typical ligands of Sra (fucoidan) or TLR4 (LPS). The independence between these two pathways may be explained by the lack of LPS binding to Sra. Similarly, Sra has been shown to affect dimerization and ubiquitination of TNF receptor-associated factor 6 (TRAF6), resulting in an alteration of the inflammatory response.<sup>23</sup> Other studies have also suggested that Sra attenuated the response of TLR4 and enhanced the activation by intracellular receptors, such as TLR3, NOD2, and NALP3.<sup>40</sup>

Although the role of Sra in the septic response remains to be established, it may be related to the modulation of TLR4 signal transduction pathways. Another alternative to explain the survival of *Sra*( $-/-$ ) mice during sepsis may be associated with the initial elevated levels of HDL in circulation that we observed in these animals (Figure 7). HDL has been shown to neutralize LPS toxicity<sup>45</sup> and it has been reported to bind free LPS, as well as to remove LPS from the cell surface.<sup>46</sup> Elevated HDL has also been correlated with protection from endotoxic shock in humans<sup>47</sup> and in animal models.<sup>48–50</sup> In addition, high levels of HDL have been described to reduce organ injury after hemorrhagic shock<sup>51</sup> and ischemia/reperfusion,<sup>52</sup> suggesting that the effect of this lipoprotein may be beyond LPS neutralization. Several reports have certainly indicated anti-inflammatory and antioxidant effects of HDL.<sup>37,38</sup> A HDL-mediated reduction in the inflammatory response has been shown during endotoxic shock.<sup>37,48,53</sup> Reduced total cholesterol, LDL and HDL levels were observed in circulation in patients after injury<sup>54</sup> and sepsis.<sup>55</sup> This reduction in HDL has been correlated with a negative outcome.<sup>55–57</sup> In fact, an increase in HDL levels over time that was not detected in non-survival septic patients was observed in septic shock survival patients in an intensive care unit.<sup>58</sup> Therefore, it is possible that the improved survival rate endotoxic and septic shock observed in *Sra*( $-/-$ ) mice as opposed to wild-type may be linked to the initial elevated HDL levels observed in the knockout animals. The higher HDL levels in *Sra*( $-/-$ ) versus B6 mice is puzzling. High levels of HDL have been associated with reduced expression of scavenger receptor B or CD36 in human studies.<sup>59</sup> This observation was clearly confirmed in investigations using CD36 knockout mice.<sup>60</sup> However, a possible role of Sra in the clearance of HDL has not been established. Plasma HDL levels have been correlated with genetic factors.<sup>61</sup> Mouse genetic studies have reported a quantitative trait locus (QTL) associated with HDL within chromosome 8.<sup>62,63</sup> We have also identified a QTL for the inflammatory response to LPS located within mouse chromosome 8, which contains Sra

(Mrs1) as a candidate gene.<sup>28</sup> However, there was no overlap between these two regions. In contrast, other genetic studies analyzing plasma fatty acid levels in Alaskan Eskimos resulted in a QTL on chromosome 8 containing *Sra* (*Msr1*), which was also considered as a candidate gene.<sup>64</sup>

In summary, our studies suggest that *Sra* is a modulator of the signal transduction pathway mediated by TLR4, altering the inflammatory process. In addition, our observations demonstrated that the absence of *Sra* in mice resulted in a better survival rate from septic shock. This increase in survival is not a result of the contribution of *Sra* to sequester LPS, but is rather associated with an increase in basal levels of HDL. The possibility that elevated HDL levels could improve survival from sepsis may open new and exciting approaches to ameliorate the detrimental consequences of sepsis.

### Funding

These studies were supported by the National Institute of General Medical Sciences (grant no. GM073825).

### Acknowledgements

We thank Professor Motohiro Takeya (Department of Cell Pathology, Kumamoto University, Kumamoto, Japan) for the generous gift of anti-*Sra* Ab.

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