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Toll Like Receptor 4 Stimulation Increases Scavenger Receptor A Expression On Murine Macrophages

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By

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ABSTRACT

Sepsis is the body's response to an overwhelming infection and is a serious consequence of critical illness. It can cause tissue damage, organ failure, and death. Sepsis continues to have an unacceptably high mortality rate, due to the lack of effective treatments. Specific therapeutic targets for sepsis remain elusive since the complex functional changes that result in a septic state remain poorly understood. Macrophage Scavenger Receptor A (SRA, CD204) is a surface receptor that binds negatively charged, endogenous and exogenous ligands. We have discovered that SRA plays a significant role in the pathophysiology of sepsis. We have shown that mice with SRA have increased inflammation, decreased survival, and increased bacterial burden compared to SRA deficient mice. We have also found an increase in the expression of SRA on monocytes and macrophages in septic wild type mice. To determine the mechanism responsible for increased SRA expression in sepsis we treated a mouse macrophage cell line, (J774a.1), with mediators that stimulate toll like receptors (TLRs), innate immune receptors which are activated in sepsis. The cells were cultured with ultra pure LPS (a TLR 4 ligand), PAM3CSK4 (a TLR 2 ligand), glucan (a Dectin-1 ligand), ultra pure LPS and PAM3CSK4, or ultra pure LPS and glucan for 24 hours. The cells were stained with an SRA antibody, and flow cytometry was used to measure the SRA expression for each treatment group. LPS treatment alone resulted in a significant increase in SRA expression when compared to control cells. Specifically, LPS increased SRA expression by 53.4% compared to media alone ($p<0.05$). PAM3CSK4 alone or in combination with LPS had no significant effect on SRA expression when compared to control cells. Glucan alone had no significant effect on SRA expression when compared to control cells, but glucan in combination with LPS did significantly increase SRA expression 47.7% ($p<0.01$).

From these data we can conclude that an increase in SRA expression on macrophages in sepsis is mediated by TLR4 stimulation, but not by TLR2 or Dectin-1 stimulation.

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INTRODUCTION

Sepsis syndrome and septic shock are the foremost causes of death in intensive care units (ICU) (1). The number of individuals that acquire sepsis continues to grow with approximately 751,000 patients developing sepsis syndrome per year in the Unites States. The average mortality rate for sepsis-infected patients is 28.6% or 215,000 individuals per year (2). Sepsis is thought to commence following a serious injury or infection and creates an overwhelming inflammatory response. Sepsis may lead to or co-develop along with multiple organ dysfunction syndrome (MODS), systematic inflammatory response syndrome (SIRS), or acute respiratory distress syndrome (ARDS). MODS occurs in approximately 30% of septic individuals (3, 4). With ARDS the lung is the most frequently damaged organ, but other organs like the kidneys, brain, and heart may also be affected (3, 4, 5). Individuals that survive sepsis are typically found to have diminished function mentally and physically (6).

The underlying mechanisms that result in a septic state are complex and poorly understood; however, it is known that the activation of pattern recognition receptors (PRR) by their cognate pathogen associated molecular patterns (PAMPs) promotes an inflammatory state (7). In the cases of overwhelming infection, such as sepsis, this results in an overwhelming stimulation of these receptors, and thus, overwhelming inflammation. The toll like receptors (TLR), including TLR2 and 4 are known to play a role in the pathophysiology of sepsis, explaining the use of PAM3CSK4, a TLR2 ligand and gram positive microbe, and LPS, a TLR4 ligand and gram negative microbe (8). Specifically, SRA is known to co-associate with TLR4 in sepsis, which is associated with the inflammatory response involving NFκB activity and cytokine production (9). TLR2 and Dectin-1 are expressed on neutrophils and other leukocytes. TLR2

stimulation has been reported to increase SRA expression in murine bone marrow. Glucan, a Dectin-1 ligand and fungal microbe, has also been found in sepsis (10, 11).

Macrophage scavenger receptor class A (SRA, CD204) is a type 2 membrane receptor classically expressed by macrophages that binds to negatively charged endogenous (oxidized LDL, apoptotic cells) (8, 12) and exogenous ligands (endotoxin, glucan) (12, 13). SRA regulation has been found to be up regulated by NF-κB in response to cytokines in macrophages and by p38 MAP kinase in response to LDL (14, 15). SRA's role in atherosclerosis is best understood; however, SRA also seems to play a role in the innate immune response to infection (14).

We have found that SRA plays a significant role in the morbidity and mortality of sepsis. Specifically, we as well as others, have discovered that wild type cecal ligation and puncture (CLP) induced septic mice have increased inflammation, increased bacterial burdens, and decreased survival, when compared to SRA knock-out mice (16, 17). Interestingly, SRA has been found to be protective in several other infections (18, 19). Furthermore, we have found that wild type mice have an increased SRA expression on spleen, bone marrow, and blood monocytes and macrophages. These findings denote the important role that SRA plays in the pathophysiology of sepsis (16). For this reason we hypothesize that mediators present in sepsis result in an increase in SRA expression that contributes to sepsis and septic sequelae. The goal of the present study is to determine a mechanism responsible for increased SRA expression on macrophages in sepsis.

METHODS

J774a.1 Cell Culture

The murine macrophage cell line, J774a.1, was cultured in a Dulbecco's Modification of Eagle's Medium (DMEM, ATCC) with 9% newborn calf serum, 1% fetal bovine serum, and antibiotics at a 0.4×10^6/ well plating density. J774a.1 cells were cultured in 6-well plates with 3mL DMEM and treated using PAM3CSK4 (Invivogen, 10 μg/ml), glucan (10 μg/ml), ultrapure LPS (upLPS, Invivogen, 100 ng/ml), a combination of PAM3CSK4 and ultrapure LPS, a combination of glucan and ultrapure LPS, or media alone and incubated at 37°C for 24 hours.

Flow Cytometry

The adhered cells were washed and covered with 500 μL Dulbecco's Phosphate Buffered Saline (DPBS) and incubated at 37°C for 5-10 minutes. The adhered cells were then scraped into DPBS using pipet tips, collected, and pelleted at 2000 rpm for 5 minutes. Cells were blocked in PBS supplemented with 5% normal rabbit serum (NRS), 0.5% bovine serum albumin (BSA), 0.1% sodium azide, and 5mM EDTA with anti-mouse CD16/CD32 (BD Pharmingen) before staining with 40 μL of mixture added to each sample. SRA was stained using rat anti-mouse SRA CD204 FITC (AbD Serotec, Oxford, UK) at 1:10 dilution in 10 μL stain buffer or the isotype control anti-body FITC rat IgG2a (BD Pharmingen) at 4:10 dilution in 10 μL stain buffer. BD Pharmingen suggested protocol was used in the staining process. After incubating 24 hours at 4°C the cells were suspended with 250 μL BD Pharmingen Stain Buffer (BSA), and SRA mean fluorescence was measured by a FACScalibur flow cytometer and analyzed using CellQuest software (BD Biosciences, Mountain View, CA).

Data Analysis

Each experiment was run in at least triplicate with an N = 3/ treatment group. The SRA mean fluorescence was recorded for each sample. The data are represented as mean and standard error of the mean for each group. Analysis of variance (ANOVA) and Holm-Sidak method were used to compare the means between the groups. P values of 0.05 or less were considered statistically significant. The SRA mean fluorescence results for each treatment group were then compiled into bar graphs.

RESULTS

The procedure of treating and staining the murine J774a.1 cells had three experimental replicates with 6 groups in each replicate and N=3 in each group. Once the SRA mean fluorescence for each replicate was recorded and a one-way ANOVA was run, the average of SRA mean fluorescence for the replicates of each treatment group were determined and bar graphs were created. Figure 1, displays the significant increase in SRA expression seen with the murine J774a.1 cells that were treated with ultra pure LPS compared to the control group. Specifically, murine J774a.1 cells treated with ultra pure LPS increased SRA expression by 53.4% compared to the media alone. Figure 1 also shows that in cells treated with PAM3CSK4 alone or PAM3CSK4 in combination with ultra pure LPS, SRA expression was not significantly affected by treatment.

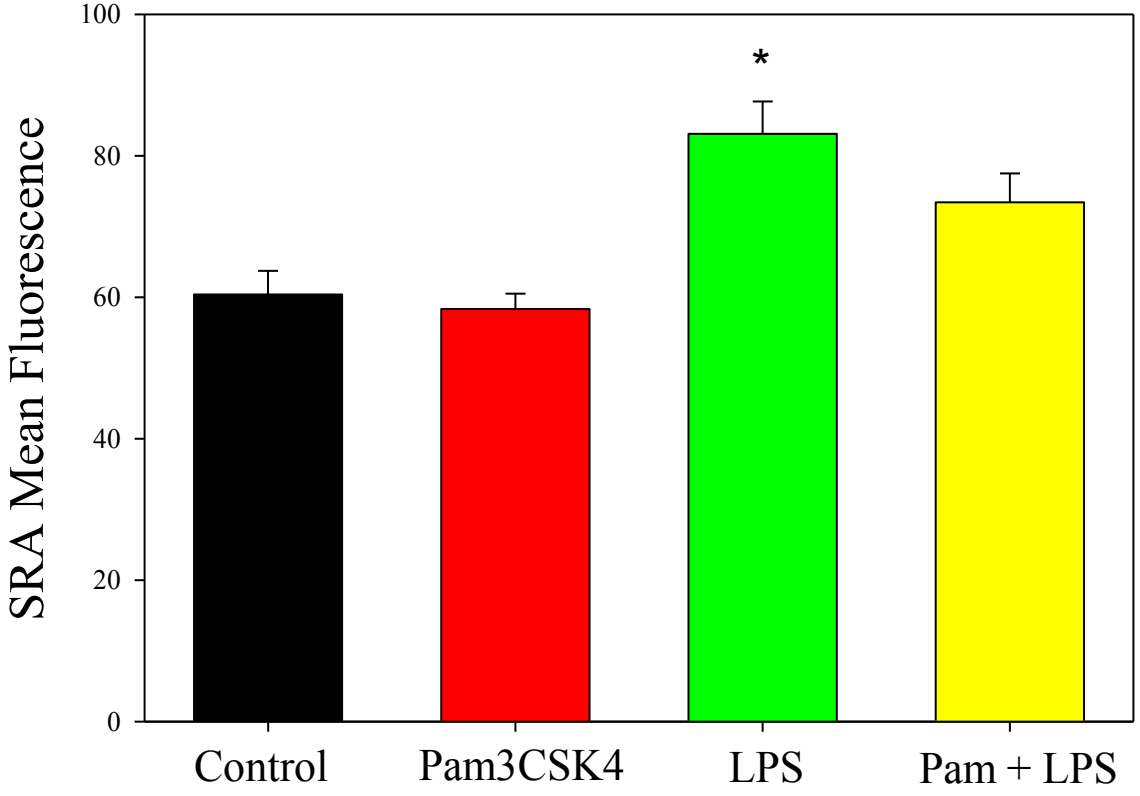

Figure 1. Murine J774a.1 cells were treated with PAM3CSK4, ultra pure LPS, or a combination of both and incubated for 24 hours. Cells were stained for SRA and analyzed by flow cytometry for SRA mean fluorescence. Cells treated with ultra pure LPS alone show a significant increase in SRA expression. (*indicates $p \leq 0.01$)

Figure 2, displays the significant increase in SRA expression seen with the murine J774a.1 cells that were treated with ultra pure LPS alone and in combination with glucan compared to the control group. Specifically, murine J774a.1 cells treated with ultra pure LPS in combination with glucan increased SRA expression by 47.7% compared to the media alone, control. Figure 2 also shows that cells treated with glucan alone were not significantly affected by treatment.

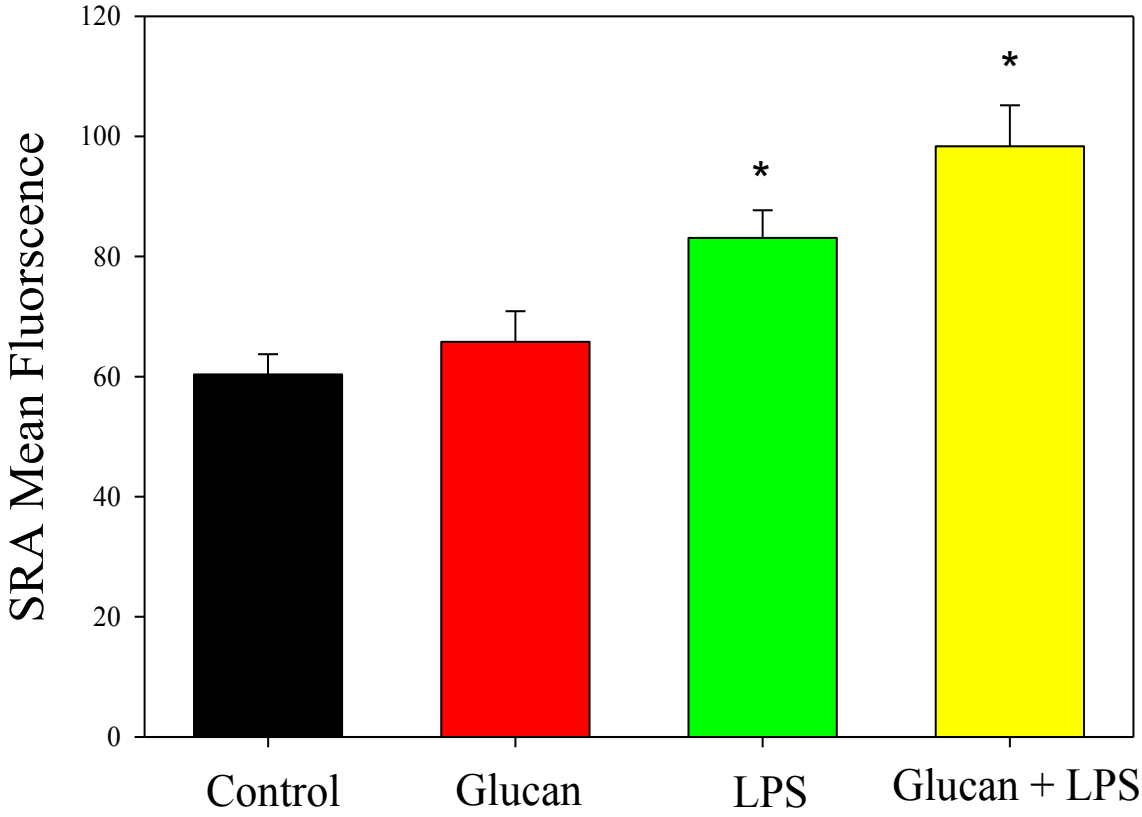

Figure 2. Murine J774a.1 cells were treated with glucan, ultra pure LPS, or a combination of both and incubated for 24 hours. Cells were stained for SRA and analyzed by flow cytometry for SRA mean fluorescence. Cells treated with LPS alone and in combination with glucan both show a significant increase in SRA expression. (*indicates $p \leq 0.01$)

DISCUSSION

This study produced a few important observations. One of the most important results observed was the 53.4% increase in SRA mean fluorescence when murine J774a.1 cells were treated with LPS, a TLR4 ligand. This suggests that TLR4 stimulation increases SRA expression on murine macrophages. This supports the idea that SRA plays a significant role in the morbidity

and mortality of sepsis via interaction or co-associating with TLR4 throughout sepsis. TLR4 is known to induce NFκB activity and to produce cytokines (14). Therefore, when SRA and TLR4 co-associate, the response TLR4 has from endogenous and exogenous ligands that are released during sepsis is heightened, which results in greater NFκB activity and greater inflammatory cytokine production. This stimulation of TLR4 is likely due to specific pathogen-associated molecular patterns (PAMPs) released during sepsis since it is known that SRA facilitates bacterial uptake like LPS and LTA (20). It has also been found that SRA deficient mice have a greater presence of bacteria in the blood in specific infections like *Listeria monocyotgenes* and *Neisseria meningitides* septicemia, but in *Pneumocystis carinii* infection SRA deficient mice were more effective at removing the organism from the lungs than the wild type mice controls (18, 19). This observation suggests that if the interaction between SRA and TLR4 or the activation of TLR4 by the LPS PAMP was blocked, then lower NFκB activity and cytokine production would be observed, thus reducing the overall inflammatory response with sepsis.

Another finding was that there was no significant increase in the SRA mean fluorescence for murine J774a.1 cells treated with PAM3CSK4, a TLR2 ligand, or glucan, a Dectin-1 ligand. This result indicates that TLR2 and Dectin-1 stimulation does not significantly increase SRA expression. However, when murine J774a.1 cells were treated with LPS and PAM3CSK4 there was no significant increase, suggesting that TLR2 stimulation or its activation by the PAM3CSK4 PAMP, prevents the increase in SRA expression seen in cells treated with LPS, stimulating TLR4. This may suggest a treatment strategy for sepsis, i.e. a treatment that stimulates TLR2 in order to prevent the significant increase in SRA expression observed in TLR4 stimulation alone. If this significant increase in SRA expression is prevented, then the overall inflammatory response to sepsis could be reduced. Further research needs to be done to

determine the role TLR2 stimulation plays in preventing the increase in SRA expression with TLR4 stimulation. In contrast, when murine J774a.1 cells were treated with glucan and LPS in combination, SRA was still significantly increased due to the probable interaction of TLR4 and SRA. Therefore, Dectin-1 stimulation in combination with TLR4 stimulation does not inhibit the significant increase in SRA expression found with TLR4 stimulation alone.

To conclude, our results support the concept that TLR4 interacts with SRA to increase expression in the inflammatory response in sepsis. Further research is needed to determine the specific interaction between SRA and TLR4, to determine a mechanism to block this interaction or activation of TLR4 by the LPS PAMP. This blocking could result in a reduced inflammatory response in sepsis. Further research is also needed to determine the mechanism by which TLR2 stimulation prevents the increase in SRA expression with TLR4 stimulation in combination. With further research and determination of the mechanism responsible for these results, a treatment for reducing the overall inflammatory response in sepsis could be developed.

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