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Short term statin treatment improves survival and differentially regulates macrophage-mediated responses to *Staphylococcus aureus*

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Abstract

Staphylococcus aureus is the most prevalent etiologic agent of sepsis. Statins, primarily prescribed for their cholesterol-lowering capabilities, may be beneficial for treating sepsis due to their anti-inflammatory properties. This study examined the effect of low dose, short term simvastatin pretreatment in conjunction with antibiotic treatment on host survival and demonstrated that pretreatment with simvastatin increased survival of C57BL/6 mice in response to *S. aureus* infection. *In vitro* studies revealed that short term simvastatin pretreatment did not reduce *S. aureus*-stimulated expression of surface proteins necessary for macrophage presentation of antigen to T cells, such as MHC Class II and co-stimulatory molecules CD80 and CD86, but did reduce both basal and *S. aureus*-stimulated levels of C5aR. Additionally, this work demonstrated the ability of simvastatin to dampen macrophage responses initiated not only by bacteria directly but by membrane vesicles shed in response to infection, revealing a new mechanism of immune modulation by statins. These data demonstrate the ability of short term simvastatin pretreatment to modulate immune responses and identify new insights into the underlying mechanisms of the anti-inflammatory properties of simvastatin that may decrease the pathophysiological effects leading to sepsis.

Keywords

COMPLEMENT; INFLAMMATION; MACROPHAGE; MEMBRANE VESICLE; SEPSIS; SIMVASTATIN; STAPHYLOCOCCUS AUREUS

Introduction

Invasion of host tissue by a pathogen results in the activation of a variety of innate immune responses. Complement is a collection of serum proteins that are activated in response to pathogens and serve to contain and destroy the pathogen as well as initiate and aid innate immune responses [1]. Upon pathogen encounter, tissue macrophages are activated, increasing the expression of proteins on their cell surface, production of cytokines,

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phagocytic ability, and killing mechanisms. Furthermore, activated macrophages initiate and coordinate an inflammatory response that induces local vascular changes, allowing fluid, proteins, and cells to accumulate at the site of infection to contain and eliminate the pathogen [2]. In addition to the activities of complement and macrophages, there is increasing evidence of membrane vesicles (microparticles, microvesicles, ectosomes, exosomes) in regulating immune processes. Membrane vesicles are released from a variety of cell types, which can be enhanced during infection. These vesicles have immunostimulatory and immunosuppressive abilities, regulating the activation and function of immune cells and subsequently influencing inflammatory immunity [3–5]. Despite the multitude of innate immune responses, not all pathogens are contained locally. The spread of a pathogen results in a systemic inflammatory response, referred to as sepsis [2].

S. aureus is a Gram-positive, opportunistic pathogen that can cause a range of health conditions, from minor skin infections to more serious conditions including pneumonia, toxic shock syndrome, and meningitis. In the United States, *S. aureus* is the most prevalent bacterial pathogen causing infections in hospital inpatients and is the second leading cause of bacterial infections in outpatients [6]. Specifically, *S. aureus* is the most prominent pathogen causing bloodstream infections that result in sepsis [7, 8], and bloodstream infections caused by *S. aureus* are associated with the highest mortality [9].

The increasing frequency of *S. aureus* infections is primarily attributed to increases in the use of invasive procedures, and numbers of immunocompromised patients [10–12]. Current antibiotic therapies are increasingly ineffective for treating *S. aureus* infections, prompting a need to develop alternative and adjunctive therapies. Recently, statins have been shown to have beneficial effects on cardiovascular processes, independent of lowering cholesterol, and these benefits extend to effects on inflammatory diseases, highlighting the potential anti-inflammatory properties of statin drugs [13]. There is mounting evidence for the prospective usefulness of statin drugs in a variety of inflammation-driven diseases. Recent studies have demonstrated a role for statin therapy in the treatment of sepsis. LPS-induced sepsis in a murine model demonstrated that pretreatment with cerivastatin increased survival [14]. Similarly, mice treated with cerivastatin 24 hours pre- and up to 72 hours post- LPS or live bacterial challenge were protected from sepsis-related death [15]. Furthermore, mice pretreated with simvastatin had improved survival in response to sepsis induced via cecal ligation and perforation (CLP) [16], and simvastatin treatment prior to CLP induction of sepsis in a mouse burn model increased survival [17]. Low-dose, long- term simvastatin treatment also has been found to increase survival in a murine model of *S. aureus*-induced sepsis [18]. Several clinical studies provide further evidence of statins as possible effective treatments for sepsis and support the findings from studies using mouse models [13]. Taken together, the results from these studies demonstrate the potential for statins as an effective treatment for sepsis. However, the data are not fully conclusive and a better understanding of how statins achieve these beneficial effects through their modulation of immune responses is necessary. Toward this end, simvastatin was examined in a murine model of systemic infection to assess effects on immune responses and survivability and macrophage-mediated innate immune responses elicited by *S. aureus* infection were assessed *in vitro*.

Materials and methods

Cell culture

HUVEC (human umbilical vein endothelial cells, Life Technologies, Carlsbad, CA) were grown in filter-sterilized M200 (Life Technologies) antibiotic-free media (M200 supplemented with low serum growth supplement (Life Technologies) at 37°C in 5% CO_2. RAW 264.7 macrophages (American Type Culture Collection, Manassas, VA) were grown in filter-sterilized DMEM (Lonza, Allendale, NJ) antibiotic-free media supplemented with 1% v/v L-glutamine (BioWhittaker) and 10% v/v FBS (Atlanta Biologicals, Lawrenceville, GA) or RPMI-1640 (BioWhittaker) supplemented with 1% v/v L-glutamine (BioWhittaker) and 10% v/v FBS (Atlanta Biologicals) at 37°C in 5% CO_2.

S. aureus preparation

Methicillin-sensitive *Staphylococcus aureus* (MSSA) (#29213, ATCC) were cultured in tryptic soy broth (TSB) (Sigma-Aldrich, St. Louis, MO) at 37°C shaking at 200 rpm. Bacteria were washed and diluted to 3×10^8 bacteria/mL. This bacterial strain was selected based upon extensive characterization of the inhibition of invasiveness by simvastatin [20].

Mice and infection model

All protocols were approved by the Ball State University Animal Care and Use Committee prior to initiation of each study. C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were housed individually in filtered cages. Mice were separated into groups based on treatments with no fewer than three mice per group. Based on average body weight of males and females separately for each study, a stock of 10 mg/ml of simvastatin (Calbiochem [EMD Chemicals], Gibbstown, NJ), dissolved in ethanol, was diluted with saline for a final dosage of 1000 ng simvastatin per gram of mouse body weight and a total injection volume of 100 μL. For each study, mice in experimental groups received simvastatin 18 and 3 hours prior to *S. aureus* infection via intraperitoneal (i.p.) injection. Control groups were given 1% ethanol in saline as a vehicle control. Mice were inoculated with *S. aureus* at 1×10^7 colony forming units (cfu) by i.p. in 1 mL of 5% mucin (BD, Franklin Lakes, NJ) diluted in 0.85% sterile saline. Based on average weight, 10 mg of gentamicin (Sigma-Aldrich) per kg of mouse body weight was administered in saline in a final volume of 100 μL. The dosage for gentamicin was at the ED30 (effective dose for 30% survival). This dosage was used in order to detect augmented protection by simvastatin, if it existed. Gentamicin was used because it is poorly permeable to host cell membranes and was previously used in the *in vitro* studies to determine inhibition of invasiveness with this strain of *S. aureus* [20]. All mice received gentamicin via i.p. injection at 3, 6, 12, 24, and 48 hours post-infection.

Analysis of *S. aureus* in the blood

Mice were euthanized at 24 or 48 hours post-infection via pentobarbital injection (5 mg in saline) and blood was collected via cardiac puncture. Whole blood (100 μL) was plated on pre-warmed tryptic soy agar (TSA) (Sigma-Aldrich) supplemented with 7.5% sodium chloride for selective growth of *S. aureus*. Following overnight incubation of plates at 37°C, cfu were counted.

S. aureus invasion assay

S. aureus was inoculated from glycerol stock in tryptic soy broth (Sigma-Aldrich) and subcultured daily for 2 days prior to running the assay (200 rpm, 37°C, 24 h). On the day of the assay, bacteria were pelleted (10000 rpm, 37°C, 3 min), washed in saline, pelleted as above, resuspended in saline, then fluorescently labeled by incubation with rabbit anti-mouse IgG Alexa Fluor 488 (final concentration 8 μg/ml, RT, 20 minutes, Invitrogen). Protein A, a *S. aureus* cell surface protein, avidly binds IgG thereby labeling the bacteria. Labeled bacteria were washed twice as above and resuspended to 2.3×10^8 cfu/ml in saline. Cell cultures of THP-1 (ATCC, TIB-202) were collected and resuspended to a cell density of 3×10^5 cells/ml in 35 mm dishes in RPMI-1640 (ATCC, 30-2001) supplemented with 10% fetal bovine serum (Atlanta Biologicals)/0.1% 2-mercaptoethanol (Life Technologies). Following incubation with simvastatin (Calbiochem [EMD Chemicals]) or DMSO (Thermo Fisher Scientific) as the negative control (37°C, 5% CO_2, 20 h), host invasion was initiated by addition of 488-labeled *S. aureus* at a multiplicity of infection of 30 (37°C, 5% CO_2, 1 h). Invasion was stopped by placing plates at 4°C (10 minutes). Extracellular bacteria were labeled by incubation with anti-Staph polyclonal antibody, raised against soluble and structural antigens to *S. aureus*, (final concentration 16 μg/ml, 4°C, 40 min, Meridian Life Science, Saco, ME) followed by incubation with goat anti-rabbit 555 (final concentration 8 μg/ml, 4°C, 40 min, Life Technologies). Cells were fixed in a final concentration of 1.5% formaldehyde and samples read using the Accuri C6 cytometer (BD, San Jose, CA).

Membrane vesicle preparation and cellular stimulation

Tissue culture plates (100 mm and 35 mm) were treated with Attachment Factor (Life Technologies). HUVEC (1.2×10^6) were seeded on 100 mm plates, and RAW cells were seeded at 2.0×10^5 cells on 35 mm plates. Cells were allowed to grow overnight at 37°C at 5% CO_2. HUVEC and RAW cells were then either treated with 1 μM simvastatin (Calbiochem [EMD Chemicals]) or 1 μM DMSO (Thermo Fisher Scientific) overnight. All HUVEC plates were infected (post-simvastatin or DMSO treatment) for 2 hours with 6×10^8 bacteria (MOI of 300) in saline to induce microvesicle formation due to MSSA infection. The HUVEC supernatant was removed from the plates and centrifuged at 1500g in 15 ml conical tubes for 10 minutes at 10°C to remove HUVEC fraction. Supernatant was incubated with 80 μg/mL gentamicin (Sigma-Aldrich), and 32 μg/mL lysostaphin (Sigma-Aldrich) for 40 minutes at 37°C in 5% CO_2, mixing every 10 minutes (previously determined to rid microvesicle preparation of MSSA). Tubes were centrifuged at 17000g for 30 minutes at 4°C, and the supernatant was completely removed. One mL of cold RAW media was used to resuspend the microvesicle pellet, and 200 μL of the microvesicle preparation was added to the corresponding simvastatin or DMSO treated RAW plates and incubated at 37°C in 5% CO_2. Following overnight incubation, supernatant and RAW cells were harvested for analysis. An additional 200 μL of the microvesicle preparation from simvastatin and DMSO groups was diluted in 2 ml cold TSB and spread on a TSA plate to confirm no MSSA remained in the supernatant.

S. aureus infection of RAW macrophages

RAW cells were plated onto 35mm plates at 2.5 X 10^5 cells/ml in supplemented RPMI (BioWhittaker). Cells were pre-treated with 1 μM simvastatin (Calbiochem [EMD Chemicals]) or DMSO (Thermo Fisher Scientific) for 18–24 hours prior to infection. Each plate received either 1 X 10^8 bacteria (MOI 400) or an equal volume of 0.85% saline and incubated at 37°C and 5% CO_2. After one hour, media from each plate was replaced with media containing 50 μg/ml Gentamicin (Sigma-Aldrich) and 20 μg/ml Lysostaphin (Sigma-Aldrich) and incubated for 12–24 hours before analysis of cell surface marker expression by flow cytometry (described below).

Flow cytometry

RAW macrophages (1×10^6 cells per sample), stimulated by microvesicle treatment or infected with *S. aureus* as described above, were washed and stained in FACS buffer (PBS containing 2% BSA and 0.1% NaN_3). Cells were first incubated with diluted normal rat serum (Jackson ImmunoResearch, West Grove, PA) for 5 minutes at 4 °C then washed once in FACS buffer. Samples were stained with antibodies (MHC Class II, CD80, CD86, CD40, and C5aR) conjugated to FITC, PE, CyChrome, or allophycocyanin (eBioscience, San Diego, CA). Staining was performed at 4°C for 15 minutes. Cells were washed and then fixed in FACS buffer containing 0.5% formaldehyde. Samples were analyzed using an Accuri C6 flow cytometer. Mean fluorescence intensity was recorded and normalized to the background fluorescence intensity of the negative control.

Enzyme-linked immunosorbant assay (ELISA)

Levels of TNF-alpha present in the supernatants of microvesicle-treated RAW cells were detected using a TNF-alpha ELISA kit (eBioscience). The procedure was performed according to the manufacturer's guidelines. Samples were analyzed in duplicate using a microplate reader (Model 680, Bio-Rad).

Statistical analyses

Results are represented as mean +/− standard error of the mean (SEM). For the survival studies, statistical differences were assessed by the Kaplan-Meier Log-Rank test. For flow cytometric analysis of cell surface marker expression on macrophages, one way ANOVA was performed followed by Student Neuman-Keuls post-hoc analysis. For the bacterial clearance assays, the Mann-Whitney Rank Sum test was performed. Student's *t*-test was used for assessing all other assays. Differences between groups were considered statistically significant at $p \leq 0.05$.

Results

Simvastatin pretreatment increases survival of mice infected with *S. aureus*

To investigate the effect of low dose (below the predicted range of therapeutic dosage [21]) and short term (less than 24 hours) simvastatin pretreatment on the induction and progression of sepsis due to *S. aureus* infection, C57BL/6 mice were pretreated with simvastatin or vehicle control prior to infection with *S. aureus*. It has been recently reported

that the resistance of C57BL/6 mice to *S. aureus* infection is dependent upon innate immune mechanisms, due in part to their ability to efficiently recruit neutrophils to the site of infection [19]. Furthermore, strains of mice that are highly susceptible to *S. aureus* infection, such as Balb/c, demonstrate impaired neutrophil recruitment that prevents them from quickly mobilizing an effective innate response to control the infection [19]. Thus, C57BL/6 mice were used for this survival study to examine the ability of simvastatin to affect immune responses under normal innate immune conditions. Antibiotic treatment at the ED30 was given post-infection to examine whether simvastatin augmented the antibiotic therapy. Survival was assessed for 14 days and demonstrated that mice pretreated with simvastatin prior to infection with *S. aureus* had increased survival compared to control mice (Figure 1).

Clearance of *S. aureus* from the blood is not enhanced by simvastatin pretreatment

At higher doses, simvastatin has been reported to have anti-microbial effects on *S. aureus in vitro* [22] and *in vivo* higher dosage [23] or longer duration [18] improved clearance. To investigate the possibility that short term, low-dose simvastatin treatment may enhance the clearance of *S. aureus*, levels of *S. aureus* in the blood were examined at 24 and 48 hours post-infection in mice pretreated with simvastatin or vehicle control. Twenty-four hours post-infection, no significant difference in numbers of bacteria in the blood between simvastatin pretreated and vehicle pretreated mice was observed (Figure 2a), and by 48 hours, almost all of the bacteria were cleared from the blood in both treatment groups (Figure 2b). These data demonstrate that the increased survival to severe *S. aureus* infection of simvastatin-treated mice is independent of bacterial clearance.

Macrophage functions are altered in response to short term simvastatin pretreatment

Macrophages are important orchestrators of inflammatory reactions and thus modulation of macrophage activation and function may affect the initiation and progression of sepsis and subsequent survival of the host to infection [2]. To investigate the ability of short term simvastatin pretreatment to modulate macrophage activities in response to *S. aureus* infection, macrophage activities were investigated *in vitro*. Following pathogen invasion of host tissue, macrophages are stimulated to ingest invading pathogens. Previous reports examining the effects of statin treatments on the phagocytic abilities of monocytes and macrophages have demonstrated the ability of statins to both enhance [24, 25] and inhibit [26, 27] phagocytosis. To examine the effects of short term simvastatin pretreatment on the phagocytic activity of macrophages in response to *S. aureus* infection, macrophages were pretreated with simvastatin or vehicle control and infected with Alexa Fluor 488-labeled *S. aureus*. Following invasion, extracellular bacteria were made distinguishable from intracellular bacteria by post-labeling of extracellular bacteria with an antibody to soluble and structural antigens to *S. aureus* raised in rabbit -followed by anti-rabbit Alexa Fluor 555. Macrophages were then analyzed by flow cytometry to assess bacterial internalization (Figure 3). The results demonstrated that macrophages pretreated with simvastatin had reduced *S. aureus* invasion compared to vehicle control treated cells.

Pathogen invasion not only enhances phagocytosis by macrophages, but also induces the production of a large amount of pro-inflammatory cytokines, which can be further enhanced by the presence of the complement protein C5a. C5aR expression is enhanced on

macrophages in response to pathogen invasion and its binding of C5a augments the production of pro-inflammatory cytokines by macrophages, contributing to a septic pathology [28]. To investigate the effect of short term simvastatin pretreatment on C5aR expression, simvastatin or control treated RAW macrophages, mock-infected or infected with *S. aureus*, were examined by flow cytometry (Figure 4). Simvastatin pretreatment resulted in lower basal expression of C5aR on mock-infected macrophages and decreased C5aR expression stimulated by *S. aureus* infection compared to controls. These data suggest that simvastatin may modulate complement activity stimulated by *S. aureus* infection by down-regulating the expression of complement receptors, decreasing the binding of complement proteins and subsequently decreasing the activation of cells such as macrophages.

In human macrophage cell lines, simultaneous treatment with simvastatin and IFN-gamma results in decreased expression of activation markers such as MHC Class II, CD40, CD80, and CD86 compared to IFN-gamma-stimulated controls [29]. To investigate the effect of short term simvastatin pretreatment on gene expression in macrophages, the expression of cell surface activation markers on simvastatin or control treated RAW macrophages mock-infected or infected with *S. aureus* was examined by flow cytometry (Figure 5). Short term simvastatin pretreatment did not reduce the expression of *S. aureus*-stimulated activation marker expression on RAW macrophages nor alter the basal expression level of the cell surface proteins. Taken together these data suggest that short term simvastatin pretreatment is sufficient to decrease phagocytic activity and subsequent uptake of bacterial pathogens as well as complement receptor expression but does not affect the expression of cell surface proteins indicative of macrophage activation.

Macrophage activation stimulated by membrane vesicles from *S. aureus*-infected cells is dampened by short term simvastatin pretreatment

Recent studies have demonstrated that the membrane vesicles, released from a variety of different cells, interact with immune cells and can modulate immune responses [4]. Specifically, membrane vesicles released from infected macrophages can interact with uninfected macrophages and induce a pro-inflammatory response [30]. *S. aureus* invasion of host tissue results in its uptake by immune and non-immune cells, and recently pretreatment with simvastatin has been shown to inhibit *S. aureus* internalization by endothelial cells [20]. To investigate the influence of short term simvastatin pretreatment on membrane vesicle release by infected non-immune cells and subsequent activation of macrophages, simvastatin or control treated HUVEC were infected with *S. aureus* and membrane particles were harvested from the supernatant. Simvastatin or vehicle control treated RAW macrophages were then incubated with the isolated membrane particles for 24 hours and levels of TNF-alpha were analyzed by ELISA (Figure 6). Simvastatin pretreatment of HUVEC and RAW macrophages resulted in decreased TNF-alpha production compared to controls, demonstrating the ability of short term simvastatin pretreatment to modulate the stimulating activity of membrane vesicle transfer to immune cells, resulting in dampened macrophage activation.

Discussion

This study demonstrated the ability of low dose, short term simvastatin pretreatment to enhance survival to *S. aureus* infection and highlighted novel mechanisms by which short term simvastatin pretreatment modulates macrophage-mediated innate immune responses to *S. aureus*. The beneficial effect of statin treatment on the ability of mice to survive sepsis-related death due to *S. aureus* infection has been investigated previously [15]. The findings from our work compliment the previous study by demonstrating the ability of simvastatin as only a short term pretreatment to induce systemic changes prior to *S. aureus* exposure that result in enhanced survivability following infection. In the previous study, cerivastatin was administered to Balb/c mice 24 hours prior to challenge with *S. aureus* and treatments continued for 72 hours post-infection. In contrast, our findings demonstrate that two doses of simvastatin (below the range of therapeutic dosage) administered to C57BL/6 mice within 24 hours prior to *S. aureus* challenge with no continuing treatments is sufficient to increase survival. Additionally, this previous study did not incorporate antibiotics into the study design. This study administered gentamicin at the ED30 for 48 hours post-infection to examine if antibiotic efficacy could be enhanced by simvastatin treatment. Possibly, the failure to observe a difference in bacterial clearance in our study (Figure 2) is due to the presence of antibiotics, lending further support that our design revealed a role for immunomodulation over and above clearance.

Our *in vitro* investigations examining macrophage-mediated innate immune mechanisms demonstrated novel mechanisms by which simvastatin may down-regulate hyper-inflammatory reactions that contribute to a septic pathology; through regulation of membrane vesicle activity and complement receptor surface expression. Our finding that simvastatin pretreatment did not reduce *S. aureus*-stimulated expression of surface proteins necessary for macrophage presentation of antigen to T cells (Figure 5), but did reduce both basal and *S. aureus*-stimulated levels of C5aR (Figure 4), which is a stimulator of macrophage inflammatory activities when engaged, demonstrates specificity and selectiveness by simvastatin to modulate these immune responses. This finding supports a previous report examining the effects of simvastatin on macrophages stimulated with opsonized *S. aureus*. Simvastatin impaired the bactericidal response (phagocytosis and oxidative burst) of macrophages to IgG-coated *S. aureus* and enhanced the production of inflammatory mediators such as TNF-alpha through a mechanism dependent upon FcgammaR since non-opsonized *S.aureus* did not elicit enhanced production of inflammatory mediators [31]. Our findings, together with Benati et al., highlight the ability of simvastatin to differentially regulate gene expression and cellular functions when encountering opsonized and non-opsonized *S. aureus* and demonstrate an important selective property of simvastatin that may be a necessary aspect of its anti-inflammatory properties and ability to increase survival of mice infected with *S. aureus*.

Interestingly, in our mouse model, C5a serum levels were not different between control and simvastatin-pretreated mice (data not shown), which would suggest that this aspect of complement activity is unaffected by simvastatin. Indeed, reports examining the effects of statin treatments on complement activity and complement-mediated inflammation in a variety of *in vivo* models have been conflicting [32–39]. However, our finding that

simvastatin treatment reduced basal and *S. aureus*-stimulated levels of C5aR on macrophages *in vitro* suggests modulation of complement activity by simvastatin is a result of alterations in receptor expression, and potentially provides an explanation for the conflicting reports found in the literature While it has been previously shown that simvastatin treatment impairs surface protein recycling [40], the demonstration of this ability applied to surface receptors intimately involved in the pathogenesis of sepsis is novel and highlights a potential mechanism through which simvastatin mediates its anti-inflammatory effects.

Importantly, our work has revealed a unique mechanism through which simvastatin regulates macrophage activation. Recent work indicated that membrane vesicles released in response to bacterial infection stimulate an inflammatory response in uninfected, recipient macrophages [30]. Therefore, we investigated whether this inflammatory response would be dampened by simvastatin treatment of the infected host cells and of the recipient, non-infected macrophage and found that simvastatin attenuated the robust pro-inflammatory response of recipient macrophages. This finding is in contrast to previous work demonstrating simvastatin's ability to enhance the production of pro-inflammatory mediators [31], while seemingly contrasting, the two studies actually provide more evidence for the very specific affects that simvastatin exerts on cellular functions such that the previous work was examining FcgammaR-stimulated functions, while our study examined membrane vesicle-stimulated functions. Potentially, simvastatin's specific dampening of the early macrophage response to not only bacterial invasion but also to membrane vesicles is a modulatory mechanism regulating the hyper-inflammatory status that is the hallmark of sepsis.

Conclusion

This study identified the ability of short term simvastatin pretreatment to differentially regulate macrophage functions: dampening activities that increase macrophage activation, such as phagocytosis, bacterial uptake, C5aR expression, and sensitivity to membrane vesicles, while leaving functions necessary for the stimulation of adaptive responses unaffected. Additionally, our finding that pro-inflammatory, macrophage-mediated responses were initiated not only by bacteria directly but by membrane vesicles shed in response to infection reveal that the immunomodulatory role of statins during infection extends beyond direct interaction with bacterial pathogens, potentially dampening the overall hyper-responsiveness through multiple mechanisms at the level of the host and potentially decreasing the pathophysiological effects leading to sepsis. Taken together these data identify new insights into the underlying mechanisms of the anti-inflammatory properties of simvastatin and highlight its ability to be a very specific immunomodulatory agent.

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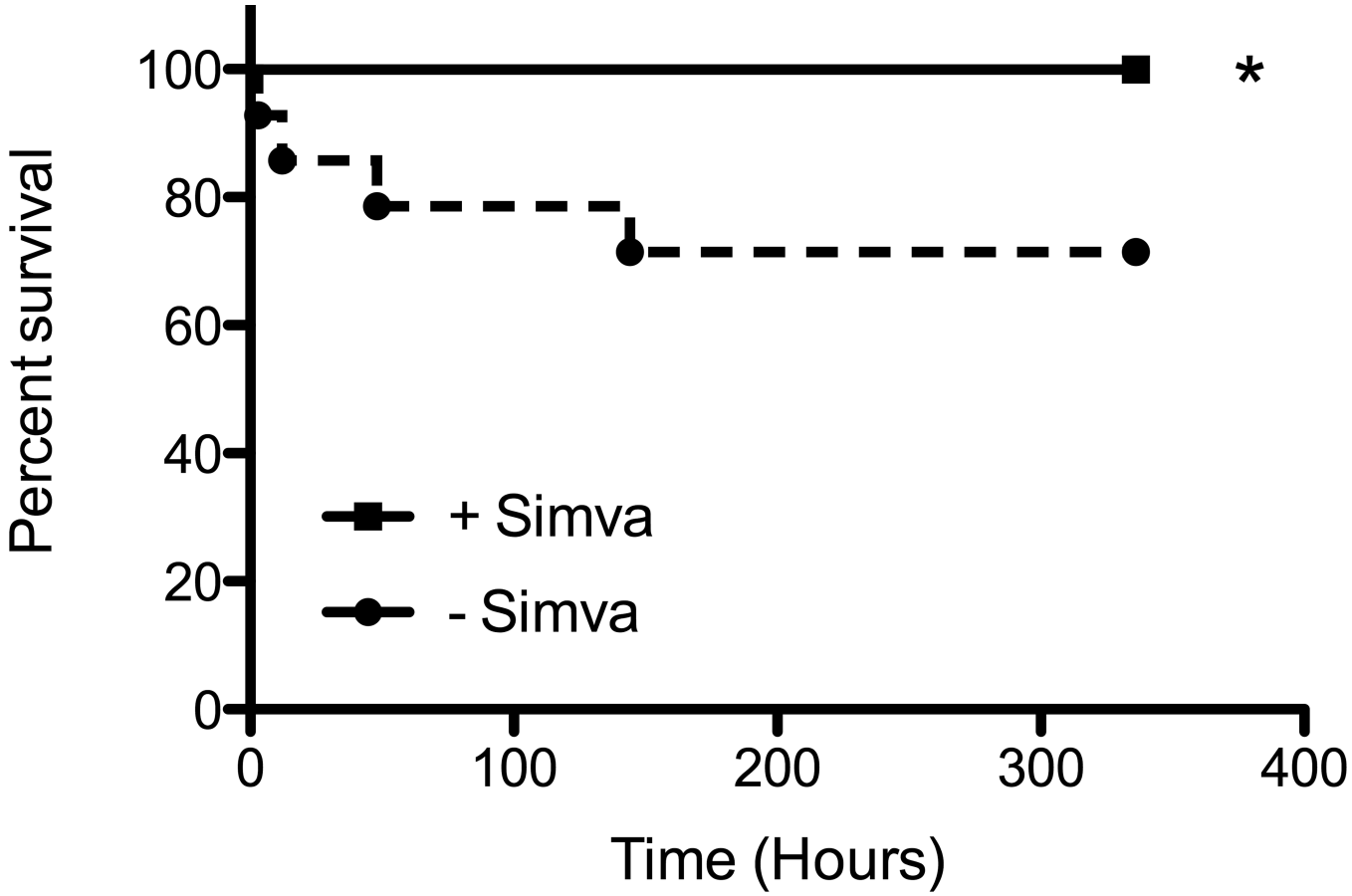

Figure 1. Simvastatin pretreatment increases survival of mice infected with *S. aureus*

Male and female mice were pretreated with simvastatin (+Simva) or vehicle control (−Simva) 18 and 3 hours prior to infection with *S. aureus*, treated with gentamicin 3, 6, 12, 24, and 48 hours post-infection and observed for 14 days. The graph represents individual data points combined from 3 replicate studies (n=13–14/group). * $p \leq 0.05$ by Kaplan-Meier Log Rank analysis.

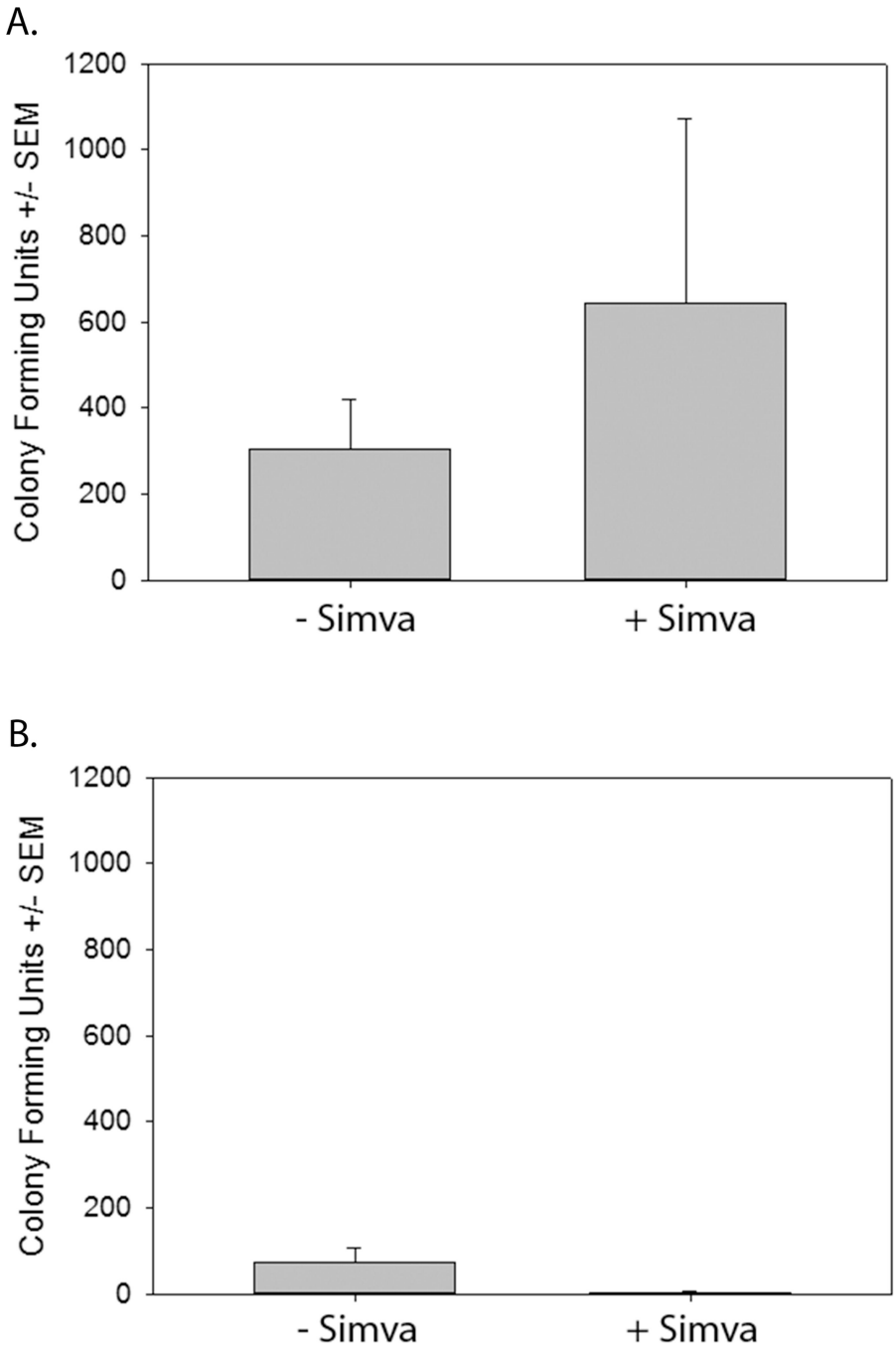

Figure 2. Clearance of *S. aureus* from the blood is not enhanced by simvastatin pretreatment

Mice were pretreated with simvastatin (+Simva) or vehicle control (−Simva) 18 and 3 hours prior to infection with *S. aureus* and treated with gentamicin 3, 6, 12, 24, and 48 hours post-infection. Whole blood was isolated 24 hours (a) and 48 hours (b) post infection and plated on tryptic soy agar. Data were pooled from 2 replicate experiments (n=5–10/group) and analyzed using the Mann-Whitney rank sum test.

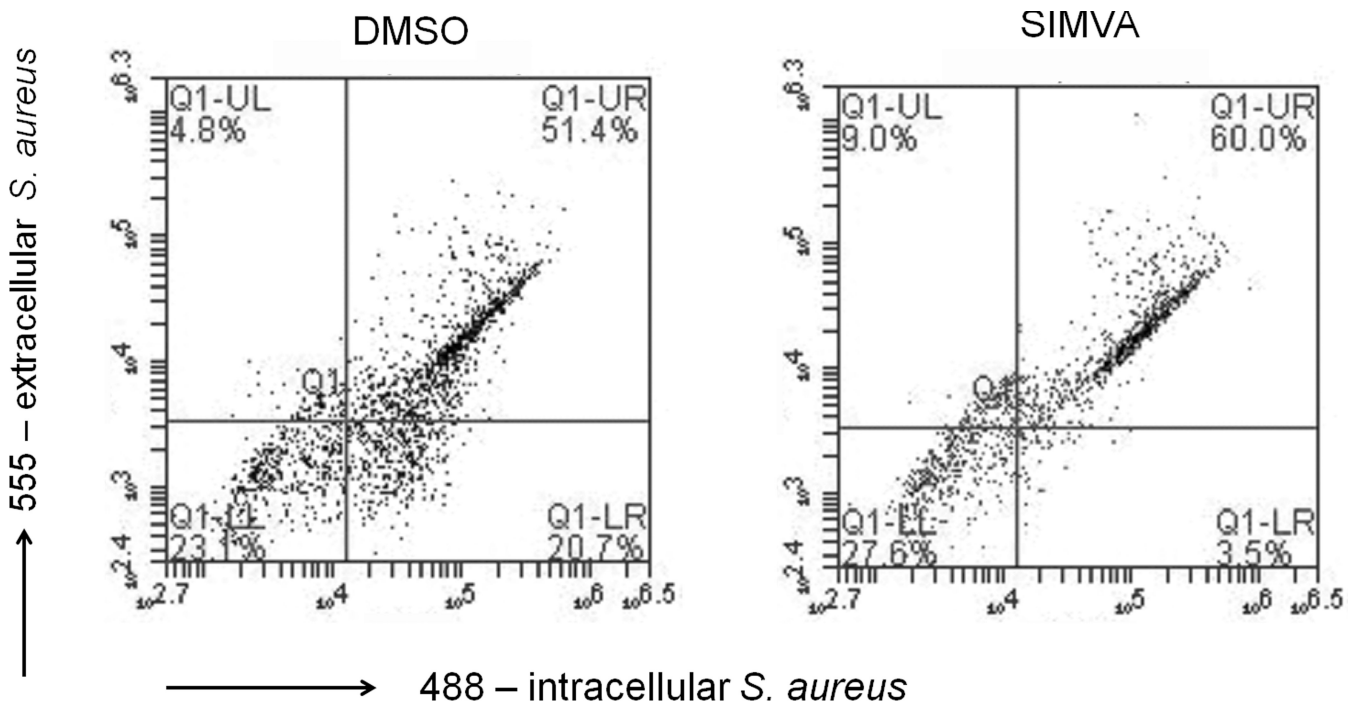

Figure 3. Simvastatin inhibits *S. aureus* host cell invasion

THP-1 were pretreated with 10 μM simvastatin (SIMVA) or dimethyl sulfoxide (DMSO) for 20 hours followed by invasion by Alexa Fluor 488-labeled *S. aureus* (1 hour). Following invasion, extracellular bacteria are made distinguishable from intracellular bacteria by post-labeling extracellular bacteria with anti-*S. aureus* followed by anti-rabbit Alexa Fluor 555. Lower right quadrant indicates intracellular, 488-labeled bacteria. Data is representative of 3 experiments.

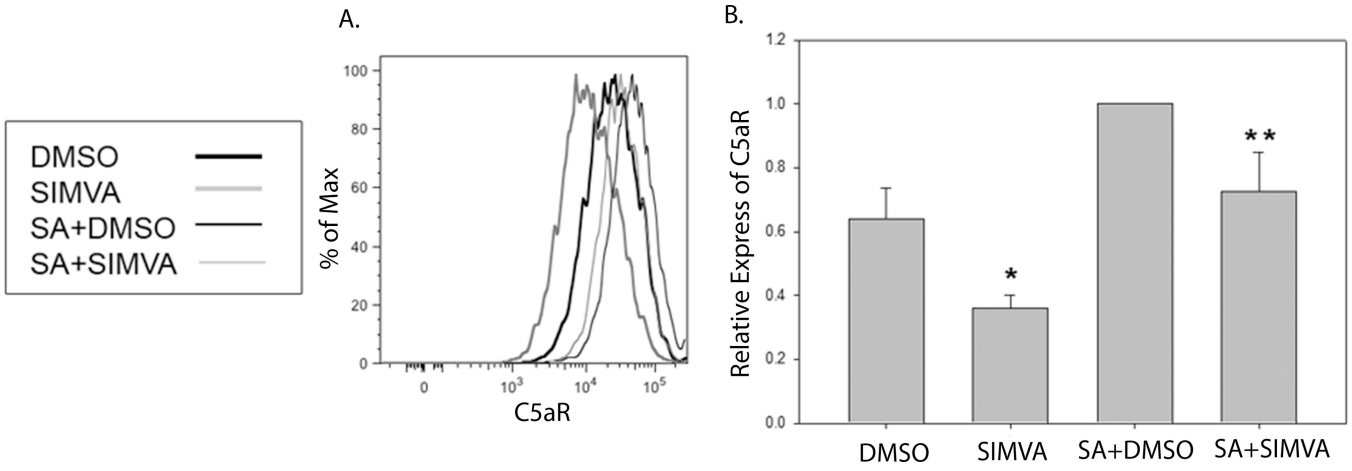

Figure 4. Simvastatin pretreatment reduces C5aR expression on macrophages

RAW cells were treated with 1 μM simvastatin (SIMVA) or dimethyl sulfoxide (DMSO) 18–20 hours prior to infection with *S. aureus* for 1 hour (SA+DMSO and SA+ SIMVA). The infection was stopped and cells were analyzed 18 hours post-infection for expression of C5aR. Histograms (a) are from one experiment that is representative of 3 total experiments. Normalized mean fluorescence intensity (b) was pooled from all experiments and analyzed by one-way ANOVA followed by Student Neuman-Keuls post-hoc analysis. *$p \leq 0.05$ compared to DMSO. **$p \leq 0.05$ compared to SA+DMSO.

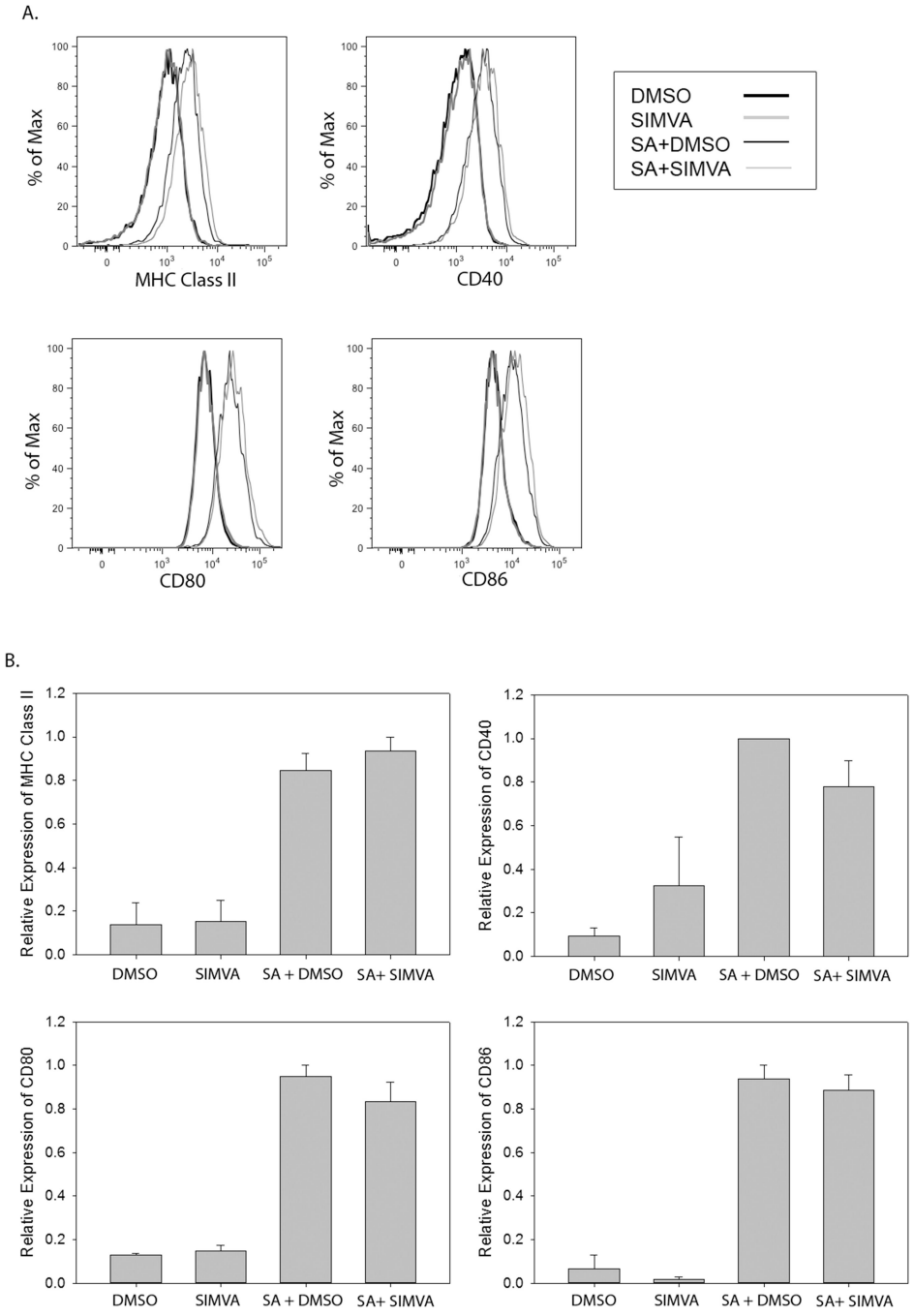

Figure 5. Simvastatin does not alter the expression of *S. aureus*-induced surface proteins on macrophages

RAW cells were treated with 1 μM simvastatin (SIMVA) or dimethyl sulfoxide (DMSO) 18–20 hours prior to infection with *S. aureus* for 1 hour (SA+DMSO and SA+ SIMVA). The infection was stopped and cells were analyzed 18 hours post-infection for expression of MHC Class II, CD40, CD80, and CD86. Histograms (a) are from one experiment that is representative of 3 total experiments. Normalized mean fluorescence intensity (b) was

pooled from all experiments and analyzed by one-way ANOVA followed by Student Neuman-Keuls post-hoc analysis.

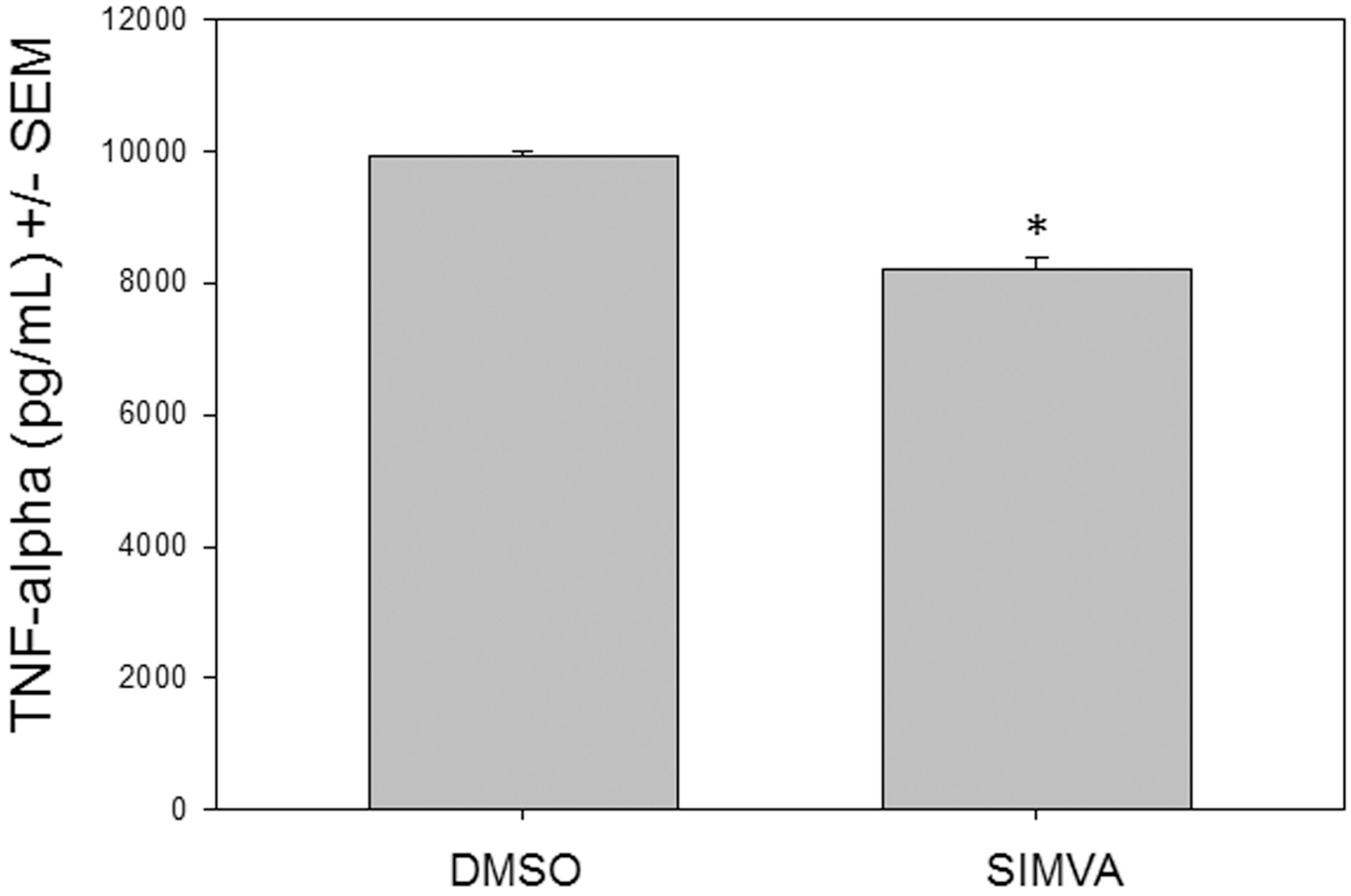

Figure 6. Macrophage activation stimulated by membrane vesicles (MV) from *S. aureus*-infected cells is dampened by simvastatin pretreatment

RAW cells were pretreated with vehicle control (DMSO) or with1 μM simvastatin (SIMVA) for 20 hours, and then incubated for 2 hours with MV harvested from pretreated, infected human umbilical vein endothelial cells. TNF-alpha was measured at 24 hours by ELISA. *$p \leq 0.05$ by Student's *t*-test.