THE EFFECTS OF SIMVASTATIN PRETREATMENT ON INNATE IMMUNE RESPONSES TO *STAPHYLOCOCCUS AUREUS* INFECTION

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RESULTS

Simvastatin does not enhance clearance of S. aureus
Complement C5a was not decreased in septic C57BL/6 mice
Downward trend in TNFα in simvastatin pretreated mice

DISCUSSION
Introduction

Sepsis is a systemic inflammatory response that causes increased heart rate and respirations, fever, and inadequate blood flow to organs. One of the most prevalent causes of sepsis is the Gram-positive bacterium *Staphylococcus aureus* (*S. aureus*). With *S. aureus* becoming increasingly resistant to antibiotics, new methods for the treatment and clearance of sepsis caused by *S. aureus* are needed. Studies have shown that the lipid lowering drug simvastatin is protective against incidence of sepsis by specifically having immunomodulatory effects and anti-inflammatory properties. Thus, it may be an alternative way to prevent sepsis due to *S. aureus* infections. Previous studies in our laboratory have shown that simvastatin pretreatment increases survival of mice infected with *S. aureus* and alters the adaptive immune response such that increased levels of IgG2c in response to *S. aureus* infection are reduced to the level of uninfected controls. To explain the survival and adaptive response alterations seen in the previous studies, we began to examine the immediate innate immune responses to *S. aureus*. This work demonstrated that 24 hour simvastatin pretreatment has no effect on bacterial clearance but does result in the reduction of serum levels of TNFα. From these studies, we hypothesized that
Simvastatin pretreatment may decrease the inflammatory response to *S. aureus* infection, as well as other innate immune responses, allowing for the clearance of *S. aureus* without widespread host tissue damage that can result in death. To investigate our hypothesis, we investigated the level of complement activity in simvastatin-pretreated mice versus control mice. Our results demonstrated that simvastatin pretreatment does not alter levels of complement component C5a. Taken together the data demonstrates that some innate immune responses are being altered, which may contribute to the increased survival of simvastatin-treated mice infected with *S. aureus*. 
*Staphylococcus aureus*

*Staphylococcus aureus* (S. aureus), discovered by Alexander Ogston in 1880, is a Gram-positive bacterium that appears in grape-like clusters [1, 2]. *S. aureus* is a part of the Micrococcaceae family and when cultured on blood agar, *S. aureus* appears as large, golden-yellow colonies [3]. *S. aureus* grows best under facultative anaerobe conditions and is catalase positive, which distinguishes it from enterococci and streptococci. It is also coagulase positive, distinguishing it from other staphylococci. *S. aureus* is commonly found within the nose and skin with twenty percent of the human population being a natural carrier [3]. *S. aureus* is typically spread by direct human contact. However, with its ability to live on fabrics such as polyester, a fabric used to make hospital curtains. *S. aureus* can also spread via materials that have been in contact with an infected individual [3].

Numerous medical conditions are caused by *S. aureus*, including many minor skin irritations such as boils, pimples, and impetigo, toxic shock syndrome, pneumonia, and meningitis [4]. It is a major cause of nosocomial infections and bacterial sepsis (bacteremia) [3]. Bacterial sepsis is the presence of bacteria or
their toxins in the blood and other tissues. The clinical manifestations, such as
organ failure or death, result from an overreaction of the immune system in
response to the bacteria causing destructive inflammation. There are an
estimated 750,000 individual cases of bacterial sepsis reported each year [3].
Epidemiological analysis found that sepsis killed 120,491 hospital patients in
2000 [5] and that the number of cases of sepsis increased from 82.7 for every
100,000 patients in 1979 to 240.4 for every 100,000 patients in 2000. Many
factors have been attributed to the increased prevalence of sepsis, such as
increased numbers of invasive procedures, and increased antibiotic use, which
has led to drug resistance in certain bacteria such as methicillin resistant S.
aureus (MRSA) [5].

*S. aureus* is known to be an exocytotic bacterium, but is now believed to
have a possible endocytotic mechanism by which *S. aureus* invades non-
phagocytic vascular host cells. *S. aureus* invades the non-phagocytic vascular
host cells by utilizing the ligand fibronectin and its integrin receptor $\alpha 5\beta 1$ located
on the host cell [6-10]. The surface of *S. aureus* has the adhesin fibronectin
binding protein (FnBP), which binds fibronectin molecules, thus coating the
bacteria with the ligand [6, 8, 11]. Once *S. aureus* is coated with the fibronectin
binding protein, the *S. aureus* can then bind to the $\alpha 5\beta 1$ receptor located on the
non-phagocytic vascular cells. $\alpha 5\beta 1$ may be the only bacterial factor necessary
for invasion of *S. aureus* [12] because it allows for *S. aureus* to be taken up by
the non-phagocytic vascular host cells. Once inside the non-phagocytic vascular
host cells, *S. aureus* can no longer be found by the immune system or seen as foreign [13]. The internalization of *S. aureus* into endothelial cells may provide the answer for why antibiotic treatment is not effective in some cases. It may also answer why more than one immune response is needed to efficiently clear the infection [10, 13].

*Inflammation*

When fluid, plasma proteins, monocytes, and neutrophils (phagocytes) accumulate locally due to infection or physical injury, an inflammatory response occurs [14]. The inflammatory response is a component of the innate immune system that serves the purpose to deliver effector cells and molecules that kill the pathogen and to form blood clots to prevent further spread of the infection [15]. The inflammatory response is activated when there is non-specific recognition of a pathogen or foreign particle, such as lipopolysaccharide (LPS), a protein on Gram-negative bacteria, or peptidoglycan, which comprises about 50% of staphylococcal cell walls by weight and lipoteichoic acid, a glycerol phosphate polymer anchored into the membrane of the cytoplasm [3], on Gram-positive bacteria [16, 17]. Within hours of the pathogen invading the extracellular spaces, the inflammatory response is induced.

Inflammation is characterized by the hallmark symptoms of pain, redness, heat and swelling which can be attributed to changes in the blood vessels. One important change is vasodilation, which is caused by tumor necrosis factor alpha (TNF$_{\alpha}$), a cytokine released from activated macrophages. The increase in blood
flow to the site of infection causes both heat and redness. TNF$\alpha$ is also a potent activator of the endothelial cells that make up the blood vessels. Once the blood vessels are activated by TNF$\alpha$, they express cell-adhesion molecules and have increased vasopermeability allowing monocytes and neutrophils to extravasate across the blood vessels into the tissues [14, 18]. Lastly, the fluid and proteins from the blood that are extravasated into the tissue along with the monocytes and neutrophils causes the symptoms of swelling and pain [17]. Taken together, TNF$\alpha$ and the changes in blood vessels are important first steps in the initiation of inflammation.

Once inflammation has begun, neutrophils are the first phagocyte to enter the site of infection. The monocytes then follow the neutrophils to the site of infection, where they mature into macrophages once inside the tissue. Macrophages and neutrophils play an important role in the innate immune system by recognizing, phagocytosing, and destroying many pathogens without the help of the adaptive immune system. Once a bacterial pathogen enters a tissue, macrophages and neutrophils recognize cell surface molecules (LPS, lipoteichoic acid) on the pathogen and begin phagocytosis. However, some bacteria, such as \textit{S. aureus}, sometimes require opsonization by complement proteins or antibodies before macrophages will recognize the bacteria for phagocytosis. The process of phagocytosis requires the macrophage to internalize the pathogen, and then enclose it within a phagosome. Both macrophages and neutrophils also have lysosomes containing enzymes,
proteins, and peptides that fuse with the phagosome. The binding of the phagosome and lysosome creates a phagolysosome where the contents of the phagosome are degraded by the degradative enzymes of the lysosome [14, 18].

During the inflammatory response, macrophages not only release TNFα, but also release interleukin-12 (IL-12), which is an activator of Th1 cells. This subset of CD4⁺ cells is activated when macrophages are chronically infected with a pathogen, such as *S. aureus* [13]. The Th1 subset activates the chronically infected macrophage through binding of MHC Class II receptor and CD40, and secreting interferon-γ (IFNγ), which enhances macrophage functions [13]. This process also recruits more macrophages to the site of infection by the release of the TNFα. TNFα signals more macrophages to the site of infection where they phagocytose bacteria that were released by the destroyed macrophage [19] (Figure 1).
In summary, the inflammatory response is a series of events that triggers activation of innate immune cells and secretion of pro-inflammatory molecules. The events of vasodilation and increased fluid into the damaged tissue allow for monocytes and neutrophils to cross the endothelial cell lining. These cells, along with macrophages (the orchestrators of the inflammatory response), begin to destroy the invading pathogen. The macrophages release cytokines and chemokines to further clear the pathogen and to promote healing of the damaged tissue/site of infection.
The Complement System

In addition to the initiation of an inflammatory response, other pathways of the innate immune system, such as the complement system, are activated in response to infection. Discovered in 1922 by Jules Bordet [20], the complement pathway is a system of plasma proteins (C1-C9), circulating throughout body fluids and tissues, and is only activated by the presence of extracellular pathogens [14]. The complement system was originally discovered as an effector arm of the antibody response, but it is now known to be activated in the early stages of infection in the absence of antibodies [14]. In the complement system, several of the complement proteins are proteases that become activated when cleaved by another specific protease and/or complement protein [14]. The complement pathway is only activated by a triggered enzyme cascade, resulting in the cleavage of zymogens (inactivated complement proteins) to their activated protein fragment [14]. Each complement protein is designated with the capital letter “C”, a number (1-9) and once cleaved, either a lower case “a” or a lower case “b”. The larger fragment (designated with the letter “b”) then cleaves other complement proteins for continuation of the complement pathway. The smaller fragments (designated with the letter “a”) travel through the blood to trigger other inflammatory mediators. For example, the first complement protein in the classical pathway is C1q, which binds to the surface of a pathogen or to an antibody, which is bound to the pathogen surface. Once bound to the surface of the pathogen or to an antibody, C1q binds to C1r and C1s, forming the C1qrs
complex that then cleaves C4 into C4a and C4b. C4b then cleaves C2 into fragments C2a and C2b. All activation of the complement system occurs on the surface of the pathogen, ensuring that all complement zymogens are only activated at the surface of the pathogen and not spontaneously [14]. The functions of the complement system (Figure 2) include lysis of the pathogen, opsonization (caused by C3b), and activation of the inflammatory response (caused by C3a and C5a) [14, 21].

Figure 2. An overview of the complement system.

There are three different activation pathways of the complement system. The classical pathway is triggered by the binding of C1q to an antibody:antigen complex on the pathogen's surface or by binding of C-reactive protein. The lectin pathway (mannose-binding lectin pathway) is the second pathway that is activated by the binding of mannose binding lectin to mannose residues on the pathogen. The last pathway is the alternative pathway, which is activated by spontaneous cleavage of C3b and its subsequent binding directly to the surface of the pathogen.

There are three ways in which the complement system protects the body against infection. One function of the complement system is the creation of a large number of complement proteins that bind covalently to pathogens, which opsonizes the pathogen for phagocytosis. Two, the small fragments of
complement proteins act as chemoattractants that recruit more phagocytes (macrophages and neutrophils) to the infection site. This process is called complement activation. The final component of the complement system involves the membrane attack complex (MAC), which damages bacteria by creating pores in the bacteria’s cell membrane, causing the bacteria to lyse [14]. To form the MAC, C5b binds C6, C7, and C8. This C5-C8 complex then binds to many C9 proteins to make the MAC. The MAC perforates the membrane of the invading organisms, causing lysis to occur (Figure 3). The smaller cleaved fragments (C3a and C5a) act as anaphylatoxins, causing the release of histamine by mast cells, amplifying the inflammatory response [21, 22].

Figure 3. The membrane attack complex is composed of C5b-9.

Once cleaved, the larger complement fragments C5b-8 join C9 together to create the MAC. The formed MAC pokes holes in the pathogen’s cellular membrane, causing it to lyse.
While the outcomes of complement are the same, there are three distinct ways in which complement can be activated [14]. The three pathways of the complement system are the classical, alternative, and mannose-binding lectin (MBL). The classical pathway is a key component in linking the innate immune system and the adaptive immune system, since it requires antibody bound to antigen to bind to complement protein C1q and activate this pathway. The binding of the antibody to the pathogen creates an antibody-antigen complex. Binding to natural antibodies on the pathogen surface can also activate the classical pathway in the innate immune system. The natural antibodies, which are of the IgM, class are produced by B-1 cells, which are a type of B cell not associated with the adaptive immune system due to their limited functions and capabilities. They are a part of the subset of lymphocytes known as the innate-like lymphocytes (ILL). ILLs have a high specificity for microbial antigens, and interact with many pathogens. B-1 cells produce IgM in, which then binds to the surface of the pathogen. C1q then binds to the IgM on the pathogen surface, which activates the classical pathway. Once bound to the pathogen by any of the aforementioned processes, C1q binds two molecules (C1r and C1s) to create the C1qrs complex (Figure 4). This C1qrs complex cleaves C4 into C4a and C4b.
C4b then cleaves C2 into C2a and C2b. The C2a fragment binds to the C4b making up the C3 convertase, which cleaves C3 into C3a and C3b [14]. C3a, an anaphylatoxin, activates the inflammatory response by binding to nearby neutrophils and monocytes/macrophages, then recruiting them to the site of infection. C3b joins the C3 convertase complex creating the C5 convertase. The C5 convertase cleaves C5 into C5a and C5b. C5a, just like C3a, recruits and activates neutrophils and monocytes/macrophages, while C5b binds to C6-9 to form the membrane attack complex (MAC) [14, 21].

The complement system can also be activated by the alternative pathway. This pathway can be activated on many bacterial surfaces in the absence of antibody existence. This pathway leads to a distinct C3 convertase designated as C3bBb. This pathway, unlike the classical pathway, depends on the spontaneous...

Figure 4. C1q binds C1r and C1s to form the C1qrs complex in the classical pathway.

C1q is made up of 6 globular heads and collagen tails. The collagen tails bind two molecules of C1r and C1s forming the C1qrs complex. The binding of the molecule to the pathogen or to antibody causes C1r to cleave C1s. The cleaved complex then cleaves C4 into C4b and C4a (not pictured).
hydrolysis of the thioester bond of C3. C3 is abundant in the plasma and is spontaneously hydrolyzed to form C3b. The C3b molecule allows the complement protein Factor B to bind. The binding of Factor B allows for another complement protein Factor D to bind, which cleaves Factor B into Bb and Ba, forming C3bBb, which is a fluid-phase C3 convertase. The C3bBb is a small C3 convertase but can cleave many molecules of C3 to C3a and C3b. Most of the C3b is hydrolyzed, but some does bind to the surface of the pathogen, resulting in more cleavage of Factor B (by Factor D) into Ba and Bb, resulting in the alternative pathway C3 convertase.

Due to the spontaneous cleavage of C3 in the alternative pathway, the stability of the C3 convertase is critical in the activation of complement. There are a few proteins that inhibit Factor B binding to C3b, including decay-accelerating factor (DAF), membrane cofactor of proteolysis (MCP), cell-surface complement receptor type 1 (CR1), and Factor H. DAF, CR1, and Factor H displace the complement protein fragment Factor Bb from C3b. CR1, MCP, and Factor H catalyze bound-C3b by Factor 1 (plasma protease) producing inactive C3b (iC3b). These inhibitory proteins, keep the C3 from being activated. This activation would initiate the inflammatory response at inappropriate times. However, when complement is activated on bacterial cell surfaces, the C3 convertase is stabilized by properdin, or Factor P. Factor P stabilizes the C3 convertase and also amplifies complement activation (Figure 5). [14]
Figure 5. The alternative pathway of complement activation only attacks pathogens and not host cells because which are protected by regulatory proteins.

C3 is spontaneously cleaved into C3(H₂O). C3(H₂O) binds to Factor B and cleaved by Factor D forming a soluble C3 convertase (panel 1). The soluble C3 convertase cleaves more C3 into C3a and C3b, which binds to both host and pathogen cell surfaces (panel 2). Bound C3b binds Factor B, which is cleaved by Factor D forming a C3 convertase (panel 3). If the C3 convertase forms on host cells, regulatory proteins specific for complement that are expressed by the host cells inactivate it. These proteins include complement receptor 1 (CR1), decay-accelerating factor (DAF), membrane cofactor of proteolysis (MCP), and Factor H. Factor H, CR1, and MCP displace the protein fragment Bb from C3b while CR1, MCP, and Factor H catalyze bound C3b by the plasma protease factor I, which produces inactive C3b (iC3b). Bacterial cell surfaces do not express any regulatory proteins specific for complement and bind factor P, which stabilizes the C3bBb convertase.
Once the C3 convertase is stabilized on the bacterium by Factor P, C3 convertase can start to cleave C5 into C5a and C5b. From this point, the complement process follows the same steps as the classical pathway.

The mannose-binding lecting pathway is the third and final pathway that can activate the complement system. This pathway is initiated by the binding of the carbohydrate-binding protein mannose-binding lecting (MBL) to the mannose-containing carbohydrates on the bacterial cell surface. MBL is a six-headed molecule that, like C1q, forms a complex with two zymogens, MASP-1 and MASP-2 (Figure 6). The role of MASP-1 is not clear. However, the MASP-2 zymogen is similar to C1r and C1s (of the C1qrs complex seen in the classical pathway). MBL only binds to the cell surfaces of bacteria due to the

Figure 6. The structures of the C1qrs complex and the mannose-binding lectin (MBL) protein are similar.

The classical pathway and the mannose binding lectin pathway of the complement system are activated by similar structures. The difference between the two pathways is that the classical pathway uses antibody:antigen complexes to bind to the surface of the pathogen while the MBL pathway uses the sugar residues on the pathogen surface to bind MBL to activate the complement system.
arrangement and spacing of the sugar residues on the pathogen’s cell surface [14]. Once MBL is bound to the surface of the pathogen, MASP-2 cleaves C4 and C2, which activates complement and forms the C3 convertase [14]. Once the C3 convertase is formed, the remaining steps of the MBL pathway are similar to that of the classical pathway and the alternative pathway. The complement system is another way for a pathogen to be recognized quickly for the purpose of triggering an inflammatory response, and to also connect the innate immune system with the adaptive immune system, via the classical pathway. No matter the pathway chosen, the complement system works to contain and destroy invading pathogens by opsonization (resulting in destruction of the pathogen) and enhancement of the inflammatory response by the anaphylatoxins C3a and C5a [14, 21, 22].

C5a is a potent activator of the inflammatory response and recent studies have linked C5a to the severity of sepsis. The effects of C5a in small amounts (less than 10 nM) are beneficial in clearing infection from the blood. These effects include a variety of biological effects such as increasing vascular permeability, inducing smooth muscle contraction, and inducing chemotaxis of neutrophils and monocytes [23, 24]. C5a also primes neutrophils so that they are enhanced to release degradative enzymes in the presence of a pathogen. C5a also interacts with endothelial cells to release P-selectin, allowing for neutrophils to extravasate across the blood vessel and into the infected tissue [23].
During sepsis, the C5a receptor (C5aR) is upregulated on neutrophils and macrophages, along with cells of the liver, lungs, and heart [23]. When C5aR is upregulated on the cells of these organs, C5a will bind to its receptor on these cells and will cause the organs to fail [23]. A study showed that primates who received an anti-human polyclonal C5a IgG antibody had decreased incidence of sepsis when compared to those primates who did not receive the anti-human polyclonal C5a IgG antibody [25]. Another study showed that septic male c57B/6 mice that had C5a or C5aR blocked had increased survival when compared to control mice [26]. Together, these studies demonstrate the importance of C5a in sepsis.

**Statins**

Statins are a class of lipid-lowering drugs that inhibit 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in cholesterol biosynthesis [27], and are used to decrease the levels of low-density lipoprotein (LDL) [28-30]. In the 1970’s, the HMG-CoA reductase inhibitor was isolated from *Penicillium citrium*, leading to the production of numerous statin drugs [31]. Under normal circumstances, the HMG-CoA reductase catalyzes the conversion of HMG-CoA to mevalonate [29, 32, 33]. Though all statins control cholesterol levels, they differ in their hydrophobicity and the effective drug dose. Atorvastatin, fluvastatin, lovastatin, and simvastatin are all lipophilic or hydrophobic compounds (soluble in fats and oils), while pravastatin and rosvastatin are hydrophilic [30, 33]. Statins are metabolized by cytochrome
P450, a family of enzymes. This metabolism by cytochrome P450 determines the half-life which then determines how long the statin drugs will interact with other drugs [33]. Lovastatin, pravastatin, and simvastatin, all derived from fungus, have half-lives of one to three hours, while atorvastatin, fluvastatin, and rosuvastatin are synthetically made and have half-lives of up to nineteen hours [29].

**Statins and Sepsis**

Independent of their lipid-lowering capabilities, statins have also been shown to reduce the development of sepsis, [34, 35]. For example, one study found patients, sixty-five years or older who were prescribed statins for medical use prior to entering the hospital for cardiovascular problems, exhibited a lower occurrence of sepsis when on statins. These patients also had a significant reduction in the incidence of severe sepsis and death [34]. In yet another study, following a twenty-eight day analysis, patients with multiple organ dysfunction syndrome (MODS) using statins demonstrated a decrease in mortality when compared to patients not taking statins. The patients taking statins also had lower hospital mortality than those patients not receiving drug treatment [36]. In contrast, one study showed patients hospitalized for pneumonia that were prescribed statins over a 28-day period also exhibited a reduction in the risk of death due to sepsis. However, after adjustments for other variables, there was no significant observational increase in survival [35]. Together, these studies show that statins have the potential of increasing survival in patients with sepsis.
Finding new treatments for sepsis is important due to there being approximately 750,000 cases of sepsis in the United States annually, with mortality rates of 30-50% [16]. Sepsis is caused by an infection that results in an overreaction of the immune system resulting in multiple organ failure and possibly death [16, 22, 37]. Bacterial products (e.g. lipotechoic acid) interact with toll like receptors (TLRs) located on the surface of immune cells. This interaction induces the inflammatory response, increases the response of macrophages and neutrophils to kinases, cytokines, chemokines, and other pro-inflammatory mediators, to clear the infection [37].

Additional studies into the effects of statins on the immune response have been done in vivo using mouse models. In sepsis, the inflammatory response of the innate immune system is what clears the pathogen from the blood. However, due to the robust response of inflammation, organ failure and death may occur in the host. Studies done with mice have shown that statin treatment has reduced the strong inflammatory response attributed to sepsis. Although no longer approved by the FDA, 10 mL/kg cerivastatin pretreatment in male CD-1 mice 12 and 1 hour prior to LPS administration reduced serum levels of TNFα [38]. In another study, simvastatin treatment, at 6 and 18 hours prior the induction of sepsis (via cecal perforation), prolonged survival in male C57 mice compared to mice who received control treatments [32]. Merx et al. also demonstrated that male C57 mice had increased survival when 0.02 mL/g [BW] of pravastatin, simvastatin, or atorvastatin was administered six and eight hours after cecal
perforation [39]. It was also demonstrated that rats pretreated with 100 µg/kg simvastatin in 1 mL NaCl 18 hours prior to α-toxin, a toxin found on S. aureus, administration had decreased serum TNFα levels along with reduced α-toxin-induced changes of decreased heart rate and blood pressure [40]. In another study, C57BL/6 mice at 24 hours and 1 hour prior to injection of LPS and then 24, 48 and 72 hours post injection of LPS (45mg/kg) had significantly reduced TNFα and IL-6 levels, as well as a reduced incidence of death after the treatment of LPS [41]. The same study showed that cerivastatin pretreatment in C57BL/6 mice 24 hours and 1 hour before injection with S. aureus and 24, 48, and 72 hours post infection with live S. aureus (5x10^7) showed a decrease (not statistically significant) in both TNFα and IL-6 [41]. These studies demonstrate that mice treated with statins may avoid the detrimental effects of sepsis, as shown by increased survival.

Statins and the Immune System

The innate immune system is responsible for clearing pathogens from the blood and does so by activating various immune system components such as inflammation, complement, and coagulation. These actions are initiated by signaling pathways and inflammatory mediators. Statins have been shown to inhibit signaling pathways by first inhibiting the HMG-CoA reductase [42]. HMG-CoA reductase inhibition by statins will, in turn, inhibit the production of mevalonate (the product of HMG-CoA reductase). This inhibition of mevalonate production then inhibits the metabolites associated with it, including the
isoprenoid intermediates, such as geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP). These intermediates serve as lipid attachments used for intracellular signaling [43]. Isoprenoid reduction inactivates GTP-binding proteins, which include Ras, Rac and Rho, limiting their ability to signal and direct cellular traffic [43-45]. In the presence of statins, Rac, Ras and Rho are gathered within the cytosol in their inactive form. Statins accumulate these proteins in the cytosol by inhibiting the prenylation of the Rho-kinase pathway [46]. These patterns of events suggest that statins have effects on protein signaling and protein-migration, in addition to their cholesterol-lowering effects.

Along with their immunomodulatory effects, recent studies have demonstrated that statins have anti-inflammatory properties [47], and many of these studies investigate the effect of statins on specific immune functions in vitro. Specifically, in the presence of statins the inflammatory response is reduced as evidence by Human hepatoma cell line (Hep3B) having reduced release of C-reactive protein (an activator of the complement system) when introduced to 50 ng/mL IL-6 and 0.1 μmol of atorvastatin or 1 μmol of simvastatin [48, 49]. In another study simvastatin has been shown to reduce the rate of neutrophil migration to the site of infection, leading to a reduced inflammatory response [48]. However, in another study, monocytes activated with TNFα for 45 minutes and then treated with 5, 100, and 500 μM of pravastatin for 24 hours had decreased levels of TNFα but had IL-6 levels that were not affected [50]. Macrophages release the cytokine IL-6 along with TNFα and interleukin-1 (IL-1),
all of which contribute to the inflammatory response associated with sepsis. These cytokines then activate T-cells, which produce pro-inflammatory cytokines and chemokines [14, 51]. Importantly, these studies suggest that statin drugs may be capable of decreasing aspects of the inflammatory response that occur during sepsis.

The effects of statins on macrophages and T cells

The ability of statins to alter signaling pathways can affect functions in immune cells, such as gene expression and cellular activation. Specifically, statins can alter the expression of MHC molecules and the activation of T cells by reducing the expression of MHC class II on antigen presenting cells and other cells of the immune system [52]. MHC class II molecules are involved in antigen-specific activation of CD4 T helper cells [53, 54]. MHC class II molecules are only expressed on a small number of cells known as antigen presenting cells (APCs). Its expression can be upregulated on APCs when the cytokine, IFN-γ is released by Th1 cells [52]. IFN-γ functions to communicate with cells to express MHC class II, and activate T-cells [1, 52, 54, 55]. Under normal circumstances, the presence of melvalonate induces IFNγ-stimulated MHC Class II expression. However, with a 3 hour incubation of 10 μM of lovastatin, IFNγ-induced MHC class II molecules are lost due to decreased levels of melvalonate [56, 57]. Statins can also affect the function of macrophages, the directors of the innate immune response that initiate the inflammatory response. Macrophages present the pathogen on the surface by way of the MHC class II molecule leading to the
activation of a subset of CD4 T helper cells called Th1 cells [14]. Under normal circumstances, CD40 and IFN-γ activate macrophages in the presence of infection. *In vitro* studies have shown that the expression of CD40 by IFN-γ has been inhibited on macrophages in the presence of lovastatin [58]. Statin use *in vitro* has also been linked to inhibition of macrophage movement and IL-6 secretion, decreasing the level of inflammation [36]. Collectively, these studies demonstrate that statins alter macrophage activities, limiting innate immune responses such as inflammation and affecting adaptive responses such as T cell activation.

*Statins and TNFα*

TNFα is a potent cytokine activator of the inflammatory response that is released by macrophages. Studies in mice have shown that LPS administered to mice pretreated with cerivastatin resulted in decreased serum levels of TNFα when compared to mice that did not receive statin treatment [41]. In a human study, 40 mg and 80 mg of simvastatin were given to patients 4 days after receiving LPS by inhalation, which led to decreased levels of TNFα compared to patients who received a placebo [59]. However, TNFα is not always decreased with statin use. In another study, patients received a 0.06 ng/kg bolus of *Escherichia coli*, then for 6 hours orally received 20 mg of simvastatin for 14 days. Serum was drawn every day for 14 days, and the plasma from all patients was analyzed for levels of TNFα using an ELISA. The results showed no difference in TNFα serum levels in patients who received simvastatin when
compared to patients who just received the bolus of *Eschericia coli* [60]. Additionally, an *in vitro* study using murine macrophages infected with *Plasmodium berghei* after a 24 hour pretreatment with 7.47 mM of simvastatin or 12.3 mM of atorvastatin secreted increased levels of TNFα [61]. Taken together, these studies show that statin treatment modulates TNFα levels, but the data is not conclusive regarding its ability to consistently increase or decrease TNFα production.

**Statins and Complement**

As stated previously, complement is an important feature of the innate immune system and can initiate an inflammatory response. Therefore, the effects of statins on complement must be considered. *In vivo* studies have shown that statins decrease the level of complement. For example, rats treated with yeast-activated rat serum (Y-act RS) and pretreated with 5 or 10 mg/kg of fluvastatin for 15 days experienced an inhibition of C-reactive protein. The rats also experienced a decreased inflammatory response, as shown by a decrease in polymorphonuclear (PMN) cells within the peritoneal cavity [62].

*In vitro* studies show that endothelial cells treated with atorvastatin, simvastatin, or mevastatin demonstrated a decrease in complement activation due to an increase in decaying-accelerating factor (DAF). DAF is a membrane-bound regulatory protein that inhibits the formation of the C3 and C5 convertases [63]. After an eight week treatment with 20 mg/daily of simvastatin, patients with hypercholesterolemia had decreased levels of serum C3c [64].
Not all studies link statin treatment and decreased complement activity. One study done on rats showed that when 2 mg/kg of simvastatin was given 2 days before disease induction and continued throughout the study there was no effect on the complement system in reference to glomerular injury as determined by no difference in glomerular disease between the control group and the group pretreated with simvastatin [65]. In an in vivo study, a four-minute pretreatment of human serum, already treated to activate the complement system, with 0.1, 1.0 or 10 μM of atorvastatin did not affect the activation of complement as shown by no change in complement proteins within the serum as analyzed by an enzyme immunoassay (EIA) of C1rs-C1-inhibitor complexes, C4bc, C3bBbP and monoclonal antibodies bH6 (specific for an epitope in C3b) and aE11 (specific for a neoepitope on C9).[66]. All together, these studies suggest that statin treatment, whether pretreatment or post-treatment have various effects on complement.

This research has shown that statins, as a group, may decrease the incidence of sepsis, induced by a variety of methods, due to having anti-inflammatory and immunomodulatory effects. Importantly, these investigations have demonstrated the importance of the innate immune system in sepsis and the alterations that may result due to statin treatment. Numerous aspects of the innate immune system have been researched including TNFα serum levels, IFNγ serum levels, and all pathways of the complement system, and macrophage and neutrophils activation. But more studies to identify the specific alterations of
innate immune mechanisms \textit{in vivo} would help clarify the role of statins as a treatment for sepsis.
To investigate whether simvastatin lessened the inflammatory response to 
*S. aureus* infection, allowing for proper clearance of *S. aureus*, without 
widespread, non-specific damage to host tissue that can result in death, our 
collaborator (Dr. Susan McDowell) did a two-week long survival study. All 
survival experiments contained three C57BL/6 mouse treatment groups: MUCIN, 
*S. aureus*/gentamicin (-Simva), and *S. aureus*/gentamicin/simvastatin (+Simva). 
The MUCIN group received saline/ethanol 18 and 3 hour prior to *S. aureus* 
infection and then 10 mg/kg [BW] gentamicin was administered by intraperitoneal 
(i.p.) injection 3, 6, 12, 24, and 48 hours post *S. aureus* infection. The *S. 
aureus*/gentamicin (-Simva) group received saline/ethanol 18 and 3 hours prior to 
infection with $1 \times 10^7$ cfu *S. aureus*, and then received 10mg/kg [BW] of 
gentamicin at the same time points as the MUCIN group. The third treatment 
group, *S. aureus*/gentamicin/simvastatin (+Simva) received 1000 ng/g [BW] 
simvastatin 18 and 3 hours prior to *S. aureus* infection and then received 
10mg/kg [BW] gentamicin at the same time points as the previous treatment 
groups. The pooled data showed a significant increase in survival of the +Simva 
group when compared to the -Simva treatment group (Figure 1).
Figure 1. Simvastatin pretreatment increases survival of mice infected with S. aureus. Mice were pretreated with simvastatin (+Simva) or vehicle control (-Simva) 18 and 3 hours prior to infection with S. aureus. All mice were given 10mg/kg [BW] gentamicin at 3, 6, 12, 24, and 48 hours post-infection. Mice were observed a total of 14 days post-infection. Data were pooled from 3 replicate studies (n=13-14/group). * p ≤ 0.05 by Kaplan-Meier Log Rank analysis.
In order to examine the immune response in these mice and determine whether a Th1 or Th2 response was being mounted, an ELISA was performed to analyze antibody levels. While elevated levels of IgG1 are indicative of a Th2 response, elevated levels of IgG2c, not IgG2a are indicative of a Th1 response in C57BL/6 mice due to an allelic isotype difference between the mice. When the data were pooled, IgG2c levels were elevated in the –Simva group, as anticipated due to infection, but were reduced in the +Simva group, while there was no difference in IgG1 levels between treatment groups (Figure 2). Importantly, the reduced IgG2c levels in the +Simva group were comparable to levels in uninfected controls.
Figure 2. Antibody responses to *S. aureus* are altered by simvastatin pretreatment. Mice were pretreated with simvastatin (+Simva) or vehicle control (-Simva) 18 and 3 hours prior to infection with *S. aureus* in mucin or mucin alone (Mucin). All mice were given 10mg/kg [BW] gentamicin at 3, 6, 12, 24, and 48 hours post-infection. Serum was collected 14 days post-infection and analyzed by ELISA for levels of IgG1 (a) and IgG2c (b). Data were pooled from 3 replicate studies (n=10-14 mice/group) and analyzed by one-way ANOVA (* p < 0.05 vs. Mucin and + Simva, # < 0.05 vs. -Simva).
These studies demonstrated that survival of *S. aureus*-infected C57BL/6 mice was increased. The decreased levels of IgG2c in simvastatin pretreated mice indicate that adaptive responses are altered such that Th1 responses are muted. This data suggests that a 24 hour simvastatin pretreatment may serve to down-regulate the adaptive immune system that would normally cause destructive, non-specific inflammatory response.

Based on these findings we hypothesized that simvastatin pretreatment may decrease the inflammatory response to *S. aureus* infection, as well as other innate immune responses, allowing for the clearance of *S. aureus* without widespread host tissue damage that can result in death. To investigate our hypothesis, we examined bacterial clearance, complement activity, and TNFα levels.
Materials and Methods

Treatment schedule and dosage calculations

For each study, the timeline of treatments will be as follows:

<table>
<thead>
<tr>
<th>Treatment Day</th>
<th>Description of treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>-1</td>
<td>Pretreatment of simvastatin or saline 18-20 hours before <em>S. aureus</em> infection.</td>
</tr>
<tr>
<td>0</td>
<td>Simvastatin or saline/ethanol pretreatment 3 hours prior to <em>S. aureus</em> infection. <em>S. aureus</em> was injected 3 hours after last simvastatin and saline/ethanol treatment, gentamicin treatments were given at 3, 6, 12 hours (clearance and TNFα studies had 24 hour gentamicin treatment) post infection.</td>
</tr>
<tr>
<td>1</td>
<td>All mice were sacrificed by CO₂ or pentobarbital (10 mg/mL; clearance and TNFα studies) and serum was isolated via cardiac puncture.</td>
</tr>
</tbody>
</table>

Dosage calculations

The calculations for gentamicin (#1397, Sigma Aldrich, St. Louis, MO) and simvastatin (#567021, Calbiochem, Gibbstown, NJ) dosages were based on average body weight of the mice. Males and females were weighed separately to obtain a more accurate dosage calculation. Gentamicin was made in saline while simvastatin was dissolved in ethanol (#E7148, Sigma Aldrich) first before being made into solution with saline. To make the 0.85% saline, 4.25g NaCl (#S7653, Sigma Aldrich) was mixed in 500 mL milli-Q water and then filter sterilized. Saline
or ethanol diluted in saline was used as the negative control for gentamicin and simvastatin treatments, respectively. The calculations for both gentamicin and simvastatin were as follows:

Gentamicin was used at a 10 mg/kg body weight (BW) per mouse in each experiment. 10 mg/kg [BW] gentamicin=1.0 mg/0.021kg (average weight of 21g), which is equivalent to 0.21 mg. 0.21 mg was the amount needed per syringe per mouse in milliliters (mL). Therefore, 0.21 mg equaled 0.21 mL per syringe, per mouse. In order to calculate the volume of gentamicin (1.0 mg/mL) solution to make, the following calculation was used for each study: 0.21 mL per syringe per mouse * 5 (time points for each gentamicin treatment) * number of mice receiving gentamicin + 3 to calculate for possible spillage. This calculation gave the volume of saline needed for the gentamicin solution. To make the volume of gentamicin needed, the working concentration of gentamicin (1.0 mg/mL) was multiplied by the volume of saline needed (mL), then divided by the concentration of the stock of gentamicin, and then multiplied by 1000 to get the volume of stock gentamicin needed in µLs.

For a concentration of 1000 ng/g of body weight per mouse for simvastatin, the following calculation was followed: 1000 ng/g [BW] simvastatin = x/21g (from average weight of 21g). X equaled 1000 * 21, which was the total nanogram needed per mouse. To determine the micrograms needed, the total nanograms were converted by taking 21000/1000=21 µg/mL. The following calculation was used to determine the volume of simvastatin (100µg/mL) needed
per dose: 21/100 * 1000=210 μL per syringe per mouse. The simvastatin concentration of 100 μg/mL was determined by the following: 2 syringes * (n mice receiving simvastatin + 3 for possible error) * 210 (μL needed per syringe)/1000 L in 1 mL. This calculation determined how many mL of saline were needed. The volume stock of simvastatin (10mg/mL in EtOH) was calculated by multiplying the volume of saline by 100 μg (simvastatin concentration needed was 100μg/mL) and dividing by 10,000 (stock simvastatin concentration was 10mg/mL, while the working concentration of simvastatin was 100 µg/mL), then multiplying by 1000.

For the vehicle control for gentamicin, the following calculation was used: 5 syringes per mouse (number of times antibiotic will be administered) * (number of mice receiving saline control + 3 for possible errors) * the volume needed per syringe per mouse in milliliters. To determine the vehicle control for simvastatin, the following calculation was used: 2 (syringes per mouse) * (n mice in control group + 3 for spillage) * (µLs per syringe per mouse) / 1000. This calculation gave the volume of saline needed in milliliters (mL). The volume of ethanol needed was calculated by multiplying the stock concentration of simvastatin (100µg/mL) by the volume of saline needed (mL), and then dividing that total by 1000.

**Mice**

C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME and Harlan Laboratories, Indianapolis, IN) were used. All mice were housed in CL277 until
each experiment was started. The mice for each experiment were housed in CL77C in individual filtered cages and had free access to food and water. BSU IACUC approved all experiments under protocols submitted by Dr. Bruns and Dr. McDowell.

For each infection study, all mice were infected with \textit{S. aureus}. Each treatment group contained no fewer than five mice. The control group was saline/ethanol (-Simva) and the treatment group was simvastatin (+Simva). At the end of each study, all contents of the cages (bedding, food, and water), water bottles and lids, cage lids, cages, and carcasses were autoclaved. The water bottles and lids, cages, and cage lids were washed with bleach-water and air-dried. The autoclaved bedding, food and carcasses was placed into black trash bags and put directly into the waste.

\textit{Preparation of S. aureus}

\textit{S. aureus} (#29213, American Type Culture Collection, Manassas, VA) was subcultured from a tryptic soy agar (TSA) (#22091, Sigma Aldrich) slant. One loop of culture from the TSA was swirled into 5 mL tryptic soy broth (#22092, Sigma Aldrich) and incubated at 37°C, shook at 225 rpm overnight. A new subculture of \textit{S. aureus} was made between 3:00 and 4:00PM the following two days with three subcultures being made on the day before infection (one was a back-up). On the day of infection, one overnight subculture of \textit{S. aureus} was mixed and 1 mL aliquots were made into four microcentrifuge tubes. The aliquots were centrifuged for 3 minutes at 10,000 rpm at 37°C. The supernatants were
discarded and the pellets were resuspended in 400 μL pre-warmed, 0.85% sterile saline. The microcentrifuge tubes were spun again at 10,000 rpm at 37°C. Using a pipetman, the supernatant was completely removed from the pellet and then one microcentrifuge tube was resuspended in 1000 μL of 0.85% saline. From the 1000 μL, 100 μL were taken and added to a new microcentrifuge tube with 900 μL of sterile 0.85% saline already in it and vortexed. The absorbance of the vortexed microcentrifuge tube was then read using a spectrophotometer. To determine how much mucin needed to be added to the dilute pellet to get $3 \times 10^8$ bacteria/mL, the following equation was used: $\text{OD reading}/X \text{ mL}=0.3/1 \text{ mL}$. So, for example, if the OD reading is 0.6 then $0.6/x \text{ mL}=0.3/1 \text{ mL}$, $x=2 \text{ mL}$. Therefore, 2 mL of 5% mucin was added to re-suspend the pellet for a final bacteria count of $3 \times 10^8$. From this 2 mL solution, 1 mL was added to 29 mL of 5% mucin in a 50 mL falcon tube. The calculation to determine this volume is as follows: $3 \times 10^8(X)/\text{mL}(1 \times 10^7/1 \text{mL})(30 \text{ mL})= 1 \text{ mL}$ of $3 \times 10^8$. So, 1 mL of the 2 mL solution was added to 29 mL of 5% mucin to get the bacterial count of $1 \times 10^7$/mL. Once the pellet was resuspended in 5% mucin, each mouse received a 1 mL injection of the $1 \times 10^7$/mL solution.

50 mL of 5% mucin (#21211, BD, Franklin Lakes, NJ) was prepared by adding 2.5 g mucin to approximately 30 mL of 0.85% sterile saline. The mucin and saline solution were mixed by vortexing, and rocked overnight at 4°C. On the day of injections the volume was brought up to 50 mL using the 0.85% sterile saline.
Clearance Study

After 24 and 48-hour post *S. aureus* injection all mice were sacrificed spell out by intraperitoneal injection of 10 mg/mL pentobarbital and serum was collected via cardiac puncture. One hundred µLs of serum from each mouse was immediately plated onto TSA/7.5% sodium chloride (NaCl) plates to select for *S. aureus* colony formation. Plates were incubated overnight (O/N) at 37°C. After incubation, the colonies were counted and the colony forming units were determined by using the following calculation: number of colonies counted * 10 [plated 100 µL out of 1000 µL; is 1/10 of the dilution] * dilution factor for plate counted (1). The colony forming units were analyzed by a two-tailed Student’s t-test using SigmaStat and SigmaPlot.

Complement ELISA protocol (Reagents)

All reagents used in each experiment were from R&D Systems and provided in a mouse complement component C5a kit (#DY2150, R&D Systems, Minneapolis, MN).

The capture antibody (#DY2150, R&D Systems) was diluted to a working concentration of 4 µg/mL in phosphate buffered saline (PBS) (#9808, Cell Signaling, Danvers, MA) without carrier protein (BSA). To get a working concentration of 4 µg/mL, 720 µg/ML of rat anti-mouse C5a was reconstituted with 1 mL PBS. Once the capture antibody was reconstituted, it was added to 10
small 0.5 mL microcentrifuge tubes in 100 μL aliquots and stored at -80°C until the day of the experiment.

The reagent diluent (#DY2150, R&D Systems) was made by adding 1g bovine serum albumin (BSA; #9998, cell signaling, Danvers, MA) to 100 mL of 1x PBS. The reagent diluent was made fresh for each experiment.

The standard (170 ng/mL; #DY2150, R&D Systems) was reconstituted with 500 μL of Reagent Diluent. The reconstituted solution was aliquoted (50 μL) into 10 small (0.5mL) microcentrifuge tubes and stored at -80°C for future use. One aliquot was stored at 4°C for immediate use.

The detection antibody (36 μg/mL of biotinylated goat anti-mouse C5a; #DY2150, R&D Systems) was reconstituted with 1 mL of reagent diluent. One hundred μL aliquots were made into 10 small (0.5 mL) microcentrifuge tubes. All but one of the aliquots was stored at -80°C for future use. One aliquot was stored at 4°C for immediate use.

Streptavidin-horseradish-peroxidase (HRP; #DY2150, R&D Systems) was made by adding 5.5 μL of the Streptavidin-HRP to 11 mL reagent diluent. This dilution could have been stored at 2-8°C for up to six months after the initial use but was made fresh for each experiment instead.

One liter of wash buffer was made by adding 500 μL of Tween 20 into one liter 1x PBS. It was then stored at 4°C and used for multiple experiments.
**Complement ELISA protocol**

The capture antibody was diluted to a working concentration of 4μg/mL in PBS. To coat one 96-well plate, 61.16 μL of reconstituted capture antibody was added to 11 mL PBS. 100 μL of the 4μg/mL working concentration of capture antibody was added to each well, covered with plastic wrap, and incubated overnight (12-18 hours) at room temperature. After the overnight incubation the 96-well plate was washed 3 times with wash buffer by tilting the plate and adding the solution to the side of the wells. After the third wash, the plate was inverted and patted on a stack of paper towels to remove any excess solution in the wells. Three hundred μLs of reagent diluent was added to each well after the 96-well plate was patted dry, covered with a paper towel, and incubated for 1-hour at room temperature. During this 1-hour incubation, the serum samples and standard were prepared for addition to the 96-well plate. After incubation, the 96-well plate was washed with wash buffer as previously described above. The standard concentration range was 0.5 pg/mL-1000 pg/mL and was added to rows A and B. To get the 1000 pg/mL concentration 100 μL of reagent diluent was added to well A3-A12 and B1-B12. Three μLs of standard was added to 0.5 mL of reagent diluent and 200 μL of this solution was added to wells A1 and A2. 100 μL from wells A1 and A2 was transferred to A3 and A4 mixed 3-5 times and transferred to wells A5 and A6. This mixing and transferring was continued through to row B. The remaining 100 μL from wells B11 and B12 was discarded so all wells in rows A and B had equal amounts of solution in them. Fifty μLs of
reagent diluent was added to rows D-H of the 96-well plate. Sixty-two µLs of the plasma samples were added to row C. Using a multi-channel pipet, 12 µL from row C was added to row D and mixed 3-5 times. After mixing, 12 µL was transferred to row E. The mixing and transferring was continued down the rows and the excess 12 µL from row H was discarded. The plate was covered with a paper towel and incubated for 2 hours at room temperature. After the 2-hour incubation, the 96-well plate was washed as previously described. A working concentration of 200 ng/mL of detection antibody (36 ng/mL) was made by adding 61.6 µL into 11 mL of reagent diluent. 100 µL of this working concentration was added to each well of the 96-well plate and incubated for 2 hours at room temperature. The 96-well plate was washed as in previous steps, and 100 µL of 1:200 diluted Streptavidin-HRP was added to each well, covered with a paper towel and incubated for 20 minutes at room temperature avoiding direct light. After the 20-minute incubation the plate was washed as before and 100 µL of substrate solution was added to each well. Adding equal parts of solution A and B together made the substrate solution. Once the substrate solution was added to each well, the plate was covered with a paper towel and incubated for 20 minutes at room temperature avoiding direct light. Fifty µLs of stop solution (#DY2150, R&D Systems) was added after incubation and the plate was gently tapped to assure mixing. The plate was read using a Bio-Rad 680 plate reader (#169-1000, Bio-Rad, Hercules, CA) at a wavelength of 450 nm.

*TNF*α *ELISA*
After mice were sacrificed by intraperitoneal injection with 10 mg/mL pentobarbital, serum was collected 24 and 48 hours post S. aureus infection via cardiac puncture. Serum was placed on ice for 15 minutes and then centrifuged 5 minutes at 5000 rpm at 4°C. The supernatant was aliquoted into a new microcentrifuge tube and centrifuged for 1-2 minutes at 10,000 rpm at 4°C. The supernatant was drawn off and placed into a new microcentrifuge tube. The supernatant was either stored at -20°C or analyzed for serum TNFα concentrations the same day using a TNFα Enzyme-linked immunosorbent assay (ELISA; #88-7324, eBioscience, San Diego, CA).

To analyze serum TNFα levels, a 96-welled plate was coated with 100 µL capture antibody (1:250 dilution) in each well for 24 hours prior to running the serum samples. After incubation, the 96-well plate was washed five times using 1X PBS/0.05% Tween-20 (#P5927-100mL, Sigma Aldrich) wash buffer. The wash buffer was kept in the wells for one minute before the next wash was completed. All washes were done so that the stream of wash buffer did not hit the wells directly. After the third wash the plate was patted dry on a stack of paper to towels to assure all wash buffer had been removed. After the fifth wash, 200 µL 1X assay diluent was then added to the all wells and incubated at room temperature (RT) for 1 hour. After incubation all wells were washed as previously described and 100 µL of standard was added so that the range was from 0.5 pg/mL-1000 pg/mL in rows A and B of the 96 well plate. Fifty µLs of sample was added to row C and then 1:2 serial dilution was completed down the plate so that
row D contained a 1:4 dilution, row E a 1:8 dilution, row F 1:16, row G a 1:32 and row H a 1:64 dilution. Once all samples were added to the plate the plate was incubated at RT for 2 hours or at 4°C overnight for maximum sensitivity. After incubation all wells were washed as previously described. One hundred µLs of detection antibody (1:250) was added to each well and the plate was incubated at RT for 1 hour. The plate was washed as previously described and 100 µL of a 1:250 dilution of Avidin-HRP was added to each well and incubated for 30 minutes at RT. The wells were dumped and soaked in wash buffer for 1-2 minutes. After the 1-2 minute incubation the plate was washed as previously described. One hundred µLs of substrate solution was added to each well and incubated at RT for 15 minutes. After incubation, 50 µL of stop solution was added to each well and the plate was read at 450 nm using a Bio-Rad microplate reader and a two-tailed Student’s t-test was run for statistical analysis.

**Statistical analyses**

Results were represented as mean +/- standard error of the mean (SEM). A Student’s t-test was performed for all analyses using Sigma-stat. P-values less than 0.05 were considered statistically significant.
Results

Simvastatin pretreatment does not enhance *S. aureus* clearance from the blood.

Previous studies done in our laboratory determined that simvastatin pretreatment increases survival in C57BL/6 mice. To provide a reason as to why the mice pretreated with simvastatin had increased survival, we looked at possible innate immune responses. We first wanted to determine whether *S. aureus* was being cleared faster in the simvastatin pretreated mice when compared to the control mice. C57BL/6 mice were sacrificed 24 and 48 hours post *S. aureus* infection and serum was extracted and analyzed for the presence of *S. aureus*. The results indicate that clearance of *S. aureus* was not enhanced in the 24 hour (Figure 3a) or 48-hour (Figure 3b), when compared to the mice not pretreated with simvastatin.
Figure 3. Simvastatin pretreatment does not enhance bacterial clearance. Mice were pretreated with simvastatin (+Simva) or vehicle control (-Simva) 18 and 3 hours prior to infection with S. aureus. 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 Student’s t-test.
Complement C5a was not decreased in C57BL/6 mice pretreated with simvastatin.

To examine the relationship between the innate immune system and survival of C57BL/6 mice we examined serum levels of complement component C5a by use of an ELISA. Our results demonstrated that serum C5a levels were not different in mice pretreated with simvastatin versus the control group (Figure 4).

Figure 4. Simvastatin pretreatment does not affect complement activity. Mice were pretreated with simvastatin (+Simva) or vehicle control (-Simva) 18 and 3 hours prior to infection with S. aureus. All mice were given 10mg/kg [BW] gentamicin at 3, 6, 12, 24, and 48 hours post-infection. Serum was isolated at 24 hours (a) and 48 hours (b) post-infection and analyzed by ELISA. Data were pooled from 2 replicate studies (n=8/group) and analyzed using Student’s t-test.
Simvastatin pretreatment induces a downward trend in serum TNF$\alpha$ levels.

To examine any alterations to the inflammatory response due to simvastatin pretreatment and the relationship between the innate immune system and survival of C57BL/6 mice, we examined TNF$\alpha$ serum levels via ELISA. The results indicate that TNF$\alpha$ serum levels trended downwards in simvastatin pretreated mice at both 24 and 48 hour time points (Figure 5a and 5b) with 48 hours having the largest decrease when compared to the control group (Figure 5b).
Figure 5. In response to *S. aureus* infection, TNF-alpha levels trend downward due to simvastatin pretreatment. Mice were pretreated with simvastatin (+Simva) or vehicle control (-Simva) 18 and 3 hours prior to infection with *S. aureus*. All mice were given 10mg/kg [BW] gentamicin at 3, 6, 12, 24, and 48 hours post-infection. Serum was isolated at 24 hours (a) and 48 hours (b) post-infection and analyzed by ELISA. Data were pooled from 2 replicate studies (n=3-10 mice/group) and analyzed using Student's *t*-test.
Discussion

Our studies demonstrate that the increased survivability of \textit{S. aureus}\textsuperscript{-infected} mice following short-term simvastatin pretreatment may be dependent upon decreased serum levels of TNF\(\alpha\), but not complement activation. Sepsis is a robust inflammatory response to a pathogen \cite{67} that affects approximately 750,000 individuals per year \cite{68}. The inflammatory response is the primary response of the innate immune system. Recent studies have shown that statins, which are used commonly to lower levels of cholesterol \cite{69} also have anti-inflammatory effects \cite{70}. These anti-inflammatory properties have been shown to reduce inflammation in the presence of bacterial infections along with other inflammation driven diseases \cite{47}. One of the pro-inflammatory cytokines causing inflammation and inducing sepsis is TNF\(\alpha\) \cite{71, 72}. Statin treatment has been shown to decrease levels of TNF\(\alpha\) both \textit{in vitro} and \textit{in vivo} \cite{73}. Reduced levels of tissue TNF\(\alpha\) were seen in rats pretreated one hour prior to receiving the immune irritant carrageenan \cite{73, 74}. A decrease in TNF\(\alpha\) was also seen in the tissue supernatant of mice with intraperitoneal inflammation that had been treated for nine days with 0.6 or 6 mg/kg of fluvastatin during the infection \cite{75}. Furthermore, patients treated with 20 mg of simvastatin per day for eight weeks have reduced levels of serum TNF\(\alpha\) \cite{76}. Our study, which examined the effect of
a low dose pretreatment of simvastatin, provides further supporting evidence on the ability of simvastatin to reduce levels of TNF$\alpha$. The reduction of TNF$\alpha$ in mice pretreated with simvastatin suggests that simvastatin modulates pro-inflammatory cytokines, increasing the survival of *S. aureus* infected mice.

Along with the activation of the inflammatory response, the clearance of bacteria also requires the activation of the complement system. Studies done to examine the effect of statin treatment on the activation of complement have demonstrated that complement activation was reduced in humans given 80 mg simvastatin a day for 5 weeks in human serum samples as evidence by a reduction in levels of complement protein C3 [77]. Activation of complement in patients treated with 10 mg of atorvastatin and 600 IU of vitamin E per day was reduced after a three months of treatment [78]. Statin treatment has been shown to also modify complement-regulating proteins such as decay-accelerating factor (DAF), causing a reduction in complement-mediated lysis of endothelial cells [79]. However, studies done with rats demonstrate that a two day simvastatin pretreatment had no effect on complement-mediated glomerular injury [80], and there was no difference on the development of complement-dependent proteinuria in rats treated with simvastatin [81]. Furthermore, a four-minute pretreatment with pravastatin and atorvastatin showed no effect on the activation of complement in human serum [66].

The complement system contributes to the initiation and progression of sepsis, specifically sepsis caused by bacteremia [82]. The complement protein C5a is specifically known to be a potent pro-inflammatory released in the
presence of sepsis [82, 83]. C5a is a strong chemoattractant for neutrophils
(granulocytes that phagocytose pathogens), monocytes, and macrophages \textsuperscript{78}.
C5a also enhances phagocytosis of the neutrophils and macrophages and
initiates the release of granule enzymes by the neutrophils. In cases of sepsis,
C5a is associated with increased multiple organ failure and death [84]. There
has been no literature to our knowledge, published on the effects of complement
protein C5 in response to pretreatment with simvastatin. Our results demonstrate
that pretreatment with simvastatin 24 hours prior to \textit{S. aureus} induced sepsis has
no effect on serum C5a levels. This finding suggests that the modulation of
complement activity may not be an important mechanism in which simvastatin
pretreatment increases survival of C57BL/6 mice with \textit{S. aureus} induced sepsis.

In conclusion, our studies demonstrate that short-term pretreatment with
simvastatin 24 hours prior to infection with \textit{S. aureus} is sufficient enough to
increase survival of mice and decrease immunoglobulin levels of IgG2c
(indicative of a Th1 response) in C57BL/6 mice. This increase in survival is
mediated through decreased production of pro-inflammatory cytokines (TNF\textsubscript{\alpha}),
but not by modulation in complement protein C5a.
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