The effect of simvastatin pretreatment on immunologic memory and survival in response to secondary \textit{Staphylococcus aureus} infection

A DISSERTATION

SUBMITTED TO THE GRADUATE SCHOOL

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE

DOCTORATE OF EDUCATION IN SCIENCE

BY

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BALL STATE UNIVERSITY

MUNCIE, INDIANA

MAY, 2013
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BALL STATE UNIVERSITY

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ABSTRACT

DISSERTATION: The effect of simvastatin pretreatment on immunologic memory and survival in response to secondary *Staphylococcus aureus* infection

STUDENT: Lisa K. Smelser

DEGREE: Doctorate of Education in Science

COLLEGE: Sciences and Humanities

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*S. aureus* is a leading cause of sepsis in the United States, which is the result of an overly robust inflammatory reaction mounted by the host’s immune system in response to microbial invasion. In cases of *S. aureus* infection, antibiotic treatment may not clear the infection quickly enough to prevent sepsis. In varying murine models of sepsis, statins have been found to be protective for sepsis. Increasing evidence suggests that statins are potent immune modulators. Previous studies in our lab demonstrated that short term simvastatin treatment was protective for sepsis-related death due to *S. aureus* infection. Analysis of antibody levels revealed that levels of total serum IgG2c were decreased in simvastatin pretreated mice, suggesting an alteration in immune function that may have contributed to the increased survival. Although altered immune function may be protective for some primary responses, the immune alteration due to simvastatin pretreatment may leave the host less prepared for subsequent exposure to a pathogen. To investigate this possibility, a secondary infection model was implemented whereby simvastatin pretreated and control mice were infected with *S. aureus* 14 days after primary infection and assessed for survival. While simvastatin pretreated mice did
not differ in survivability to secondary *S. aureus* exposure compared to control mice, memory B and T lymphocyte functions were altered. Simvastatin pretreatment significantly increased levels of serum IgM following secondary infection. In addition, IgG1 secretion was dampened from memory B cells that had been stimulated with lipoteichoic acid and peptidoglycan or lipopolysaccharide at 48hrs post-isolation. Simvastatin also decreased proliferation of stimulated memory B cells and memory T cells compared to controls. These findings demonstrate the potent ability of short term simvastatin treatment prior to primary infection to be an immune modulator of both primary and secondary responses resulting in altered memory immune function.
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Introduction

Humans have two problems to control when infected with pathogenic bacteria. The first is controlling the damage done to the host by the invading organism itself. The second is to limit the damage done by the host’s immune system as it mounts an attack to contain and destroy the invading pathogen. When the immune system cannot control and clear the pathogenic bacterial infection locally, the infection can spread, activating a systemic immune response and causing wide-spread damage. This systemic damage is a result of the host’s inflammatory response to the bacteria and is commonly called sepsis [1]. *S. aureus* is a major cause of bacterial sepsis, an inappropriate hyper- or hypo-immune response causing severe inflammation or immune unresponsiveness [2, 3], often resulting in organ failure and death [2, 4].

Recently, a class of drugs inhibiting 3-hydroxy-3-methyl-glutaryl-CoA reductase activity to decrease cholesterol synthesis, collectively called statins, has been shown to have anti-inflammatory and immunomodulatory properties separate from their ability to reduce cholesterol levels and cardiovascular disease [5]. A variety of studies, by us and others, have demonstrated the ability of simvastatin to enhance survival to sepsis [5-11]. Increasing evidence suggests that statin treatment may prevent cellular invasion of pathogenic bacteria and down-regulate immune responses that initiate sepsis [12, 13].
However, while alteration of immune responses may be beneficial in one aspect of a pathogenic infection, there is potential for the altered immune response to be a detriment in fighting subsequent infections.

In an unaltered immune response, upon activation, T cells are influenced to become specialized cells that direct immune responses with specific characteristics that are effective for fighting the invading pathogen. Depending on the types of cytokines that T helper cells secrete, they are categorized as Th1 or Th2 cells. Importantly, T cells regulate the production of specific antibody classes that are produced during an infection. IL-4, a Th2 cell cytokine, induces class switching to IgG1, while IFNγ, a Th1 cell cytokine, induces class switching to IgG2a (IgG2c in C57BL/6 mice) [14-16]. Through their secretion of IFNγ, Th1 cells enhance inflammatory responses. Th2 cells, via IL-4, down-regulate Th1 responses as well as inflammatory reactions [14, 17-19]. Together, specifically directed lymphocyte functions result in the clearance of infecting pathogens while concurrently activating a set of memory lymphocytes, which facilitates efficient clearance of the pathogen during future exposures.

We have data demonstrating that short term simvastatin pretreatment results in significant reduction of serum levels of total IgG2c, an effective opsonizing antibody necessary to fight pathogenic infections, which was concomitant with increased survival of mice infected with 
\( S. aureus \). This result may be a necessary change that contributes to the increased survivability to 
\( S. aureus \) infection, but may also create a state of immunodeficiency should an individual have later encounters with other pathogenic bacteria or subsequent encounters with 
\( S. aureus \).

It was the goal of these studies to examine how simvastatin pretreatment alters immune responses, potentially limiting the generation of an effective memory response and subsequently inducing a state of immunodeficiency to secondary infection.
Literature review/background

Innate Immune Response

The immune system is divided into two parts, innate and adaptive. Innate immunity is the non-specific defense system that your body naturally has before exposure to a specific antigen or outside pathogen. It provides the first defense reaction against the invading pathogen such as *S. aureus*; however, this immune branch lacks specificity and memory. Innate immune cells recognize foreign invaders and respond quickly because they have pattern recognition receptors (PRR) that are effective in recognizing molecular patterns present on pathogens. PRRs are genetically encoded and evolutionarily conserved. When bound to pathogens, these receptors trigger phagocytosis of the pathogen for destruction or trigger cytokine production for cell signaling. The major cells involved in the innate immune response include granulocytes (neutrophils, basophils, and eosinophils) and macrophages in addition to dendritic cells, mast cells, and natural killer (NK) cells [17]. Granulocytes store antimicrobial particles in granules that are released to destroy bacteria in the event of an infection. Neutrophils, the most prominent granulocyte in tissues infected with bacteria, actively engulf bacteria via a process known as phagocytosis [20-22]. Eosinophils are also phagocytic, but play a more apparent role in the clearance of parasitic infections. Basophils, a nonphagocytic granulocyte, and mast cells are responsible for allergic reactions but also contribute to inflammatory responses. Monocytes are immature macrophages that travel in the blood, secrete proinflammatory cytokines such as TNFα, IL-1, IL-6, and IL-12, and are shuttled into infected tissue where they mature and function as phagocytic macrophages. Macrophages are the orchestrators of inflammation, a classic innate response that is characterized by redness, swelling, pain, and heat to clear an invading foreign pathogen [17, 18, 22, 23]. Through identification of bacteria using PRRs and recruitment of innate
immune cells to infected tissue, inflammation is triggered by the innate immune system to ultimately contain and destroy an invading pathogen.

Macrophages are the conductors of inflammation using neutrophils as the workhorses. Inflammation delivers effector molecules and cells to the site of the infection for pathogen clearance, promotion of tissue repair, and induction of blood coagulation. Vascular changes are also evident to increase blood flow and to recruit cells to the infected tissue to destroy the pathogen [17, 22, 24]. Endothelial cells express cell adhesion molecules (CAMs), and vascular permeability increases to induce cellular influx to the infected tissue. Neutrophils, monocytes, then eosinophils and lymphocytes enter the infected tissue. The endothelium then becomes activated, which induces local blood coagulation to contain the pathogen for destruction. Macrophages are responsible for producing cytokines and chemokines for cellular communication and trafficking. Cytokines, small proteins secreted by a variety of cells following stimulation, produce very robust responses, but act locally due to their short half-life. Chemokines control the cellular location of immune cells [17, 24]. Macrophages produce proinflammatory cytokines, chemokines, lipid communicators, and oxygen radicals. Major inflammatory cytokines produced by macrophages include IL-6, TNFα, IL-1, CXCL8, and IL-12 [17, 24]. Up-regulation of cytokines such as interleukins (IL) and tumor necrosis factor-α (TNFα) can cause inflammation, fever, and vascular changes as described above [17, 25]. CXCL8 acts as an attractant to bring neutrophils and basophils to the infection. IL-1 activates the vascular endothelium and lymphocytes, which allows greater cellular access to the infected tissue. IL-1 also promotes IL-6 production. IL-6 specifically induces fever to increase body temperature to denature pathogenic enzymes to aid in pathogen destruction and promotes T cell activation. IL-12 produced by macrophages induces NK cells to make interferon-γ (IFNγ), which further
activates macrophages to be more aggressive. TNFα is a classic pro-inflammatory cytokine that activates endothelial cells and dilates blood vessels. Through these actions, TNFα increases cellular movement to the site of infection, and fluid movement into the lymph nodes [17, 24, 26]. It is imperative that inflammation does not become chronic as tissue destruction by inflammation can lead to decreased function, and white blood cells can be chronically stimulated and recruited to the site of damage due to persistent infection [22]. Macrophage products maintain control of innate immune processes such as inflammation in addition to aiding in adaptive immune activation.

If the innate immune processes cannot contain and destroy a pathogen, the adaptive immune system is initiated 5-7 days after initial exposure to deploy additional responses to the invading pathogen including activating innate process specifically for that pathogen [17, 26]. Macrophages and dendritic cells (DCs) are key players in the activation of B and T cells. Dendritic cells are able to engulf bacterial particles and are directed to lymph nodes by chemokines where there are large populations of T lymphocytes. DCs receive signals from Toll-like receptors or other pattern recognition receptors during migration inducing maturation. This results in the loss of the DC phagocytosis ability; however, it increases the DC ability to interact with T lymphocytes [17, 26, 27]. DCs post specific bacterial proteins on the cell surface by processing the proteins into small peptides that act as antigens. The antigenic peptides are combined with major histocompatibility complex (MHC) molecules on the surface of the DC for antigen presentation. Macrophages can also present antigen when activated by engulfing and processing bacteria. Macrophages respond to cytokines produced by T cells for inflammatory regulation and during activation have larger amounts of MHC II on the cell surface increasing their ability to present antigen to T cells. Antigen presenting cells, DCs, B cells, and macrophages, all have both MHC I and II molecules on their cell
surface for antigen identification by T cells. The MHC-antigen complex is identified by helper T cells for adaptive immune response activation [26, 28]. The activation of T and B lymphocytes in response to injury and infection is the adaptive component of an immune response.

**B and T lymphocyte development and activation**

The adaptive immune system involves a response to an antigen or pathogen that is controlled by B and T cells, which are specialized leukocytes (white blood cells) called lymphocytes. B cells direct humoral immune responses, and T cells are involved in cell-mediated immune processes. While both B and T lymphocytes originate in the bone marrow, T cells migrate and develop in the thymus. B cells remain in the bone marrow and continue their development. Lymphocytes move into the location of infected tissue once activation has been initiated by pathogen identification. Lymph nodes provide an organized location for B cells to be in contact with T cells, which aide in activation. While the lymph nodes are the primary sites for initiating responses to pathogens found in the lymph and localized tissues, the spleen provides an opportunity for cells to recognize pathogens in the blood for B cell interaction with T cells to activate the adaptive responses. The adaptive immune system is specific and orchestrates a response within a week of exposure to the pathogen due to the activity of B and T cells [17, 25].

B and T cell activation occurs in the blood, spleen, or lymph nodes and results from interaction of lymphocytes with foreign antigen (small molecules from pathogens) via receptors. B and T cells both have stationary receptors on the cell surface, which have a transmembrane region to anchor the receptor to the cell membrane as well as a constant and variable region. The end of the receptor is the variable region with the tip being specific for antigen binding. B cell receptors (BCRs) have two antigen binding sites and T cell receptors (TCRs) have one. The variable region of the receptors is
generated through DNA gene rearrangement as these receptors are not directly inherited like innate PRRs but must be generated through rearrangement of inherited genes. BCRs and TCRs have great diversity because of the unlimited gene rearrangement that results in the antibody variable region, thus allowing for variety and specificity to foreign antigens [17, 26].

T cells are activated through the interaction of TCRs and MHC molecules on antigen presenting cells. Intracellular antigens are presented to CD8+ cytotoxic T cells via MHC I interaction with the TCR and CD8. Extracellular antigen peptides are presented in MHC II molecules to CD4+ helper T cells (Th) with CD4 to stabilize the interaction [17, 26, 29]. In addition to antigen binding the TCR, naïve T cells also need CD3 and zeta chains for proper activation and cell signaling. Proliferation of T cells also relies on CD28 interaction with CD80 or CD86 [17]. While TCR are more diverse than BCR, they are not able to increase binding affinity for a specific pathogen like BCR. The increase in binding affinity increases the reaction between the BCR and the antigen for a stronger binding capacity [17, 30].

B cells identify whole pathogens when the BCR binds to a specific foreign, naïve antigen [31]. Igα and Igβ are proteins essential for cell signal communication when the BCR binds to an antigen. The B cell will then engulf the BCR and antigen through receptor-mediated endocytosis to process the antigen into peptides for antigen presentation and further immune activation. MHC class II molecules aide B cells in antigen presentation to T cells. The corresponding Th cell will be attracted by the MHC II/antigen complex to activate the B cell resulting in a thymus-dependent (TD) interaction. This mechanism of B cell activation requires CD40/CD40L interaction between the B cell and the helper T cell [32, 33]. Another mechanism for B cell
activation is from a thymus-independent (TI) antigen. TI-1 antigens bind a receptor other than the BCR such as a TLR. TI-1 antigens are also called mitogens due to their ability to non-specifically activate B cells without T cell activation. Bacterial cell wall components (LPS, peptidoglycan, and lipoteichoic acid) activate in a TI-1 manner [24, 34-36]. TI-2 antigens have the capacity to bind to several BCRs at the same time and are often polysaccharides with repeating binding sites. In addition to BCR and signaling molecules Igα and Igβ, B cells need co-receptors (CR2/CD21, CD81, and CD19) for enhanced signaling. The activated B cells will undergo clonal selection and expansion so that pathogen-specific B cells divide to produce millions of copies of cells that produce antibodies specific to the pathogen [33, 37]. Once B cells are activated, they carry out specific effector functions through their secretion of specific antibody types.

**B lymphocyte effector function**

B cells can differentiate into antibody-secreting plasma cells or survive the down-regulation of immune responses following activation and become memory cells, but the primary effector functions ascribed to B cells are actually carried out by the antibodies they secrete. When activated through their BCRs in response to contact with foreign substances such as pathogenic bacteria, B cells produce antibodies, like BCRs, also known as immunoglobulins [17, 25, 33, 38]. Each B cell produces one specific antibody at a time. Antibodies are important for marking foreign substances for destruction, neutralizing pathogens, and other immune functions. Circulating in the lymph and the blood, the antibodies will bind to any pathogens with the specific antigen initially recognized. This will mark the pathogens for destruction by cell lysis or phagocytosis. Pathogen neutralization can also occur through direct antibody binding to bacterial toxins or interference with bacterial receptors advantageous for infection [37]. The type of
antibody class determines the function of the immunoglobulin. There are five antibody classes, IgM, IgG, IgD, IgA, and IgE. All isotypes of immunoglobulins share a basic common structure of light (MW 25kD) and heavy chains (MW 50-77 kD). The light chains are comparable in all isotypes, but the immunoglobulin structure differs in the formation of the heavy chain. Unique to the antibody isotype, immunoglobulins are responsible for several functions such as binding to an antigen or bacterial toxin or being a messenger in the cascade of molecular events that regulate the immune system [38]. Depending on the nature of the foreign substance, B cells switch their antibody production from one class to another while keeping the same antigen specificity [16, 17, 25]. IgM is the first antibody produced in an adaptive response as it is the best at activating mechanisms to destroy the pathogen. Through antibody gene rearrangement and splicing, IgM can class switch to IgD, IgG, IgE and IgA. The purpose of class switching is to control the type of immune response necessary to clear a pathogen and restore homeostasis as a function of each individual antibody isotype [16, 17].

Each antibody isotype has distinct functions for optimal immune function. IgA is necessary for neutralizing pathogen attachment and subsequent invasion at mucosal surfaces. IgE induces immune responses necessary for fighting parasitic infection and induces allergic reactions. IgD has no known effector function but is necessary for B cell development. IgG and IgM antibodies are the most effective at clearing foreign pathogens. Due to its pentameric structure, IgM is most effective in binding foreign pathogens to mark them for destruction. IgM is also the initial antibody produced in response to a foreign molecule identified in the human body [17]. IgG has subclasses that differ based on their specialized effector functions that allow them to regulate processes such as phagocytosis, bacterial neutralization, cellular activation, cytotoxicity, and the release of inflammatory mediators [17, 30, 39, 40]. Subclasses of IgG
antibodies can be potent stimulators of effector functions (IgG2a) or act as regulators (IgG1) of immune cell activity due to their interactions with specific antibody receptors called Fc receptors (FcR). Immune system effector functions are executed primarily through the interactions of antibodies with FcRs, such as FcγRI (which binds IgG2a with high affinity and enhances macrophage function), and FcγRIIB (which predominantly binds IgG1 initiating inhibitory signals) [41, 42]. Interactions of antibodies with signaling receptors aide in promoting immunoglobulin class switching for stimulation of effector functions to operate within the adaptive immune response alongside T lymphocytes.

**T lymphocyte effector function**

There are different types of T cells that can be helper, regulatory, or cytotoxic cells. T helper (Th) cells (CD4+) facilitate immune communication and direct immune responses by recognizing antigens and secreting cytokines that regulate the strength and extent of an immune response. T regulatory cells, a variety of Th cells, repress the humoral and cell-mediated immune function through antigen recognition to prevent unnecessary and harmful autoimmune reactions. Cytotoxic T cells (CD8+) have the ability to directly kill target cells. Once T cells have interacted with the MHC/antigen complex, the activated cytotoxic T cells search for additional cells that match the antigen. The cytotoxic T cells then secrete cytotoxins (perforin and granulysin) to destroy the pathogen via cell membrane perforation or apoptosis [17, 25, 43]; however, recent studies have suggested CD4+ T cells can also participate cytotoxic cell killing [44].

A primary function of T cells, particularly CD4+ Th cells, is to secrete large amounts of cytokines to direct the type of immune response generated. Depending on the types of cytokines that T helper cells secrete, they are categorized as Th1 or Th2
cells. Th1 and Th2 cells are identified by their secretion of large amounts of IFNγ and IL-4, respectively. Because of the cytokines they secrete, T cells influence the function of all cells of the immune system and direct the type of immune response. Importantly, T cells, through their secretion of cytokines, regulate the production of specific antibody classes that are produced during an infection [14-16, 27, 44]. IL-4, a Th2 cell cytokine, induces class switching to IgG1, while IFNγ, a Th1 cell cytokine, induces class switching to IgG2a, 2b, and 3 [14-16, 44]. Importantly, through their secretion of IFNγ, Th1 cells enhance inflammatory responses, primarily through the effects of IFNγ on macrophage activation. Th2 cells, via their secretion of IL-4 and the antagonistic activity of IL-4 towards IFNγ–stimulated functions, down-regulate Th1 responses such as inflammation. Additionally, IL-4 is important in B cell activation [3, 14, 17-19, 27, 44, 45]. Together, specifically directed lymphocyte functions result in the clearance of infecting pathogens while concurrently activating a set of immune cells that retain their specificity for the pathogen, which facilitates efficient clearance of the pathogen during future exposures.

**Immunologic memory**

Adaptive immune responses are necessary for the generation of immunologic memory. Immunologic memory is the ability of the immune system to respond more quickly, robustly, and effectively to each repeat exposure to a pathogen. During an initial, primary infection, immunologic memory is established by the generation of pathogen-specific memory B cells as well as memory T cells for long-term protection without additional antigen exposure [17, 46, 47]. Memory cells are vital in protection against a second exposure to the infecting organism (secondary infection) resulting in a memory response [48, 49].
Memory B cells prevent naïve B cells from becoming activated by a second encounter with a pathogen resulting in the memory response superseding an initial response by naïve B cells. Memory B cells participate in antibody class switching with the potential to produce IgG, IgA, or IgE in addition to IgM [17, 33, 47], and can account for up to 30% of peripheral blood B cells [33]. In the peripheral blood, evidence supports roughly 50% of memory B cells being IgM positive and the other half expressing IgG or IgA. Human IgE memory B cells have not currently been identified circulating in the blood, but possible evidence exists in murine models [33]. Memory B cells produce high affinity antibodies through somatic mutations introduced during proliferation and specific selection resulting in affinity maturation. High affinity antibody producing cells are selected to become memory cells once the initial effector responses are down-regulated because they exhibit the strongest attraction to the antigen. B cell memory responses are thought to occur in a thymus-dependent (TD) manner. TD-antigens have the ability to induce immunologic memory cells to reactivate. Interaction with stromal cells aids memory cells in maintaining homeostasis and determines the magnitude of the memory response generated [17, 33, 47, 49]. Human memory B cells are usually characterized by expression of CD27, CD19, CD20, CD22^{high}, CD44^{high}, and CD45^{high}, but the CD27 marker is not present in murine models. Traditionally memory B cells do not express CD5, CD23, CD95 (Fas). In addition, TLR 9 is very active on memory B cells compared to naïve B cells, owing to its potential involvement in secondary immune events as TLR 9 identifies bacterial DNA and activates B cells for bacterial destruction [17, 33]. In addition to the role memory B cells contribute to the memory response, memory T cells also perform vital functions in the memory response to secondary infections.

Memory T cells express different surface markers compared to naïve T cells [17, 44, 46]. Main identifying markers for memory T cells include CD44+, FasL+, LY6C+,
CD45RO+, CD45RA-, and CD127+, [17, 44]. CD127 is important for IL-7 production to induce effector T cells to become memory cells. For activation, memory T cells only need CD3 stimulation unlike naïve T cells that need both CD3 and CD28. Usually, naïve T cells have CD62L on the cell surface [17], but some memory cells has been shown to re-express CD62L and CCR7 [44]. CD4+ T memory cells interact with IL-17 stromal cells in the bone marrow during times of inactivity and are LY6C positive. LY6C on T cells is thought to be indicative of a “true” memory T cell [17, 46, 47]. Specific CD4+ memory T cell functions that contribute to an effective memory response include the quick production of copious amounts of cytokines, up-regulating cytotoxic functions of T cells, and directing B cell activities [44].

Memory cells result from the initial immune response to pathogen invasion that survive the induced apoptosis necessary to down-regulate immune responses after the pathogen has been cleared. While memory cells may not actively divide or perform effector functions, they do divide to replenish the memory population without antigen stimulation [17, 46, 47]. Disagreement does exist in the literature surrounding the protective nature of the memory response such that “memory” protection may be gained from persisting neutralizing antibodies or T cells that have been constantly activated by persistent antigen rather than from effector lymphocytes that have survived the down regulation of the immune response [44]. Both B and T cells are required in a memory response due to the synergistic nature of the interactions between B and T cells in the adaptive immunologic memory response to fight infection [17, 46, 47].

The generation of memory cells is necessary for protecting a host from subsequent, secondary pathogenic invasion. In addition to the presence of memory cells, elevated levels of pathogen-specific antibodies, primarily IgM and IgG isotypes,
remain in the blood and interstitial fluid following a first encounter with a pathogen [17, 33, 44, 47]. Specifically, IgM memory B cells are an important defense against gram-positive, encapsulated bacterial infections [50]. High affinity antibodies from memory B cells respond quickly with strong binding capabilities and high specificity in subsequent encounters [17, 33, 44, 47]. A primary infection is usually marked by higher levels of IgM compared to IgG, and more IgG compared to IgM is usually characteristic of a secondary infection due to effective class switching [33, 51]. Diminished total immunoglobulin (IgG, IgM, and IgA) levels must be quite substantial to decrease protection against an infectious pathogen, and IgG and IgM deficiencies result in a greater reduction in immune protection compared to IgA deficiencies [33, 51]. Decreases in immunoglobulin concentrations or memory B cell quantities have been associated with people taking rituximab, corticosteroids, anti-seizure medications, and over-the-counter medications like Ibuprofen, but does not usually create critical levels as to immunocompromise the patient [51]. However, intravenous antibody treatment (mainly IgG) has been used in patients with a lowered immune function to combat potential reduction in protection against infection [38]. Aging can also impact secondary immune responses such that memory B cell counts are lower in individuals over sixty years old. Potentially, this could contribute to a decreased protection from new pathogens if the memory response is not as responsive as it once was [33]. The combined presence of the pathogen-specific antibodies with properly functioning B and T cells is necessary for the generation and maintenance of protective immunity against subsequent exposure to the specific invading pathogen [17, 44, 46].

**Sepsis**

Sepsis is clinically diagnosed in patients with an infection plus systemic inflammatory response syndrome (SIRS). Indicators of SIRS include abnormal body
temperature, increased heart rate, and an altered white blood cell count \cite{52, 53}. Sepsis plus organ failure leads to severe sepsis, and septic shock adds acute circulatory dysfunction with unexplained low blood pressure to the list of problems. Clinical symptoms vary among patients especially those with additional medical conditions \cite{18, 53}. As the number of elderly and immunocompromised patients increases, so does the occurrence of sepsis. In 2000, 240 septic cases were reported per 100,000 people in the United States with a 30% or greater mortality rate in patients with severe sepsis \cite{22, 53-55}. Sepsis induces $17$ billion in health care costs each year with the number of cases increasing by 9% each year \cite{56}, and it is the primary cause of death in intensive care surgical units. Unfortunately, the prevalence of sepsis has not decreased with the vast increase in medical treatments available \cite{22, 23, 57}. Risk factors for sepsis include prior illness, hospitalization with invasive procedures, wide-spread use of antibiotics, decreased immune function, and genetic variations in inflammatory regulatory genes \cite{23, 52}. Risk factors for sepsis-induced death include the location of infection, the type of infecting organism, multiple organ failures, and secondary infection \cite{3, 58}. Early diagnosis of sepsis is critical for current therapies focused on bacterial clearance via antibiotic treatment, blood pressure and blood glucose homeostasis, and hydration to be effective \cite{23}.

Sepsis is a unique condition due to the initial hyper-inflammatory response followed by a hypo-immune state, which is increasingly identified as the main cause of death in septic patients \cite{2, 3, 18, 19, 23, 53, 59}. While contained, local inflammation is beneficial for infection clearance, chronic, wide-spread inflammation can lead to the development of sepsis \cite{3, 23}. Controlling extreme inflammation without inducing a hypo-immune state continues to elude effective treatment of the condition \cite{22, 59}. Cytokine communication with white blood cells and the vascular endothelium are the
major molecular mechanisms involved in the development and progression of sepsis because the inflammatory nature of sepsis damages the endothelium, increases endothelial cell activation, and causes coagulation dysfunction [3, 23, 53, 58]. Peptidoglycan, a component of gram-positive bacteria cell walls, has been shown to directly activate the endothelium adding to the dysfunction [57]. During sepsis, bacterial pathogens activate neutrophils, dendritic cells, and macrophages in addition to promoting cross talk between dendritic cells, macrophages, and CD4+ T cells [2, 18, 24, 60]. Bacterial antigens interact with TLRs on cell surfaces, specifically TLR2 in the case of *S. aureus* [24, 34, 57]. Kinases are activated and transcription of cytokines, chemokines, and other pro-inflammatory mediators like C-reactive protein, IL-6, and TNFα are upregulated. Both macrophages and neutrophils respond to this cellular cascade and are highly activated [3, 19, 54, 57, 61, 62].

Neutrophil activation promotes inflammation to clear the bacteria, but tissue damage is induced with the clearance [2, 18, 19, 23, 24]. Malfunction of neutrophils during sepsis can also lead to interference with phagocytosis mechanisms [23]. Macrophages, being the conductors of inflammation, are responsible for regulating the inflammatory response. Macrophages are efficient regulators of inflammation because their products have multiple targets to induce similar effects, and macrophage products act as antagonists of each other for subtle regulation [2, 18, 19, 23, 24].

Cells undergoing necrosis signal macrophages to produce IL-12 and induce Th1 inflammation, which drives CD4+ T cells to make IFNγ, TNFα, and more IL-12 to further promote macrophage activation and inflammation [2, 18, 19, 24]. The production of IFNγ by CD4+ T cells also induces IgG2a B cell class switching [16]. In the initial onset of septic shock, patients were found to have decreased levels of IgG and IgM during days 1-4 with IgA concentrations unchanged. However, by days 5-7, 61% of patients
had IgG and IgM levels return to acceptable ranges. The initial IgG and IgM decreases did not correspond with a spike in death rates or disease severity, but did correlate with a decrease in serum protein levels [63]. Changes in immunoglobulin production during sepsis could also be affected by a decrease in B cell number [3, 23]. In sepsis, T cell proliferation can be depressed in conjunction with an increased expression of cell surface proteins responsible for inducing a down-regulatory response and decreased expression of CD3 and CD28 necessary for activation [23]. Keeping the immune system in homeostasis is crucial in balancing the initial hyper-inflammatory response with the hypo-inflammatory state found as the disease progresses.

Excessive anti-inflammatory signaling or down-regulation of inflammatory signaling and apoptosis of immune cells can cause a patient to have an unresponsive, hypo-immune condition called compensatory anti-inflammatory response syndrome (CARS), which is the state of most patient mortality [3, 18, 19, 23, 59, 62, 64]. When monocytes circulating in the blood are activated during sepsis, inflammatory cytokines are decreased, IL-10 is upregulated, and programmed cell death of monocytes is induced [23]. HLA-DR, a surface protein on monocytes and member of the MHCII family, could develop into an important clinical factor indicating suppressed inflammation, the presence of secondary infections, and an immunoparalysis state as HLA-DR is influenced by several cellular signaling molecules in sepsis [23]. A recent study found dampened monocyte, B cell, and T cell counts in patients dying from sepsis. In addition, these patients had significantly lower levels of TNFα, IFNγ, IL-6, and IL-10 indicating immunoparalysis [65]. The reversal of the Th2 anti-inflammatory response has been shown to increase survival as high levels of IL-10, a Th2 cytokine, are seen in patients with high mortality rates [2, 18, 23]. Other predictors of mortality include low levels of TNFα and IL-6, a decrease in Th1 cells, and decreased neutrophil and
monocyte activation [18, 23, 62, 66]. In a murine model of S. aureus sepsis, TNFα and IL-1 production culminated at 4 hours post-infection with IL-6 peaking at 72 hours post-infection. In addition, non-leukocyte cells were found to add to the secretion of IL-1 and IL-6. Even though bacteria may be cleared by 48 hours post-infection, inflammatory damage and signaling can still persist [67]. During bacterial infection, levels of procalcitonin (PCT) are known to be elevated and may be linked to death. Alternatively, decreased levels of PCT may indicate a timing point to cease antibiotic therapy as lower levels of PCT may suggest a trend towards immunoparalysis. However, identification of a single clinical marker to guide medical treatment, predict disease state, or mortality risk has remained elusive as immunologic status and progression lacks homogeneity in septic patients [23].

In inflammatory diseases, one treatment is to block TNFα production by macrophages to decrease the inflammatory response; however this treatment and other anti-inflammatory treatments (glucocorticosteroids and neutralizing antibodies) have not been effective in the treatment of sepsis. The ineffectiveness of the TNFα treatment may be due juxtaposing TNFα levels in beginning stages of sepsis compared to late progression [17, 23, 24]. Several additions to current therapeutic treatment for sepsis have been investigated with little overall success as immune modulations seen in patients are not homogenous and require frequent monitoring to determine the appropriate timing for administration [23]. Current evidence does not support regular use of glucocorticoids, intravenous immunoglobulins, heparin, or rhAPC (Xigris) into treatment regimens due to variable clinical results. Each of these drugs acts to decrease inflammation or suppress the immune system, which might not be the appropriate approach because most patients are dying in a state of immunoparalysis, which indicates lack of inflammatory homeostasis is a major contributing factor to
mortality. Specifically, Xigris was previously used in clinical practice and was recently removed from the market due to refuting evidence of efficacy in the PROWESS-SHOCK trial [23]. Alternatively, immune stimulants like IFNγ, granulocyte colony-stimulating factor (G-CSF), and granulocyte macrophage colony-stimulating factor (GM-CSF) are being investigated to restore immune balance [23]. Alternative treatments that are under current investigation include macrolides such as clarithromycin to strengthen the Th1 response during immunosuppression and high-volume hemofiltration to decrease cytokine concentrations at their height to minimize inflammatory chaos [23]. Other current therapeutic approaches under investigation are focused on the effects from the infecting pathogen, the immune response to infection (activation and suppression), and endothelial regulation [3]. When investigating immune treatments for sepsis, it is vital to continually monitor immune status to determine which treatment is most appropriate as current studies have not separated patients and their treatment protocols based on immune function during disease progression [23]. In addition to immune disturbances driven by sepsis, the type of infecting pathogen can also have an impact on the progression of sepsis.

**S. aureus**

*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 [68]. Specifically, *S. aureus* is the most prominent and deadly pathogen causing bloodstream infections that result in sepsis causing 40-50% of all cases [24, 57, 68-71]. Increasing frequency of *S. aureus*
infections is partially attributed to the increase in elderly, immunocompromised patients whom are ill or in the hospital [69-71].

Gram-positive organisms have unique characteristics and effects on infection progression that differ from Gram-negative infectious organisms like *E. coli*. Gram-negative organisms have peptidoglycan (PepG) and lipoteichoic acid (LTA) in the inner cell wall, but it is lipopolysaccharide (LPS) found on the outer membrane of gram-negative species that causes an endotoxic, inflammatory effect. The outer cell wall of gram-positive organisms is comprised of peptidoglycan and lipoteichoic acid, which up-regulates inflammation [26, 72, 73]. PepG is crucial for activating mechanisms for bacterial destruction to communicate with monocytes for subsequent activation and cytokine secretion. PepG is also thought to interact with IgG. Specific to inflammatory cytokines implicated in sepsis, PepG in the blood can activate monocytes and specific proteins promoting inflammation with LTA via TNFα, IL-1, and IL-6 up-regulation. IL-12 was also found to be induced from macrophages by PepG, which makes macrophages more aggressive through IFNγ. In a rat model, injections of PepG and LTA together induced septic shock and organ injury with increased levels of TNFα and IFNγ in the plasma [57].

Being a gram-positive organism, *S. aureus* also produces exotoxins that further induce inflammation as seen in sepsis [24]. The purpose of exotoxins is to scavenge the host for nutrients the bacteria can utilize for survival and growth as well as to evade human immune defenses. The exotoxins can also act as superantigens activating large numbers of T cells through MCHII/TCR interaction and CD28, which results in immense, non-specific T cell proliferation and cytokine secretion (IFNγ) contributing to septic shock [3, 24, 57, 74-78]. Specific superantigen genotypes, like enterotoxin A gene (*sea*), have been shown to increase the virulence of *S. aureus* infections leading to a more severe
clinical manifestation. Another genotype, enterotoxin gene cluster (egc), corresponds with a less severe infection [77]. The immune system and bacteria have several ways to interact with each other for identification, infection clearance, and bacterial evasion of the immune response.

Immune cells have pattern recognition receptors (PRRs) for identifying pathogens to initiate cell signaling for proper pathogen clearance. One such type of PRRs are toll-like receptors (TLR), which are found both inside the cell and on the cell surface. TLR2, along with TLR6 or TLR1, specifically recognizes LTA and PepG, cell wall components of gram-positive bacteria [3, 17, 19, 26, 34, 57, 74]. TLR2 knockout mice were found to have an increased susceptibility to gram-positive, staphylococcus infections [57, 74, 79]. Recently, PepG recognition proteins (PGRPs) have been implicated in host protection from gram-positive infections. In addition to TLRs and PGRPs, CD14 has involvement in signaling during bacterial infections, specifically in gram-positive organisms [57]. While the immune system is efficient and effective in identifying invading bacteria, pathogens have their own means to evade immune mechanisms for survival.

*S. aureus* has the means to resist and avoid immune defense mechanisms. Immune defense tools that can be thwarted by *S. aureus* include enzymatic bacterial lysis, respiratory burst, antimicrobial fatty acids, cationic antimicrobial peptides (CAMPs), and white blood cell activity [80]. In initial identification of *S. aureus* infection, the bacteria can evade recognition and thus destruction by having a capsule or biofilm that hides the features used by the immune system for bacterial identification. *S. aureus* can also hide from the immune system by recruitment of non-specific antibodies to coat the bacteria. *S. aureus* can deactivate antimicrobial peptides released from granulocytic neutrophils. Neutrophil and macrophage phagocytic capabilities are also thwarted by *S.
*aureus* as the bacteria blocks receptors and produces toxins, which kill other leukocytes and red blood cells. Additionally, phagocytosis of the bacteria is decreased by the blockade of cytotoxic and cell lysis capabilities [74, 81]. While *S. aureus* is a prevalent infecting organism in sepsis with mechanisms for immune avoidance, the risk of a secondary infection increases with patients who are chronically immunocompromised and require frequent hospitalizations [59].

**Secondary infections**

With increasing chronic conditions that require multiple hospital visits and cause a decrease in immune function, the prevalence of subsequent infection is high. Whether the cause of the infection is from the initial infecting organism or a different species, additional infections can cause complex complications. Due to the nature of chronic illnesses, immune surveillance must be vigilant as the disease compromises immune function creating a susceptibility and sensitivity to infection. In immunoparalysis during the later progression of sepsis, secondary infections may be the ultimate cause of death due to immune failure in controlling the subsequent infection [59]. Mice infected with a respiratory virus exhibited a decrease in *S. aureus* clearance and increased inflammation with heightened susceptibility to an additional bacterial challenge [82]. Patients with influenza are also susceptible to contaminate or secondary infection, particularly *S. aureus* [83]. Sepsis disease progression into a state of immunoparalysis can increase susceptibility to secondary infections and death, current therapeutic measures have not shown great efficacy [59]; thus, alternative and adjunctive therapeutic strategies such as statin treatment are currently being investigated.

**Statins**

Statins are HMG-CoA reductase inhibitors, which are small molecules that compete with the natural substrate, HMG-CoA, for binding of the enzyme HMG-CoA
reductase, ultimately blocking the synthesis of cholesterol [84]. Thus, they are a group of commonly prescribed lipid-lowering drugs, and simvastatin was the first of these lipid-lowering drugs. Simvastatin, as with other statins, is administered to control cholesterol levels and cardiovascular disease contributing to a 30-45% decrease in cardiac events [85, 86]. Simvastatin (generic name) was discovered by Merck scientists and named Zocor [87] and is a synthetic derivate of a fermentation product of Aspergillus terreus [88]. While statins were initially marketed as cholesterol lowering drugs, additional uses and effects of the drug class have been investigated.

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 anti-inflammatory properties of statin drugs [5, 22, 89]. Initial investigations of statins in inflammatory disease were based on cardiac patients taking statins showing a decreased risk of becoming septic [90, 91]. In one study, patients on statins had a 19% decrease in sepsis prevalence [90]. Recently, two comprehensive reviews of clinical statin usage supported a decrease in infection-related deaths and morbidity in patients on a variety of statins [54, 56]. While most studies support statins having a beneficial effect on sepsis recovery and survival, the available clinical studies are very heterogeneous in type, duration, and dose of statin treatment, and they utilize a varied definition of “statin users” because studies included patients previously on statins for varied time periods and patients continuing statin treatment during hospitalization. In a prospective cohort study, non-specific statin use was found to decrease hospitalizations due to sepsis in chronic kidney disease patients participating in long-term dialysis [92], and adult patients medicated with statins previously or during hospitalization for severe sepsis were correlated with an enhanced survival rate [93]. In addition, patients admitted to the hospital with multiple organ failure
due to infection and continuing their previously prescribed statin treatment for heart conditions exhibited an increased survival rate compared to patients not taking statins [94]. Similarly, statin treatment started at hospital admission for infection enhanced survival from sepsis [95], and patients continuing their statin treatment during hospitalization for sepsis were correlated with improved survival compared to non-statin counterparts [96]. A double-blind clinical trial showed *H. pylori*-infected patients receiving traditional medication treatment plus 20 mg of simvastatin twice per day for seven days exhibited increased clearance of the bacterial infection compared to non-simvastatin controls [97]. Also, patients previously medicated with statins, but not continuing with statin treatment during hospitalization, showed a decrease in intensive-care hospitalization and diagnosis of severe sepsis compared to their non-statin counterparts [98]. In pneumonia patients requiring ventilation and not previously taking statins, treatment with pravastatin for thirty days during hospitalization showed a more efficient recovery with enhanced survival compared to non-statin control patients [99].

While pre-hospital statin treatment has been shown to decrease the incidence of severe sepsis, survival during hospitalization was not different between pre-hospital statin and non-statin patients [100]. However, elderly, male patients previously taking statins within 90 days of hospital admission for sepsis did exhibit enhanced survival, but neither statin dose nor continuation of statin treatment during hospitalization was taken into account [55].

In contrast, Danish and Taiwanese patients previously taking statins and admitted to the hospital for sepsis did not receive any survival benefit up to one month post-hospital admission [101, 102], but Danish patients previously on statins did exhibit increased survival one to six months post-infection [101]. Interestingly, a Spanish study was unable to verify the protective nature of statins on sepsis mortality and even found
evidence of diminished survival in statin treated patients; however, it was the researchers’ opinion that they were unable to properly adjust for underlying differences in the patients due to secondary conditions and age [103]. Infection rates were similar between statin and non-statin medicated patients who underwent cardiac surgery; thus, not supporting adjunctive therapeutic use for statins as an infection preventative [104].

On-going clinical trials are examining the efficacy of statins to enhance survival from ventilator-associated pneumonia with concurrent antibiotic treatment [105] as well as the ability of early statin treatment to benefit clinical recovery from sepsis [106]. Two studies are currently investigating the effects of statin treatment on immune modulation in addition to clinical recovery, specifically in elderly patients diagnosed with pneumonia [107] and a double-blind, randomized trial in patients with septic shock [108]. Given the recent increase in statin use [104], it is important to investigate pleiotropic effects of statins, especially on immune function.

It has been demonstrated that statins (including simvastatin) can modulate both innate (inflammation) and adaptive (B and T cell function) immune responses independent of their lipid-lowering activities [5]. Lovastatin was found to diminish neutrophil movement into affected tissue, possibly decreasing inflammatory damage [109]. In addition to neutrophil movement and stimulation, monocyte stimulation and infiltration can be dampened with statin treatment [23]. Statins can also down-regulate the synthesis of pro-inflammatory cytokines such as TNFα, IL-1, and IL-6 [54, 109-112] and decrease the expression of genes necessary for promoting inflammatory reactions [113] possibly through squelching TLR4 and TLR2 signaling [114], owing to their ability to decrease sepsis-related mortality. Additionally, statin treatments have been demonstrated to skew Th development towards the Th2 phenotype [115-117] and prevent cytokine-induced up-regulation of cell surface proteins on antigen presenting
cells necessary for their full maturation and activation of T cells [113, 117]. Furthermore, in murine models of inflammatory-mediated diseases, statin treatment resulted in decreased disease progression due to increases in Th2 cytokines or suppression of Th1 cytokines and cell development [117-119]. Because T cells are components of the adaptive response and regulate immune function via their cytokine secretion, effects on T cell function affect the current immune response and also dictate the type of memory cells that will remain to provide protective immunity for future infections. While this immune modulation by statins may be beneficial for the treatment of inflammatory-driven diseases or autoimmune disorders resulting from aberrant Th1 responses, the alteration of the adaptive response may affect the memory response that is generated, making an individual more susceptible to a secondary pathogenic infection. Together, these findings demonstrate the ability of statins to be potent immune regulators with the possibility to decrease memory responses promoting severe secondary infections.

**Simvastatin alters adaptive immune parameters in a primary septic infection**

Prior work done in our collaborator’s lab demonstrated the ability of short term simvastatin pretreatment to protect against septic death resulting from *S. aureus* infection [120]. To examine the immune effects that may have contributed to this increased survival, we examined serum antibody levels in mice surviving primary infection. Specifically, serum levels of IgG1 and IgG2c (the alternate gene for IgG2a that is expressed in C57BL/6 mice [121-123]) were examined. Because antibody production is influenced by the presence of cytokines secreted by T cells, relative levels of antibodies can demonstrate the type of immune response that was mounted. Fourteen days post-infection, serum was collected and analyzed for the presence of total IgG1 (indicative of a Th2 response) and total IgG2c (indicative of a Th1 response). The results demonstrated no difference in levels of IgG1 between treatment groups.
However, while IgG2c levels increased in S. aureus-infected vehicle-pretreated mice, serum IgG2c was reduced in infected, simvastatin pretreated mice to levels comparable to those seen in uninfected controls.

Increases in IgG2a (IgG2c in C57BL/6 mice) are expected during pathogenic invasion, such as S. aureus, to enhance proper immune responses to clear the pathogen. The finding that total serum levels of IgG2c are reduced in simvastatin pretreated mice may suggest that the Th1 inflammatory response to S. aureus infection is muted, similarly to what is seen in murine models of inflammatory-mediated diseases, where statin treatment resulted in decreased disease progression due to increases in Th2 cytokines or suppression of Th1 cytokines and cell development [117-119]. While statin modulation of T cell differentiation may be beneficial for the treatment of inflammatory-driven diseases, its downstream effect on antibody production may pose a potential immune-risk to healthy individuals infected by a pathogen.

**Rationale**

It is vital to understand the cumulative effects of medications such as simvastatin so that alternative and proper uses of the drug can be implemented. Understanding can be gained through basic scientific research models such as a mouse model to mimic the drug effects in the complex human body. Mice have 99% homology with the human immune system, which makes them ideal animal models for scientific research [17]. Different mouse strains are genetically unique and can react differently to antigen stimulation. Depending on the strain, Th1 or Th2 activity could be more prominent depending on the genetic code of chromosome 11. Th1 dominant mice strains include C57BL/6 and B10.D1. Th2 skewed strains include Balb/c and Balb/cBy [124]. Immunoglobulin isotype differences are also found in C57BL/6 mice compared to humans. C57BL/6 mice produce IgG2c instead of IgG2a due to genetic allele variations.
While mice have a few subtle differences in immune function, immunologic memory is still intact.

Simvastatin has been investigated in our model of a primary septic infection; however, the effect of simvastatin on the immune system’s ability to protect an individual from any type of subsequent infection has not been addressed, which is the main goal of this study. This is vital due to a potential increased risk for secondary infection in individuals who have recently been hospitalized and ill who may also be taking statin medication. It is important to examine the effects of simvastatin not just on specific immune reactions but on primary and secondary responses as a whole to identify the underlying mechanisms of its immunomodulatory properties.
Materials and Methods

The goal of these studies was to examine how simvastatin pretreatment alters adaptive immune responses, potentially limiting the generation of an effective memory response and subsequently inducing a state of immunodeficiency to secondary infection.

**Hypothesis:** Simvastatin treatment prior to primary infection with *S. aureus* impairs the generation of effective memory immune responses, increasing mortality due to secondary *S. aureus* infection.

To investigate this hypothesis survival to secondary infection, serum antibody levels, total and memory B and memory T cell proliferation and *ex vivo* production of antibodies by total and memory B cells following secondary infection were assessed.

**Overview**

**Day -1:** Simvastatin (in EtoH) pretreatment or control 18 hours (3p) before *S. aureus* infection

**Day 0:** Simvastatin pretreatment or control 3 hours before infection (8a), *S. aureus* infection at 0

Hour (11a), Gentamicin (in saline) treatments at 3 (2p), 6 (5p), and 12 (11p) hours post-infection

**Day 1:** Gentamicin treatment 24 (11a) hours post-infection

**Day 2:** Gentamicin treatment 48 (11a) hours post-infection
Day 14: S. aureus infection at 0 hour (11a) of surviving mice, Gentamicin treatments at 3, 6, and 12 hours post-infection

Day 15: Gentamicin treatment 24 hours post-infection

Day 16: Gentamicin treatment 48 hours post-infection

Day 28: Surviving mice sacrificed with serum and lymphoid tissues isolated for proliferation and ELISA analyses

**Mouse Infection and Drug Treatment**

For each study, there were 2 treatment groups (Control and Simva [experimental]) of C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) (4 complete studies were run using a total of 28 Control mice and 16 Simva mice equally male and female). Mice were housed separately in filtered cages. Drug treatments were based on average weight of males and females in each group. A stock of 10 mg/ml of simvastatin (Calbiochem [EMD Chemicals], Gibbstown, NJ) was dissolved in ethanol and diluted with saline for a final concentration of 1000 ng simvastatin per gram of mouse body weight and a total injection volume of 100 μL. For each study, mice in the Simva experimental group received simvastatin 18 and 3 hours prior to S. aureus infection via intraperitoneal (IP) injection. The control group (Control) was given 1% ethanol in saline as a vehicle control. Based on average body weight, 10 mg of gentamicin (Sigma, St. Louis, MO) per kg of mouse body weight was dissolved in saline for a total injection volume of 100 μL. All mice received gentamicin via IP injection at 3, 6, 12, 24, and 48 hours post-infection. All mice were infected with S. aureus (#29213, American Type Culture Collection, Manassas, VA) at 1x 10^7 colony forming units (cfu) in 1mL of 5% mucin (BD, Franklin Lakes, NJ) diluted in 0.85% sterile saline.
Survival Analysis

Mice surviving the primary infection with S. aureus were re-infected with the same inoculum of S. aureus and given gentamicin as described above. No additional simvastatin treatment was administered. Mice were closely monitored for the entire 28 day study with body weight and health score status being recorded each day. Mice surviving at the end of 28 days were sacrificed with blood and tissue harvested for analysis. Data was analyzed using the Kaplan-Meier Log-Rank analysis (survival) or a t-test (body weight change), SigmaPlot (Systat Software, San Jose, CA). Differences between groups were considered statistically different at $p < 0.05$.

Isolation of Blood Serum

To assess levels of IgM, IgG1, and IgG2c in the blood, serum was isolated and analyzed by ELISA. Following carbon dioxide asphyxiation, blood was drawn via cardiac puncture. After clotting occurred, the samples were centrifuged at 5000 rpm at 4°C to isolate the serum fraction.

Ex vivo Stimulation of B Lymphocytes

Total B cells and memory B cells were isolated from the spleen via magnetic sorting kits (Miltenyi Biotec, Cambridge, MA). Total or memory B cells $1 \times 10^6$ cells/ml were stimulated with 100 μg/ml peptidoglycan and lipoteichoic acid (LTAPepG) (InvivoGen, San Diego, CA) or 10 μg/ml lipopolysaccharide (LPS) (Sigma-Aldrich, St. Louis, MO). Supernatants were harvested after 48 hours.

Enzyme-linked Immunosorbant Assay (ELISA)

ELISA kits (Bethyl Labs, Inc., Montgomery, TX) were used to analyze levels of IgM, IgG1, and IgG2c in serum and ex vivo supernatants. The assay was performed according to manufacturer's instructions and absorption due to color change was determined using a BIO-RAD plate reader, model 480 (BIO-RAD, Hercules, CA) at 450
nm. Concentrations of antibodies were determined using a standard curve generated from the controls provided in the kit. Samples were plated in duplicate and the mean antibody concentration (+/- SEM) was pooled from 1-3 experiments (n= 3-16 number of mice/group) and analyzed by Student’s t-test for differences between control and simvastatin pretreated samples or a one-way ANOVA using SigmaPlot (Systat Software). p < 0.05 demonstrated a significant difference.

Assessment of Memory B and T Lymphocyte Proliferation

A MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) Cell Proliferation Assay Kit (ATCC, Manassas, VA) was used to determine the level of cellular proliferation. Memory B cells were isolated from the spleen via magnetic sorting kits (Miltenyi Biotec). T cells were isolated from inguinal lymph nodes, and memory T cell proliferation was assessed by stimulating only with anti-CD3 in the absence of anti-CD28. For T cells, wells of a 96-well plate were pre-coated with 1 μg/ml anti-CD3 (Bio X Cell, West Lebanon, NH) and incubated at 4°C. Following overnight incubation, 1.0 x 10^5 T cells/well were added. Similarly, 1.0 x 10^5 memory B cells/well were added to uncoated wells and stimulated with 100 μg/ml LTAPepG (InvivoGen) or 10 μg/ml LPS (Sigma-Aldrich). Cells were incubated at 37°C with 5% CO₂ for 24, 48, and 96 hours. MTT Reagent was added to each well, and the plate was observed after 2 hours for the appearance of a purple precipitate. Following the appearance of precipitate, the cells were lysed with the detergent reagent and incubated at room temperature in the dark for 2 hours. The 96-well plate was read at 570 nm using a BIO-RAD plate reader, model 480 (BIO-RAD). The level of proliferation was assessed by subtracting the negative control, non-stimulated value from the sample absorbance value to get a net absorbance reading. Samples were plated in triplicate. The average net absorbance value (+/-SEM) was analyzed by Student’s t-test for differences in proliferation between control and
simvastatin pretreated samples using SigmaPlot (Systat Software). $p \leq 0.05$ demonstrated a significant difference.
Results

The goal of this study was to investigate the effect of simvastatin on survivability and memory immune function in response to secondary infection by *S. aureus*.

**Simvastatin diminishes serum IgG2c levels in response to *S. aureus* infection**

Previous work in our lab demonstrated the ability of short term simvastatin pretreatment to increase the survival of mice infected with *S. aureus* [13]. To investigate possible immune alterations resulting from simvastatin pretreatment that may contribute to the enhanced survival, serum antibody levels were analyzed via ELISA at day 14 post-infection. Relative levels of IgG1 and IgG2c can be indicators of the type of immune response (anti-inflammatory or pro-inflammatory, respectively) mounted. Thus, serum levels of IgG1 and IgG2c were examined. Data demonstrated that while IgG1 levels were not different between control and simvastatin pretreated groups (Figure 1), serum levels of IgG2c were significantly decreased in simvastatin pretreated mice compared to controls (Figure 1). The decrease in IgG2c in the simvastatin pretreated mice is indicative of a dampened Th1 immune response, potentially reflective of a lessened degree of inflammation in the simvastatin pretreated mice.
Survivability to secondary *S. aureus* infection was not altered by simvastatin pretreatment

Quieting of the inflammatory response by simvastatin may be necessary to allow proper clearance of *S. aureus* without the widespread, damaging effects of an inflammatory response resulting in sepsis; however, this dampening could possibly contribute to a hypoimmune state whereby the immune system is not able to properly generate an effective memory response, subsequently inducing a state of immunodeficiency to secondary infection. To assess the effects of simvastatin on survivability and memory immune function in response to a secondary infection by *S. aureus*, C57BL/6 mice were pretreated with simvastatin or an ethanol control 18 and 3 hours prior to the initial infection only. After 14 days, mice were re-infected with *S. aureus* but no subsequent simvastatin treatment was given. Mice were again observed and weighed for 14 days following the second infection. In contrast to the results from our survival study following primary infection [120], mice pretreated with simvastatin had similar survivability compared to the control mice in response to secondary infection with *S. aureus* (Fig 2A). Mice pretreated with simvastatin had a survival of 77%, and the control mice had a survival of 73% (Fig 2A). There were also no differences in percent body weight change over the entire study between simvastatin pretreated and control mice supporting a similar rate of survival following secondary infection between the two groups (Fig 2B). These data demonstrate that although total serum antibody levels altered by simvastatin pretreatment following primary infection could have increased the host’s susceptibility to secondary infection, host survivability to secondary infection was not altered compared to controls.
Simvastatin pretreatment significantly increased serum IgM levels after secondary S. aureus challenge

To investigate the effect of simvastatin pretreatment on serum antibody levels following secondary infection, serum was collected 14 days after secondary infection and levels of IgM, IgG1, and IgG2c were analyzed via ELISA. While serum levels of IgG1 and IgG2c were similar in simvastatin pretreated and control mice, levels of serum IgM were significantly elevated in mice pretreated with simvastatin compared to control mice (Fig 3).

Simvastatin pretreatment alters antibody production by ex vivo stimulated memory B cells but not total B cells exposed to secondary S. aureus infection

To identify alterations in total B cell function that resulted from simvastatin pretreatment, splenic B cells were isolated from control and simvastatin pretreated mice following secondary infection with S. aureus and stimulated ex vivo with bacterial cell wall components LTAPepG or LPS. Levels of IgM, IgG1, and IgG2c were measured by ELISA 48 hours post-stimulation. Levels of IgM (data not shown), IgG1, and IgG2c from total B cells were similar in simvastatin pretreated and control mice stimulated ex vivo with LTAPepG or LPS (Fig 4A), demonstrating that mice pretreated with simvastatin did not exhibit alterations in antibody production by total splenic B cell populations.

To investigate the effect of simvastatin pretreatment on memory B cell function, memory B cells were isolated from simvastatin pretreated and control mice 14 days after secondary infection with S. aureus and stimulated ex vivo with LTAPepG or LPS. Levels of IgM, IgG1, and IgG2c were measured 48 hours post-stimulation using an ELISA. In contrast to the findings regarding antibody production by total B cells stimulated ex vivo,
levels of IgG1 were significantly elevated in supernatants harvested from memory B cells from simvastatin pretreated mice stimulated with LTAPepG compared to controls. However, levels of IgM (data not shown) and IgG2c levels were not different from controls (Fig 4B). Paradoxically, \textit{ex vivo} stimulation of memory B cells with LPS did not reveal any significant difference in antibody production between simvastatin pretreated and control mice 14 days following secondary infection, but data trends in favor of an increase in IgG1 production (Fig 4B). Taken together these data suggest an enhanced IgG1 production by memory B cells harvested from simvastatin pretreated mice, potentially indicative of a heightened anti-inflammatory or Th2 response. This \textit{ex vivo} data supports the \textit{in vivo} serum data from the primary infection (Fig 1) that suggested B cells from simvastatin pretreated mice were skewed away from producing the Th1-indicative IgG2c antibody signifying a decrease in pro-inflammatory signaling.

**Simvastatin pretreatment alters proliferation by \textit{ex vivo} stimulated memory B cells in response to \textit{S. aureus} secondary infection**

Given the differences in antibody production following \textit{ex vivo} stimulation, memory B cell proliferation was examined. Proliferation of memory B cells in response to LTAPepG \textit{ex vivo} stimulation was slightly diminished in mice pretreated with simvastatin compared to the control in mice (Fig 5A), and when stimulated with LPS, memory B cells from simvastatin pretreated mice had significantly decreased proliferation compared to control mice (Fig 5B). These data provided further evidence of the alterations in memory B cells as a result of simvastatin pretreatment prior to infection.
Simvastatin dampens memory T cell proliferation at 96 hrs post ex vivo stimulation

Many B cell functions are dependent upon T cell interactions. Given the dampening effect of simvastatin on memory B cell proliferation, alterations in memory T cell proliferation were investigated. T cells harvested from simvastatin pretreated and control mice after secondary S. aureus infection were stimulated with anti-CD3 in the absence of anti-CD28 to stimulate proliferation of only memory T cells. Although no difference in proliferation was observed at 24 hours, T cells from simvastatin pretreated mice had slightly reduced proliferation compared to control mice by 48 hours, and significantly decreased proliferation by 96 hours (Fig 6).

Taken together, these results demonstrate the ability of short term simvastatin treatment prior to primary infection to alter the functions of memory B and T cells following secondary infection, highlighting the potent immunomodulatory capabilities of simvastatin.
Discussion

This study demonstrated alterations in memory B and T lymphocyte function following secondary infection by *S. aureus* as a result of short term simvastatin treatment prior to primary infection. To the best of our knowledge, this study was a novel investigation into the effect of simvastatin on the generation of immunologic memory that contributes to the understanding of the diverse immune-modulating properties of simvastatin. Increasing evidence has demonstrated that statins (including simvastatin) can modulate both innate (inflammation) and adaptive (B and T cell function) immune responses independent of their cholesterol-lowering activities [5]. While immune modulation by statins may be beneficial for the treatment of inflammatory-driven diseases or autoimmune disorders resulting from aberrant Th1 responses, the alteration of the adaptive response may affect the memory response that is generated, making an individual more susceptible to a secondary pathogenic infection. Previous work done by our lab found that simvastatin pretreatment enhances survival to primary *S. aureus* infection [13]. This finding supports published data showing that simvastatin protects against sepsis-related death giving further rationale to investigate the utility of simvastatin treatment in sepsis [10, 11, 54, 56, 90, 91, 125]. In the current study,
simvastatin treatment prior to primary infection with *S. aureus* decreased serum levels of total IgG2c (Fig 1). Increases in IgG2a (IgG2c in C57BL/6 mice) are expected during pathogenic invasion, such as *S. aureus*, to enhance proper immune responses like inflammation to clear the pathogen [14-16]. The finding that serum levels of IgG2c are reduced in simvastatin pretreated mice may suggest that the Th1 inflammatory response to *S. aureus* infection is muted, similarly to what is seen in murine models of inflammatory-mediated diseases, where statin treatment resulted in decreased disease progression due to increases in Th2 anti-inflammatory cytokines or suppression of Th1 inflammatory cytokines [117-119]. While statin modulation of T cell differentiation may be beneficial for the treatment of inflammatory-driven diseases, its downstream effect on antibody production may pose a potential immune-risk to healthy individuals infected by a pathogen.

The serum antibody levels following primary infection suggest that the B cell response was skewed away from a Th1 pro-inflammatory response (indicated by reduced IgG2c) (Fig 1). Thus, memory cells, which dominate secondary responses [46, 49], generated from this reaction may be less effective in fighting a secondary infection, leaving the host potentially immune compromised. These findings demonstrate that simvastatin pretreatment did not diminish survival to secondary infection with *S. aureus* nor did it further protect against death compared to control mice (Fig 2). This implies that simvastatin may have no effect on secondary infection survival in septic patients with a previously healthy immune system. In contrast to the decrease in serum IgG2c seen in the primary infection (Fig 1), total serum antibody levels of IgG2c and IgG1 were not different between control and simvastatin pretreated mice in the secondary infection model (Fig 3). Although there was a significant increase in serum IgM in simvastatin pretreated mice, this finding did not correspond to any decrease in serum IgG levels that
would suggest simvastatin hampered class switching. This data shows unique IgG profiles between primary and secondary infection models, which suggest that simvastatin can affect serum antibody levels to dampen the primary Th1 inflammatory response, which could cause downstream consequences for B cell memory functions necessary to combat a subsequent infection [46, 49].

These data examining ex vivo lymphocyte functions in simvastatin pretreated mice following secondary infection with S. aureus suggest that simvastatin treatment prior to primary infection alone alters memory immune function, which to our knowledge is a novel finding. While total IgM, IgG1, and IgG2c production was similar from total B cells isolated from control and simvastatin pretreated mice stimulated with either LTAPepG or LPS (Fig 4A), memory B cells from simvastatin pretreated mice stimulated ex vivo with LTAPepG exhibited significant increased IgG1 production and an increasing trend in IgG1 production by LPS-stimulated memory B cells was also observed (Fig 4B). This finding is of interest because IgG1 production is counterproductive to fighting a bacterial infection due to its anti-inflammatory nature; however, this could be a result of the dampened Th1 inflammatory response as demonstrated by the serum decrease of IgG2c in the primary infection study (Fig 1), which was protective against septic mortality [11]. While antibody production by memory B cells elicits effector functions important to controlling an infection and gaining inflammatory homeostasis [30, 39, 40], proliferation of memory lymphocytes can give insight into the functional ability of the memory response. Simvastatin treatment prior to primary infection decreased proliferation of memory B cells stimulated with LTAPepG and LPS (Fig 5). Our ex vivo stimulation data in conjunction with the in vivo serum antibody data suggests simvastatin acts to dampen the primary Th1 inflammatory response, which possibly results in the “default” generation of memory B cells secreting IgG1 instead of IgG2c.
In inflammatory-driven diseases, homeostasis is garnered through effector functions of B and T cells acting synergistically. Memory T cells from simvastatin pretreated mice showed diminished proliferation starting at 48 hours post-stimulation and significantly diminishing by 96 hours (Fig 6). If memory T cell proliferation is hindered during secondary infection, T cell (memory T cells aide in memory B cell activation and proliferation) interactions with memory B cells could be dampened and could hinder the induction of antibody production and B cell proliferation.

In conclusion, this study provides evidence of the potent ability of simvastatin to act as an immune modulator, such that only two doses prior to primary infection results in altered memory responses. While simvastatin pretreatment did not enhance nor diminish the chance of survival from secondary *S. aureus* infection in our C57BL/6 mouse model compared to control mice, noteworthy immune modulations such as decreased memory B and T cell proliferation combined with a dampened Th1 skewed response were documented. With increasing chronic conditions that require multiple hospital visits and cause a decrease in immune function, the prevalence of subsequent infection with complex complications is high. Due to the nature of chronic illnesses, immune surveillance must be vigilant as the disease compromises immune function creating a susceptibility and sensitivity to infection. For example, mice infected with a respiratory virus exhibited a decrease in *S. aureus* clearance and increased inflammation with heightened susceptibility to additional bacterial infection [82]. In addition, patients with influenza are more susceptible to simultaneous infection, particularly with *S. aureus* [83]. Future studies investigating continual statin treatment on immune function during primary and secondary *S. aureus* infection will give more clinical insight on how statins modulate the immune system during infection.
References


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Figure 1. Simvastatin dampens IgG2c during primary S. aureus infection.
Mice received either the control (black bar) injection of saline/ethanol or simvastatin (Simva, grey bar) (1000ng/g [BW]) pretreatment 18 and 3 hours pre-infection with S. aureus (at 1x 10^7 colony forming units (cfu) in 1mL of 5% mucin diluted in 0.85% sterile saline). Both the Control and Simva groups were given gentamicin at 3, 6, 12, 24, and 48 hours post-infection. Mice in the uninfected (white, hashed bar) group received control injections of saline/ethanol at 18 and 3 hours prior to infection, an injection of 5% mucin at the time of infection, and saline as the antibiotic control at 3, 6, 12, and 48 hours post-infection via intraperitoneal injection. On day 14, serum samples were obtained by cardiac puncture and analyzed for levels of IgG1 and IgG2c via ELISA. Data were pooled from 3 replicate studies (13-14 mice per group). *Simva significantly lower than Control, one-way ANOVA, p<0.05.
Figure 2. Simvastatin pretreatment did not alter survivability to secondary S. aureus infection. Mice received either the control injection (saline/ethanol) or simvastatin (1000ng/g [BW]) pretreatment 18 and 3 hours pre-infection with S. aureus (at 1x 10^7 colony forming units (cfu) in 1mL of 5% mucin diluted in 0.85% sterile saline). Both the control and Simva groups were given gentamicin (10 mg/kg) at 3, 6, 12, 24, and 48 hours post-infection. Mice were exposed to secondary infection with no additional administration of simvastatin at day 14 and assessed for survival at day 28 (Control, solid line; Simva, dashed line) (A). Mice were weighed each day of the study and percent body weight change was calculated (Control, black circles; Simva, white circles) (B). Data were pooled from 3 replicate studies (n=13-25 mice/group) and analyzed using either the Kaplan-Meier Log-Rank analysis (survival) or a t-test (body weight change).
Figure 3. Simvastatin pretreatment significantly increased serum IgM levels after secondary *S. aureus* infection. Serum was harvested from mice surviving at day 28 and assessed for serum levels of IgM, IgG1, and IgG2c via ELISA. Data were pooled from 3 replicate studies (*n*=10-16 mice/group). *Simva* (grey bars) significantly higher than control (black bars), t-test, *p*≤0.05.
Figure 4. Simvastatin pretreatment increased IgG1 production by memory B cells stimulated with LTAPepG. Total (A) or memory (B) B cells were isolated from the spleens of control and simvastatin pretreated mice surviving at day 28 and stimulated ex vivo for 48 hours with either 100 μg/ml LTAPepG or 10 μg/ml LPS. Levels of IgG1 and IgG2c in the supernatant were determined by ELISA. Data were pooled: (total B cell groups n= 3-4 mice/group; memory B cell groups n=7-11 mice/group). *Simva (grey bars) significantly higher than control (black bars), t-test, p≤0.05.
Figure 5. Simvastatin pretreatment dampened memory B cell proliferation in response to ex vivo LPS stimulation. Memory B cells purified from the spleens of control and simvastatin pretreated mice surviving at day 28 were stimulated ex vivo for 24, 48, and 96 hours with either 100 μg/ml LTAPepG (A) or 10 μg/ml LPS (B) to examine proliferation. Data were pooled from 2 replicate studies (n=6-11 mice/group). *Simva (white circles) significantly lower than control (black circles), t-test, p≤0.05.
Figure 6. Simvastatin pretreatment dampens memory T cell proliferation at 96 hrs post stimulation. Memory T cells were isolated from inguinal lymph nodes on day 28 to examine proliferation of cells stimulated ex vivo for 24, 48, and 96 hours with 1 μg/ml anti-CD3. Data was pooled from 2 replicate studies (n=7-11 mice/group). *Simva (white circles) significantly lower than control (black circles), t-test, p<0.05.