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The Effect of Statin Therapy on Innate Immune Clearance of Bacteria

A dissertation submitted in partial satisfaction of the requirements

for the degree Doctor of Philosophy

in

Biomedical Sciences

by

Ohn Aaron Chow

Committee in Charge:

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Professor Christopher Glass
Professor Donald Guiney
Professor Sharon Reed
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2010

The Dissertation of Ohn Aaron Chow is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California San Diego

2010

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Abstract of the Dissertation

The Effect of Statin Therapy on Innate Immune Clearance of Bacteria

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Phagocytic leukocytes, such as neutrophils and macrophages, play a key role in host defense against bacterial pathogens. Classically, they are thought to kill bacteria through a combination of phagocytosis, oxidative burst and degranulation. Recently, leukocytes have been found to produce extracellular webs of DNA, known as extracellular traps (ETs). These structures play an important role in host defense, and represent a fundamentally new mechanism of bacterial clearance.

Serum has been found to inhibit ETs in a concentration dependent manner. We have found that serum contains heat-stable nucleases that are capable of degrading traps. These findings have important implications for the visualization and identification of extracellular traps.

Statins are amongst the most widely-used chronic medication in the world. They act by inhibiting 3-hydroxy 3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in cholesterol biosynthesis. Several recent clinico-epidemiologic studies have revealed that patients receiving statin therapy have reduced mortality associated with severe bacterial infection. We studied the pharmacological effect of statins on the innate immune capacity of phagocytic cells, focusing on the leading human bacterial pathogen *Staphylococcus aureus*. These studies revealed a beneficial effect of statins on *S. aureus* clearance using *in vivo*, *ex vivo* and *in vitro* models of phagocyte function, although paradoxically both phagocytosis and oxidative burst were inhibited. Probing instead for an extracellular mechanism of killing, we found statins boosted the production of ETs by human and murine neutrophils and also monocyte/macrophages, representing the first report of the ET phenomenon in the latter cell type. The effect of statins to induce phagocyte ETs was linked to sterol pathway inhibition by RNA interference and specific pharmacologic inhibitors. We conclude that statins, a drug therapy taken chronically by tens of millions of individuals, alters the functional behavior of phagocytic cells, which could have ramifications for susceptibility and response to bacterial infections in these patients.

In sum, our findings have important implications for our understanding of ETs, the cell biological processes that lead to their production and their clinical relevance in the context of bacterial infections.

Chapter I
Extracellular Traps and the “Ins and Outs”
of Innate Immunity

INTRODUCTION

Neutrophils are key effectors of the innate immune system. They are the most abundant white blood cell, comprising ~50-70% of leukocytes in circulation. Normally, they are short-lived, having a half-life of only 8-20 hours (Akgul, 2001). This high turnover rate may be used to prevent the overaccumulation of the potent antimicrobial components, which could potentially damage surrounding tissue (Akgul, 2001; Koedel, 2009). During an infection, neutrophils become activated by inflammatory stimuli such as bacterial components or certain pro-inflammatory cytokines. These signals cause neutrophils to delay apoptosis, extravasate from the blood and migrate toward the site of infection (Elbim, 2009). There, they use a potent arsenal of antimicrobial effectors to kill foreign invaders and clear the infection.

Classically, neutrophils are thought to kill bacteria primarily through phagocytic killing. When a phagocyte recognizes a bacterium as non-self, it engulfs it, trapping it in the phagosome (Nathan, 2006). The phagosome then matures, fusing with intracellular granules and lysosomes to form the phagolysosome. Within this organelle, bacteria are killed by a combination of antimicrobials, including oxidants, such as peroxide and superoxide, granule proteases, such as lysozyme, and antimicrobial peptides, such as LL-37. In addition to phagocytic clearance, many phagocytes release granules into their surrounding environment upon encountering a bacterium, allowing them to attack pathogens extracellularly.

In 2004, Brinkmann et al. discovered that neutrophils have the ability to release DNA into their extracellular environment in response to inflammatory stimuli, resulting in the production of chromatin webs (Brinkmann, 2004). These structures, which were

called neutrophil extracellular traps (NETs), trapped and killed the bacterial pathogens *Staphylococcus aureus* and *Shigella flexneri*. Since this landmark discovery, NETs have been found to be a key antimicrobial effector of the innate immune system. This review focuses on the recent advances in our understanding of NET biology and its importance in innate immune defense against pathogens.

What are NETs?

NETs are composed of a network of DNA, proteins and peptides that forms an expansive structure much greater in volume than a neutrophil (Brinkmann, 2004). The structural backbone of NETs is composed of chromatin, and this is decorated with a variety of proteins and peptides. Recently, Urban et al. used mass spectrometry to profile proteins bound to NETs (Urban, 2009). Histones were found to be the most abundant proteins in NETs. In contrast to intact neutrophils, where the core histones are present in similar amounts, the core histones in NETs are present in strikingly different molar amounts. Histones H2A and H2B were found to be the most abundant histones and present at similar levels. Histone H3 was present at roughly half the level of H2A and H2B and histone H4 was found to be the least abundant core histone in NETs.

NETs were also found to contain a variety of other proteins, although they were generally much less abundant than the core histones. Granule-derived proteins, such as neutrophil elastase and myeloperoxidase, were highly enriched in NETs. In fact, more NET-associated proteins were found to come from granules than from any other cellular source. Furthermore, NETs were associated with several cytoskeletal proteins, two glycolytic enzymes, catalase, a peroxisomal protein, and calprotectin, a cytoplasmic

protein. In addition to the proteins identified by Urban et al., antimicrobial peptides (AMPs) and NADPH oxidase have also been found to localize to NETs (Cogen, 2010; Jann, 2009; Munafo, 2009).

NETs are organized as stretches of smooth regions with interspersed globular regions (Brinkmann, 2004). The smooth stretches are further punctuated by regions correlating in size to nucleosomes (Urban, 2009). It is now believed that the smooth stretches are composed primarily of DNA and histones organized as stacked cylindrical nucleosomes while the globular regions likely contain granule-derived and other NET-associated proteins.

Cell Death and NET Formation

Neutrophils possess multiple strategies by which they can produce NETs. In 2007, Fuchs et al. reported that neutrophils could produce NETs through a unique and novel cell death mechanism (Fuchs, 2007). Interestingly, neutrophils that underwent NET production were morphologically dissimilar from those undergoing apoptosis or necrosis. Furthermore, neither induction of necrosis nor apoptosis with staphylococcal pore forming toxins and anti-FAS antibodies, respectively, resulted in production of extracellular traps. As such, NET formation was determined to be the result of a previously-unrecognized form of cell death that was distinct from both apoptosis and necrosis, and this process was termed “NETosis”.

At the beginning of NETosis, neutrophils begin to decondense their chromatin, unwinding their DNA from histones. This event is dependent on histone PAD4-mediated histone hypercitrullination, and inhibition of this process using the PAD4 inhibitor, Cl-

amidine, inhibits formation of NETs (Wang, 2009). Simultaneous to chromatin decondensation, neutrophils disintegrate their nuclear and granular membranes, allowing their granular and cytoplasmic material to mix. During this process, the neutrophils remain viable, as measured by retention of the vital dye calcein blue. NETs are ultimately formed when the plasma membrane ruptures and the DNA is released, resulting in the production of NETs and the death of the neutrophil.

The molecular mechanics underlying NETosis-induced NET production are still being elucidated. The cytoskeleton appears to play an important role in NET production. Neeli et al. recently showed that disruption of either microtubules using nocodazole inhibits NET production induced by LPS (Neeli, 2009). Interestingly, nocodazole treatment of neutrophils prevented nuclear breakdown and caused the cells to remain in their naïve state. In dividing cells, microtubules have been shown to facilitate nuclear enveloped breakdown by “tearing” the nuclear lamina (Beaudouin, 2002). It is possible that microtubules play a similar role in nuclear breakdown in cells undergoing NETosis.

The potential contribution of actin filaments to NET production is currently unclear. While Neeli et al. have reported that cytochalasin D inhibits NET production, Brinkmann et al. have shown that it has no effect (Brinkmann, 2004; Neeli, 2009). Thus, the effect of actin filament disruption on NET production is still unclear, and will require further investigation.

Mitochondria and NETs

In addition to NETosis, neutrophils have two alternate strategies with which they can produce NETs while still remain viable. In the first process, described by Yousefi et

al., neutrophils primed with GM-CSF and subsequently stimulated with C5a or LPS released their mitochondria in a catapult-like fashion into the extracellular environment (Yousefi, 2009). NETs formed in this process were derived from mitochondrial rather than nuclear DNA. After release of mitochondria, the neutrophils remained viable. Similar to NETosis, mitochondrial release was dependent on NADPH oxidase activity and could be blocked with DPI. Furthermore, as with NETs of nuclear origin, mitochondria-derived NETs contained granule proteins, such as myeloperoxidase and elastase. However, since mitochondrial NETs have not yet been proteomically-profiled, it is unclear whether their components differ from nuclear-derived NETs.

The other strategy for producing NETs involves platelets. Under shear stress conditions, platelets activated by the Toll-Like Receptor 4 (TLR4) agonist, LPS, bind to neutrophils, stimulating them to produce NETs (Clark, 2007). After release of NETs, the neutrophils do not stain with Sytox Green, a cell-impermeant dye. *In vivo*, this process was found to occur in liver sinusoids and pulmonary capillaries, where it contributed to trapping of *E. coli*. The exact molecular mechanism by which this event occurs and the cellular origin of these NETs is currently unknown.

While it is still unclear how the three aforementioned strategies for generating NETs are connected, it may be possible to make inferences about their relationships based upon their kinetics. Whereas PMA-induced NETosis generates NETs over the course of 2-4 hours, mitochondrial release and platelet-induced NETs seem to occur within 15 and 10 minutes after stimulation, respectively (Clark, 2007; Fuchs, 2007; Yousefi, 2009). The similarities in kinetics and viability between platelet-induced and mitochondria-derived NETs suggest that they actually represent a related process thereby

raising the possibility that activated platelets and shear stress actually induce mitochondrial release.

It is also interesting to consider the relationship with mitochondria release and NETosis. It is currently not known whether cells that have released their mitochondria can undergo NETosis. However, if this is possible, mitochondrial NETs may be a mechanism to produce NETs relatively quickly while NETosis may be used to produce NETs if mitochondrial NETs are not sufficient to control an infection. The prevention of unnecessary NETosis would allow neutrophils to undergo apoptosis, thereby preventing additional tissue damage caused by lysed cells and NETs.

NET Induction

NET production can be stimulated by a variety of inflammatory signals. Several studies have demonstrated that stimulation of neutrophils with bacteria or individual bacterial components, such as LPS, strongly induces NET production (Brinkmann, 2004; Fuchs, 2007; Yost, 2009). More complex pathogens, such as fungi (*Candida albicans*, *Aspergillus nidulans*) and parasites (*L. amazonensis*, *Plasmodium falciparum*), have also been shown to stimulate NET release (Baker, 2008; Bianchi, 2009; Bruns, 2010; Guimaraes-Costa, 2009; Urban, 2006). In addition to microbes, NETs can also be produced in response to certain host factors. These include interleukin-8 (IL-8), a chemokine, and LPS-activated platelets (Brinkmann, 2004; Clark, 2007). In addition to these factors, the complement factor C5a has also been shown induce NET release, but only in neutrophils primed with interferon- α (Ifn- α), interferon- γ (Ifn- γ) or granulocyte/macrophage colony-stimulating factor (GM-CSF) (Martinelli, 2004).

Although NETs can be stimulated by a variety of inflammatory signals, different stimuli appear to stimulate different forms of NET production. NETs production via mitochondrial release appears to be dependent on priming. Indeed, both LPS and C5a could stimulate mitochondria-derived NETs in GM-CSF primed, but not naïve, neutrophils (Yousefi, 2009). In addition, GM-CSF priming was also not sufficient to induce mitochondria release, suggesting that the integration of multiple signals stimulates the production of mitochondria-derived NETs. Interestingly, Yost et al. showed that LPS-stimulated neutrophils can release NETs without priming over a 60 minute time period (Yost, 2009). As the mitochondria release demonstrated by Yousefi et al. occurred within the course of 15 minutes, the NETs produced by naïve neutrophils may have resulted from NETosis, which would suggest that a single signal can induce different modes of NET formation in primed and naïve contexts.

ROS and NETs

NADPH oxidase is a membrane-bound enzyme complex that transfers electrons from NADPH to oxygen to produce superoxide (Lassegue, 2010). Superoxide can then be converted to hydrogen peroxide by superoxide dismutase. The activity of this enzyme appears to be a critical mediator of the formation of extracellular traps. Treatment of neutrophils with DPI, a chemical inhibitor of NADPH oxidase, has been shown to block both PMA-induced NET formation and GM-CSF/LPS-induced mitochondrial release (Fuchs, 2007; Yousefi, 2009). Fuchs et al. showed that the addition of glucose oxidase, an enzyme that generates peroxide, could overcome the inhibitory effect of DPI on NET production (Fuchs, 2007). Conversely, the addition of catalase, an enzyme that breaks

down hydrogen peroxide, was shown to inhibit NET production. Collectively, these data give strong evidence for the idea that hydrogen peroxide is a necessary step leading to NET production. However, it is important to note that while ROS is necessary, it is not sufficient to induce NETs, as evidenced by the inability of neutrophils from neonates to generate NETs in response to ROS (Yost, 2009).

The relationship between ROS and NET production can also be seen in the clinical setting. Chronic Granulomatous Disease (CGD) is a congenital disease that is characterized by the lack of a functional oxidative burst. Patients suffering from CGD have impaired innate immune function, and are susceptible to both recurrent opportunistic infections (Holland, 2010). Fuchs et al. demonstrated that patients with CGD are unable to produce NETs (Fuchs, 2007). *In vitro*, the addition of glucose oxidase restored both NET production and NET-mediated killing of *S. aureus*. Recently, Bianchi et al. showed that gene therapy restoration of NADPH oxidase activity in a CGD patient restored his ability to produce NETs (Bianchi, 2009).

In addition to ROS, reactive nitrogen species (RNS) may also contribute to NET formation. Patel et al. recently showed that nitric oxide (NO) donors stimulate the production of NETs (Patel, 2010). Interestingly, this was dependent on the actions of both NADPH oxidase and myeloperoxidase (MPO), as both DPI and ABAH, an MPO inhibitor, inhibited NO-induced NETs. Furthermore, sodium nitrite acted synergistically with MPO and hydrogen peroxide to enhance production of free radicals. These results suggest that NO may react with other ROS to produce more potent RNS, such as ONOO, that stimulate NET production.

Extracellular Traps in Other Cell Types

Since their initial discovery in neutrophils, extracellular traps have been found to be produced by other immune cell types. In 2008, von Kockritz-Blickwede et al. demonstrated that mast cells produce extracellular traps, called MCETs, in response to both PMA and bacteria (von Kockritz-Blickwede, 2008). As the first example of an extracellular trap produced by a non-neutrophil cell type, the discovery of MCETs changed the notion of trap formation as a neutrophil-specific process. Like NETs, MCETs play a key role in mast cell clearance of bacterial pathogens. They contain histones, granule proteins and antimicrobial peptides, and their antimicrobial activity can be abrogated by treatment with a combination of DNase and myeloperoxidase. MCETs were produced in a cell death process similar to NETosis. As such, NETosis was subsequently re-named “ETosis” to include non-neutrophil cell types (Wartha, 2008). Interestingly, mast cell production of extracellular traps was found to be dependent on the presence of granules, as treatment of mast cells with compound 48/80, a degranulating agent, impaired MCETs formation. This suggests that granules components may play an important role in in the generation of ETs. It is currently unknown whether the presence of granules is required for the production of NETs.

Shortly after the discovery of MCETs, Youefi et al. showed that eosinophils can also produce extracellular traps (Yousefi, 2008). In contrast to mast cells, eosinophil ETs were derived from mitochondria, as opposed to nuclear DNA. As in both neutrophils and mast cells, ETs contributed significantly to the bactericidal capacity of eosinophils. However, it is important to note that, prior to this landmark study, the role of eosinophils in innate immune defense against bacteria was largely unrecognized. As such, the

discovery of ETs served to elucidate the enigmatic role of eosinophils in host pathogen defense.

The presence of ETs in both mast cells and eosinophils raises the possibility that ET formation may play a role in an even wider variety of immune cells. Recently, we demonstrated that fetal calf serum contains (FCS) nucleases that degrade ETs, which impairs both their visualization and quantification (von Kockritz-Blickwede, 2009). These nucleases retained their activity when heated at 55C, but not at 70C. As such, it is possible that certain cell culture methodologies have limited the detection of ET formation *in vitro*. By adopting new techniques for culturing immune cells, it is possible that ETs will be detected in an even wider array of immune cell types.

NETs in Infection

It is becoming increasingly clear that ETs contribute to host defense against a wide variety of gram positive bacteria, gram negative bacteria, mycobacteria, fungi and parasites (Baker, 2008; Brinkmann, 2004; Bruns, 2010; Guimaraes-Costa, 2009; Ramos-Kichik, 2009; Urban, 2006). Due to their ability to bind and kill bacteria, it has been postulated that they function both to kill microbes and to limit the spread of infections. To date, ETs have not been shown to contribute to host antiviral defense.

DNA itself is not directly antimicrobial. As such ETs derive their antimicrobial activity from their ability to bring bacteria and antimicrobial components into close proximity. Histone H2A has been shown to play an especially important role in NET killing of bacteria and parasites, and blocking antibodies against H2A significantly reduce NET antimicrobial activity (Brinkmann, 2004). Interestingly, while *C. albicans* is

effectively killed by NETs, it is not sensitive to killing by histone H2A (Urban, 2006). Although numerous granule-derived ET proteins have been shown to be antimicrobial, their contribution to ET activity is currently unknown. To date, no studies have correlated ET activity to the presence of a specific granule protein. The cytoplasmic protein calprotectin plays a role in NET clearance of *C. albicans* (Urban, 2009). Calprotectin has also been shown to have antibacterial activity, although it is not known how this impacts ET clearance of bacteria (Corbin, 2008; Steinbakk, 1990).

While antimicrobial peptides (AMPs) have potent antimicrobial activity and are also localized to NETs, their contribution to NET activity is currently unclear. A study from Jann et al. showed that while murine CRAMP, an antimicrobial peptide, contributes to phagocytic killing of *S. aureus*, it has diminished anti-staphylococcal activity when interacting with DNA (Jann, 2009). Indeed, while purified CRAMP could effectively kill *S. aureus in vitro*, addition of DNA to this system effectively inhibited CRAMP's activity. It is possible that this effect is the result of DNA neutralizing the cationic charge of CRAMP. Ultimately, these results suggest that cationic antimicrobial peptides may have limited contribution to the overall antimicrobial properties of ETs. As such, the role of AMPs in the activity of ETs, if any, remains unclear. Furthermore, these results raise the interesting possibility that other proteins and peptides may have different properties when bound to ETs.

It is currently unclear how ETs originating from different cell types and different organelles differ in composition. However, the difference in their associated components may give them slightly different activities against pathogens. For example, while DNase is sufficient to abrogate NET antibacterial activity, myeloperoxidase must be added in

conjunction with DNase to inhibit the activity of MCETs (Brinkmann, 2004; von Kockritz-Blickwede, 2008). A more detailed understanding of ETs from different cell types and different subcellular origins should provide insight into both their antimicrobial activities as well as their individual roles in innate immunity.

The link between ET production and phagocytosis is currently not well understood. Fuchs et al. showed that, *in vitro*, neutrophils initially use phagocytic killing as their primary means of clearing *S. aureus*, but that the mode of killing shifts toward ET-based killing over time (Fuchs, 2007). During an infection, ETosis is likely in balance with other types of cell death. Neutrophils have previously been shown to undergo phagocytosis-induced apoptosis, also referred to as phagocytosis-induced cell death, after engulfing *E. coli* (Watson, 1996). While the purpose of this mode of death is not fully known, it has been speculated that the apoptotic cells may help generate a greater T-cell response (Hacker, 2002). As neutrophils undergoing apoptosis do not produce NETs, it is likely that phagocytosis-induced cell death inhibits overall NET production. During an infection, it is likely that NETosis and phagocytosis-induced apoptosis are in balance, with each contributing to different facets of the immune response.

Recently, Munafo et al. reported that opsonized *E. coli* were less able to induce NET production relative to non-opsonized controls, suggesting that phagocytosis decreases NET production (Munafo, 2009). However, this is in contrast to work from Urban et al. which suggested that opsonized *C. albicans* induced greater NET formation than non-opsonized forms (Urban, 2006). It is possible that different classes of pathogens have different effects on ET production.

ETs and Sepsis

In addition to their effect of bacterial clearance, ETs may also play a role in the pathogenesis of sepsis. In clinical studies, circulating free DNA (cf-DNA) levels have been shown to be a marker for a variety of inflammatory conditions, including infections, septic arthritis and sepsis (Logters, 2009; Margraf, 2008; Moreira, 2010). In addition, increasing levels of systemic inflammation have been correlated to higher levels of circulating nucleosomes (Zeerleder, 2003). While these studies suggest a link between ET production and sepsis, the role of ETs in sepsis is currently unclear. Several lines of evidence suggest that ETs may actually contribute to the progression of sepsis.

One of the important consequences of sepsis is tissue damage and organ failure. During ET production, granule proteins and peptides are likely released into the extracellular environment with ETs. Some of these components, such as matrix metalloproteases and myeloperoxidase, have the potential to contribute to tissue damage. Thus, the overproduction of ETs could conceivably lead to massive tissue injury and, in extreme cases, organ failure. In support of this, Clark et al. showed that the interaction between LPS-stimulated platelets and neutrophils leads to both NET production and tissue damage in liver sinusoids (Clark, 2007). It is important to note that, as postulated by Papayannopoulos and Zychlinsky, the DNA backbone of ETs may also serve to limit this collateral damage by confining antimicrobials to a particular location (Papayannopoulos, 2009).

In addition to granule components, histones may also contribute to tissue damage during ETosis. Xu et al. showed that the circulating levels of H3 increased following an

inflammatory challenge and that histone H3 and H4 were cytotoxic (Xu, 2009). Furthermore, they showed that a blocking antibody against histone H4 was protective in multiple models of sepsis. Interestingly, histones H3 and H4 are in much lower abundance relative to H2A and H2B in NETs (Urban, 2009). Because histones are present in similar quantities in intact nuclei, these data suggest that NETosis releases H3 and H4 into circulation.

ET production may also play a role in sepsis by stimulating the inflammatory response. A recent study by Zhang et al. demonstrated that mitochondrial contain damage-associated molecular patterns (DAMPs) that can elicit an inflammatory response (Zhang, 2010). As mitochondrial components are likely released into circulation during the production of both nuclear and mitochondria-derived ETs, ET production may lead to greater inflammation. In addition, mitochondrial ETs may serve a dual purpose as both an antimicrobial effector and a pro-inflammatory signal. Thus, the pleiotropic effects of mitochondria in inflammation and ET release have many potential links and implications for sepsis.

Conclusions

Since their discovery in 2004, ETs have been shown to play an important role as an effector of antimicrobial activity. The unique mechanisms by which these structures are formed have the potential to advance the way we view the cell biological processes of neutrophils and other leukocytes. However, molecular mechanisms underlying this process are still not well understood. Furthermore, the interplay between different forms of ET production and their relationship to “classical” effectors of innate immune function

is still unclear.

The release of ETs may also have important clinical implications. Not only are they an important innate immune defense mechanism, but they may also play a role in the development of inflammation and sepsis. Furthermore, ETs have been associated with certain autoimmune diseases (Hakim, 2010; Kessenbrock, 2009). Thus, future research will be necessary to fully evaluate the clinical ramifications of ET production and its potential as a therapeutic target.

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Chapter II

**Fetal calf serum contains heat-stable nucleases that
degrade neutrophil extracellular traps**

Extracellular traps (ETs) have been recently discovered as a new paradigm in the innate immune function of leukocytes (Brinkmann, 2004). Released by neutrophils, mast cells or eosinophils in response to various infectious or proinflammatory stimuli, ETs represent complexes of nuclear or mitochondrial DNA together with proteins such as histones, cell-specific proteases (e.g. myeloperoxidase) and antimicrobial peptides (e.g. cathelicidins) (Brinkmann, 2004; von Köckritz-Blickwede, 2008; Yousefi, 2008). DNA is the major structural component of ETs, since treatment with nucleases leads to their dissolution. ETs exhibit strong antimicrobial and/or immunomodulating properties and play an important role *in vivo* during several infectious or non-infectious diseases (von Köckritz-Blickwede, 2009).

Interestingly, formation of ETs has consistently been found to be inhibited in a concentration-dependent fashion by serum, as we corroborate with neutrophil ETs (NETs) in Figure 2.1 (von Köckritz-Blickwede, 2008; Fuchs, 2007). Since reactive oxygen species are an essential trigger in the formation of ETs, it was previously hypothesized that fetal calf serum (FCS) impairs the formation of ETs based on its antioxidant activity. Here, we demonstrate that FCS contains heat-stable nucleases that can degrade ETs.

In general, DNase I is regarded as the major serum nuclease. A recent report by Napirei et al. showed an additional DNase I-like nuclease to be present in serum; however, detailed information about the heat-stability of these nucleases is currently unavailable (Napirei, 2009). As shown in Figure 2.2, medium supplemented with 10% FCS, that was inactivated in the standard fashion at 56°C for 30 min, showed the ability to degrade calf thymus DNA, indicating the presence of heat-stable nucleases. Similarly,

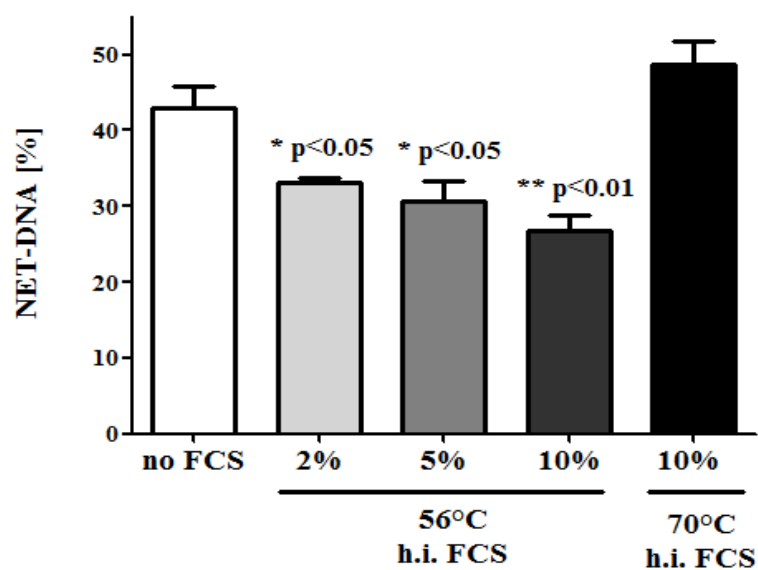


Figure 2.1 Fetal calf serum degrades NETs. Quantification of percentage of NET-DNA (using Picogreen, Invitrogen; as previously described⁵) released by human blood-derived neutrophils (isolated using PolymorphPrep system as recommended by the manufacturer, Axis-Shield) after stimulation with 25 nM PMA for 3 h at 37°C and 5% CO₂ in the presence of different concentration with FCS. Data are mean and SEM of three independent experiments. Significant differences analyzed by unpaired t-test.

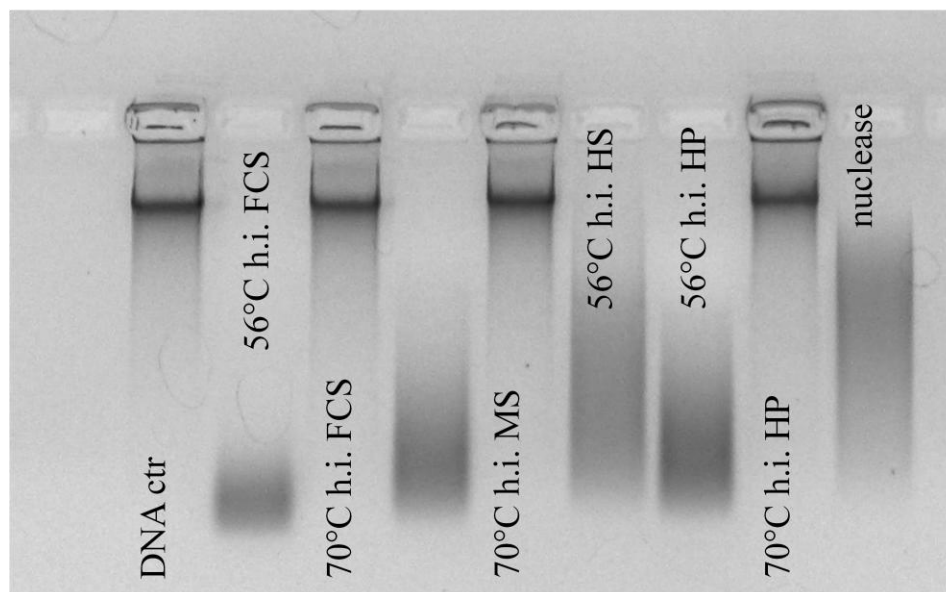


Figure 2.2 Serum from multiple sources contain nucleases. Activity of RPMI medium (Invitrogen) containing 10% FCS (Invitrogen), mouse serum (MS), human serum (HS) or human plasma (HP) in degrading 150 $\mu\text{g/ml}$ calf thymus DNA (Sigma) as determined by agarose gel electrophoresis. Serum or plasma was heat-inactivated at 56°C or 70°C for 30 min prior to experiments. Note that medium containing 56°C heat-inactivated serum or plasma showed degradation of DNA similar to micrococcal nuclease used as a positive control. In contrast heat-inactivation of serum or plasma at 70°C completely abolished this nuclease activity.

Segal et al. reported that serum contains heat-stable nucleases that have the ability to degrade oligodeoxynucleotides (ODNs) (Segal, 1992). We observed that heat-inactivation of at least 70°C for 30 min was required to prevent FCS degradation of DNA (Figure 2.2). Similar nuclease activity was found in 56°C-treated, but not 70°C-treated, mouse serum, human serum or human plasma (Figure 2.2)

Accordingly, we tested whether FCS can degrade ETs. Human blood-derived neutrophils were stimulated with 25 nM phorbol-12-myristate-13-acetate (PMA) for 2 h to release NETs and 10% FCS (heat-inactivated at 56°C or 70°C) was then added to the medium for an additional hour. The presence of NETs was visualized by confocal immunofluorescence microscopy. As shown in Figure 2.3, addition of 10% FCS, inactivated in the standard fashion at 56°C for 30 min, resulted in a distinct degradation of NETs, while FCS inactivated at 70°C did not. This finding was corroborated in a NET quantification assay (Figure 2.1). Fixation with 4% paraformaldehyde, which is often used as a blocking agent prior to immunostainings, did not prevent degradation of ETs by 56°C-inactivated FCS (Figure 2.3).

The presence of heat-stable nucleases in serum might explain why visualization of ETs remains a challenge. It may simply be that existing cell culture protocols (which normally contain 5-10% FCS) hamper their discovery. Interestingly, nuclease activity is also present in aged solution of bovine plasma albumin Fraction V (Anai, 1972), which is widely-used during in culture experiments as an alternative to FCS. Based on this knowledge, investigators should reconsider which medium and supplements are used to perform *in vitro* experiments studying the role of ETs. Furthermore, it opens the question

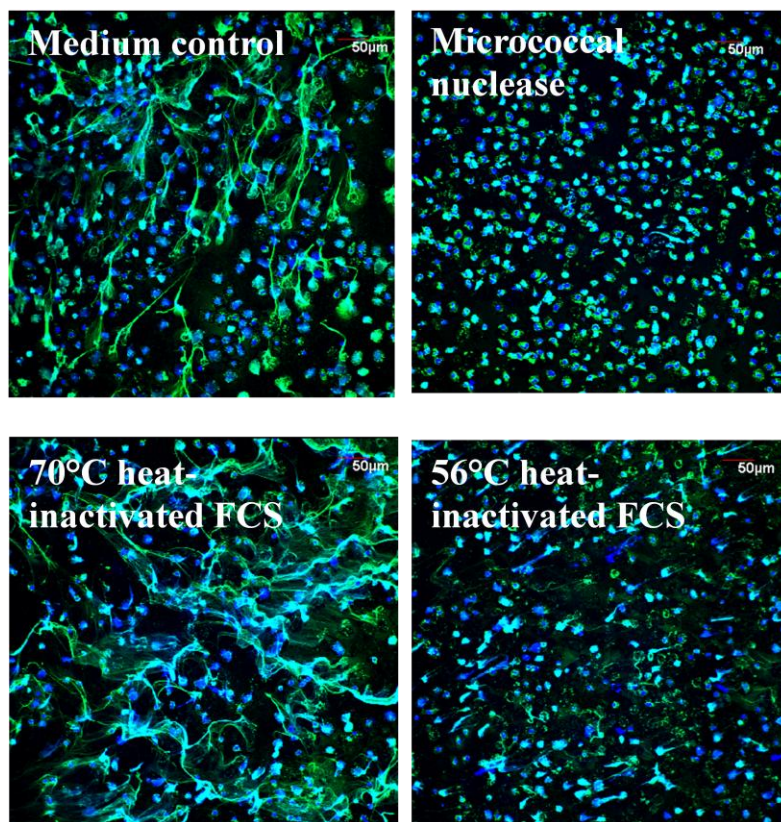


Figure 2.3 Visualization of NET degradation. Confocal immunofluorescence microscopy to visualize degradation of NETs by FCS. Human blood-derived neutrophils were stimulated with 25 nM PMA for 2 h at 37°C and 5% CO₂ in serum-free RPMI to release NETs. Then, 10% FCS, heat-inactivated at either 56°C or 70°C was added to the medium for an additional hour. As control, NETs were degraded by adding 500 mU/ml micrococcal nuclease (Worthington Biochemical Corporation) to the medium. NETs were visualized by immunofluorescence microscopy using a rabbit anti myeloperoxidase-antibody (1:300; 1 h at room temperature; Dako), followed by a secondary Alexa488-labelled goat-anti-rabbit antibody (1:500; 1 h at room temperature; Invitrogen); samples were embedded in ProlongGold+Dapi (Invitrogen) to counterstain nucleus and extracellular DNA in blue. Mounted samples were examined using an inverted confocal laser-scanning 2-photon microscope Olympus Fluoview FV1000 with Fluoview TM Spectral Scanning technology (Olympus) and a 20x/0.75 UPlanSApo Olympus objective. Note that addition of 56°C-heat-inactivated FCS to the cells, in contrast to FCS heat-inactivated at 70°C, produced degradation of NETs. Fixation of cells with 4% paraformaldehyde for 15 min at room temperature did not prevent NET degradation by 56°C heat-inactivated FCS.

of whether formation ET formation by additional leukocyte lineages, such as monocyte/macrophages, or in response to additional environmental stimuli, might also be detected under improved cell culture conditions using nuclease-free medium supplements.

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Contribution

M.v.K. designed and performed research, analyzed and interpreted data, and wrote manuscript; O.C. performed research and provided critical proof-reading of the manuscript; V. N. designed research and wrote manuscript.

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Chapter III

Statins Enhance Formation of Phagocyte Extracellular Traps

Abstract

Statins are inhibitors of 3-hydroxy 3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in cholesterol biosynthesis. Several recent clinico-epidemiologic studies have revealed that patients receiving statin therapy have reduced mortality associated with severe bacterial infection. Here we study pharmacological effect of statins on the innate immune capacity of phagocytic cells, focusing on the leading human bacterial pathogen *Staphylococcus aureus*. These studies revealed a beneficial effect of statins on *S. aureus* clearance using *in vivo*, *ex vivo* and *in vitro* models of phagocyte function, although paradoxically both phagocytosis and oxidative burst were inhibited. Probing instead for an extracellular mechanism of killing, we found statins boosted the production of anti-bacterial DNA-based extracellular traps (ETs) by human and murine neutrophils and also monocyte/macrophages, representing the first report of the ET phenomenon in the latter cell type. The effect of statins to induce phagocyte ETs was linked to sterol pathway inhibition by RNA interference and specific pharmacologic inhibitors. We conclude that a drug therapy taken chronically by tens of millions of individuals alters the functional behavior of phagocytic cells, which could have ramifications for susceptibility and response to bacterial infections in these patients.

INTRODUCTION

Statins are inhibitors of 3-hydroxy 3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in cholesterol biosynthesis. The mainstay of current hyperlipidemia treatment, an estimated 30 million individuals were prescribed statins in 2005 in the U.S. alone (Stagnitti, 2008.). The prevalence of statin usage has stimulated great interest to identify and understand significant biological effects of these drugs beyond cholesterol lowering (Merx and Weber, 2008). One remarkable observation to emerge from several clinico-epidemiological studies is that patients receiving statin therapy may experience reduced infection-associated mortality due to pneumonia (Thomsen et al., 2008), bacteremia (Kruger et al., 2006; Liappis et al., 2001) or sepsis (Almog et al., 2007; Kopterides and Falagas, 2009; Martin et al., 2007). Consistent with this finding, statin therapy improved survival in mouse sepsis models including lipopolysaccharide (LPS) administration (Ando et al., 2000), cecal ligation and perforation (CLP) (Merx et al., 2005; Merx et al., 2004), or systemic challenge with Gram+ or Gram- bacteria (Catron et al., 2004; Chaudhry et al., 2008).

The mechanism(s) by which statin treatment may protect against lethal bacteremia and sepsis are not yet understood. Certain statins exhibit modest inhibitory activity against bacterial growth *in vitro*, e.g. MIC 30 mg/L vs. *Staphylococcus aureus* for simvastatin (Jerwood and Cohen, 2008), but the drug level required for this effect (30 mg/L = 70 μ M) exceeds by ~300-fold the serum level reported in the experimental mouse model following high dose therapy (220 nM with 100 mg/kg/day simvastatin orally)

(Thelen et al., 2006). Rather, more attention has focused on immunomodulatory effects of statins that could reduce the pro-inflammatory “cytokine storm” of sepsis. Statin-treated mice had decreased tumor necrosis factor- α (TNF α) and interleukin-1 β (IL-1 β) or IL-6 levels upon LPS challenge (Ando et al., 2000; Chaudhry et al., 2008), and statin-treated human volunteers receiving LPS also had lower serum TNF α and IL-1 β levels, coupled with diminished monocyte Toll-like receptor-4 (TLR-4) and TLR-2 expression (Niessner et al., 2006). Peripheral blood mononuclear cells (PBMC) isolated from statin-treated humans showed diminished TNF α and IL-6 responses to LPS *ex vivo* (Rosenson et al., 1999), and statin exposure blunted LPS-induced TNF α , IL-1 β , IL-6 and inducible nitric oxide synthase (iNOS) expression by rat macrophages *in vitro* (Pahan et al., 1997).

Here we consider an alternative mechanism by which statins could influence the outcome of bacterial infection – by altering the intrinsic innate immune (bactericidal) capacity of phagocytic cells. We find that statin therapy *in vitro*, *ex vivo* and *in vivo* increases the ability of phagocytes to kill the leading human pathogen *S. aureus*, a phenotype we correlate to increased production of antimicrobial DNA-based extracellular traps. This alteration in immune cell behavior is mediated by inhibition of the sterol pathway.

RESULTS

Statin Induction of Phagocyte Antimicrobial Activity *In Vitro*

We first tested the effect of *in vitro* stimulation with the classical HMG-CoA reductase inhibitor mevastatin on the ability of various phagocytic cell types to kill *S.*

aureus. Statin pre-treatment significantly increased the anti-staphylococcal activity of human neutrophils, human U937 monocyte/macrophages, and murine RAW 264.7 macrophages (Figure 3.1). At the concentration used, mevastatin had no direct inhibitory effect on *S. aureus* growth (Figure 3.2). Additionally, statin treatment enhanced RAW 264.7 macrophage clearance of strains of methicillin-resistant *S. aureus* (MRSA), group B *Streptococcus* and *Salmonella typhimurium*, suggesting the induction of a generalized mechanism(s) for bacterial killing (Figure 3.3).

In beginning to probe the mechanism by which statin treatment could enhance leukocyte bactericidal function, we were presented with a paradox. Previous literature suggested that statin treatment of neutrophils and macrophages was associated with reduction in phagocytosis and oxidative burst, two key effectors of bacterial killing (Benati et al., 2009; Bokoch and Prossnitz, 1992). We confirmed that mevastatin treatment reduced phagocytic uptake of fluorescently-labelled *S. aureus* by human neutrophils as measured by FACS (Figure 3.4) and reduced the magnitude of the neutrophil oxidative burst elicited by *S. aureus* infection (Figure 3.5). These observed inhibitory effects on phagocytosis and oxidative burst suggested statin treatment was stimulating a mechanism for extracellular bacterial killing. Consistent with this hypothesis, we found that enhanced killing of *S. aureus* by statin-treated neutrophils was maintained in the face of cytochalasin D treatment, an actin microfilament inhibitor that blocks bacterial uptake (Figure 3.6).

Statin Induction of Neutrophil Extracellular Traps (NETs)

A key emerging concept in neutrophil biology is antimicrobial activity through

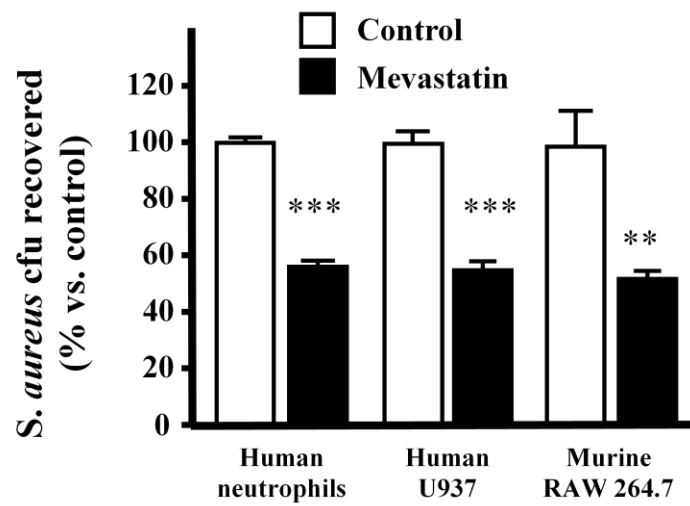


Figure 3.1 Mevastatin enhances leukocyte clearance of *S. aureus*. *In vitro* killing of *S. aureus* by primary human neutrophils or RAW 264.7 and human U937 cells treated with mevastatin or vehicle control. ** $P < 0.01$, *** $P < 0.005$ by two-tailed Student's t-test comparing control versus statin-treated group.

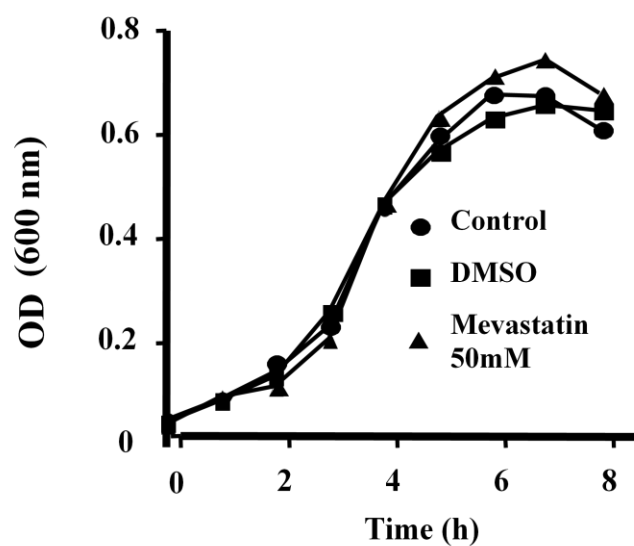


Figure 3.2 Mevastatin does not inhibit *S. aureus* growth. Growth of *S. aureus* strain Newman in RPMI with or without mevastatin (50 μ M) or DMSO vehicle control.

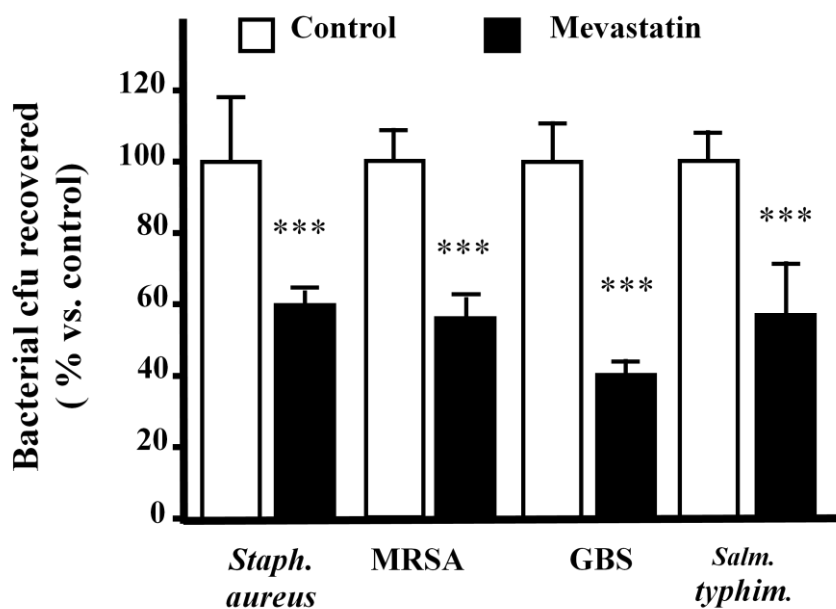


Figure 3.3 Mevastatin enhances macrophage clearance of several types of bacteria. *In vitro* killing of *S. aureus* strain Newman, *S. aureus* strain Sanger (MRSA), *S. agalactiae* strain COH1 (GBS), and *S. typhimurium* by RAW 264.7 cells treated with mevastatin or vehicle control. *** $P < 0.005$ by two-tailed Student's t-test comparing control versus statin-treated group.

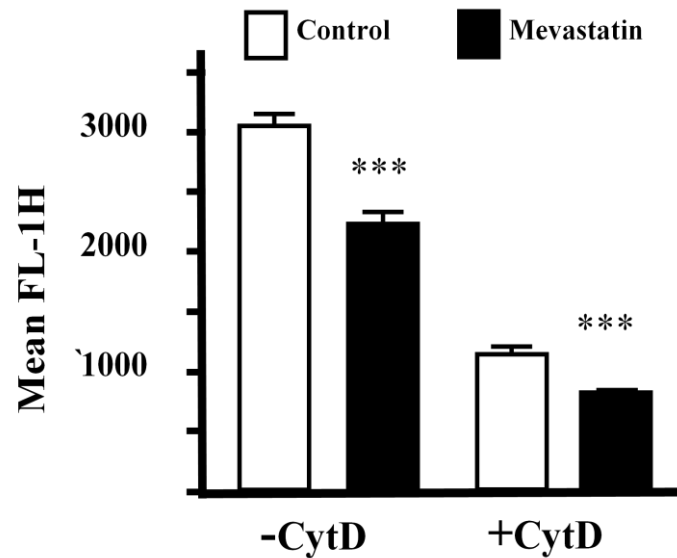


Figure 3.4 Mevastatin reduces neutrophil phagocytosis. Mean fluorescence intensity as parameter for phagocytosis of neutrophils after infection with FITC-labelled *S. aureus* Wood strain bioparticles measured by flow cytometry. As a control, 10 $\mu\text{g/ml}$ cytochalasin D was added to the samples 10 min prior to infection to prevent phagocytosis. *** $P < 0.005$ by one-way ANOVA with Tukey's post test comparing control versus statin-treated group.

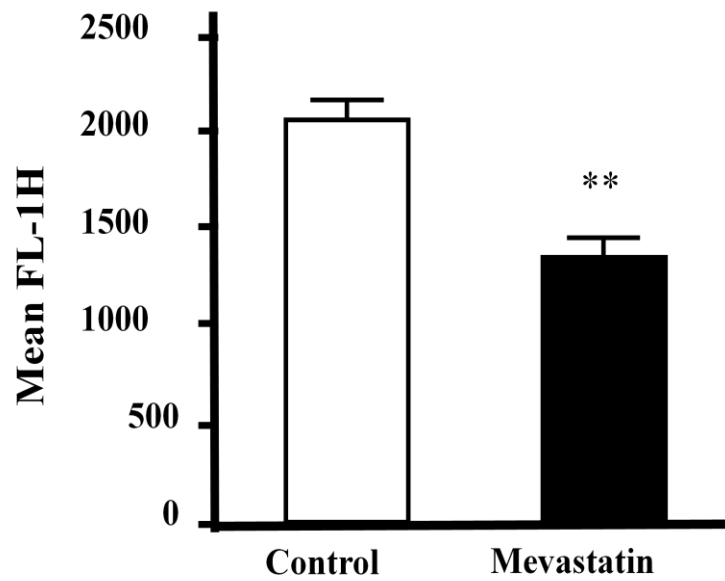


Figure 3.5 Mevastatin reduces neutrophil oxidative burst. Oxidative burst of primary human neutrophils stimulated with mevastatin or vehicle control measured by flow cytometry after 30 min incubation in the presence of 2',7' dichlorofluorescein. ** $P < 0.01$ by two-tailed Student's t-test comparing control versus statin-treated group.

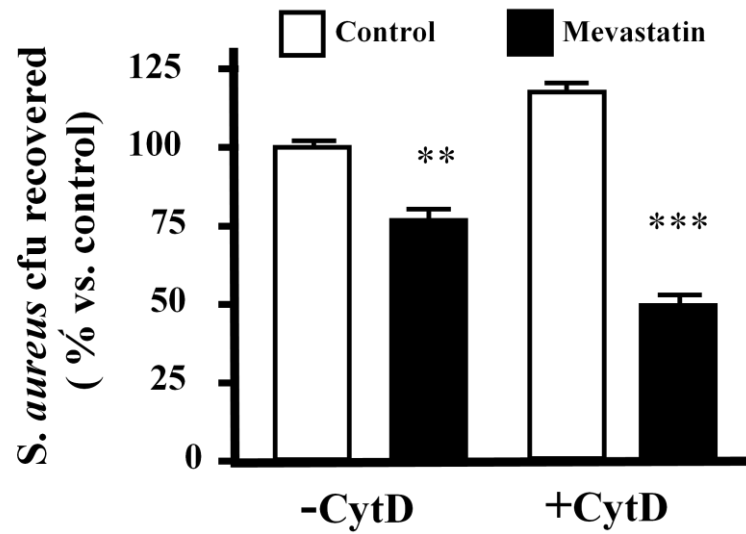


Figure 3.6 Cytochalasin D does not abrogate statin enhancement of neutrophil killing. Extracellular killing of *S. aureus* by primary human neutrophils treated with mevastatin or vehicle control. To prevent phagocytosis, 10 $\mu\text{g/ml}$ cytochalasin D was added to the samples 10 min prior to infection. ** $P < 0.01$, *** $P < 0.005$ by one-way ANOVA with Tukey's post test comparing control versus statin-treated group.

the elaboration of neutrophil extracellular traps (NETs) (Brinkmann et al., 2004). The product of a novel cell death pathway, NETs are composed of nuclear DNA, histones, antimicrobial peptides and proteases, and are capable of entrapping and killing a wide variety of bacteria and other microbes (Brinkmann and Zychlinsky, 2007; von Kockritz-Blickwede and Nizet, 2009). We found that mevastatin treatment *in vitro* strikingly enhanced NET production by freshly-isolated and PMA-stimulated human neutrophils (Figure 3.7A and 3.7B). Release of histones and cathelicidin antimicrobial peptide LL-37, two of the key antimicrobial effectors present in NETs (Brinkmann and Zychlinsky, 2007; von Kockritz-Blickwede and Nizet, 2009), was also increased following statin treatment and co-localized within the NETs (Figure 3.7A and 3.7B). Quantifying NETs by direct assessment of extracellular DNA release (Fuchs et al., 2007), we calculated mevastatin-treated neutrophils produced 2.5-fold more NETs (Figure 3.8), leading to greater entrapment of fluorescently labeled *S. aureus* within these structures (Figure 3.9). Similarly, we found that lovastatin, simvastatin and fluvastatin also enhanced NET production, demonstrating that this phenomenon is not limited to mevastatin and is common across the statin class of drugs (Figure 3.10).

NET release has previously been reported to depend upon reactive oxygen species (ROS) generation through the NADPH oxidase. When we blocked neutrophil ROS production using the NADPH oxidase inhibitor diphenylene iodonium (DPI), the level of NET generation was clearly reduced, yet still significantly greater in statin-treated cells vs. controls (Figure 3.11). Combined with the observation that statin treatment reduced overall ROS production in the neutrophils (Figure 3.5), the evidence suggests that statins may predispose cells to enter the NET cell death pathway in response to a lower threshold

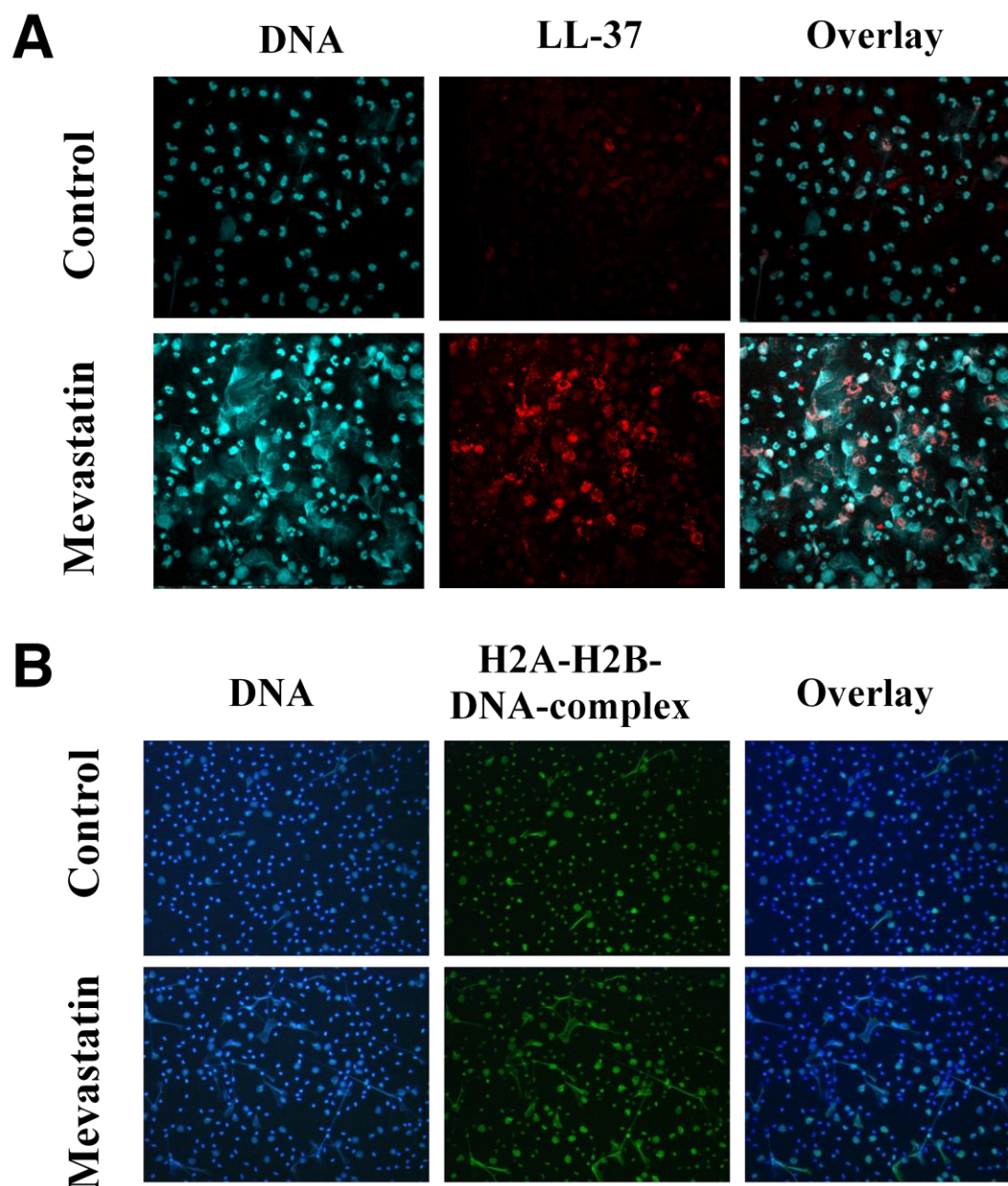


Figure 3.7 Mevastatin enhances NET production. (A) Representative fluorescent images of neutrophils stimulated with mevastatin or vehicle control and PMA to induce NETs. NET-formation was visualized in blue (Dapi) and LL-37-expression was visualized by Alexa red-immunostaining. (B) NET-formation was visualized in blue (Dapi) and H2A-H2B-DNA-complexes were visualized by Alexa green-immunostaining.

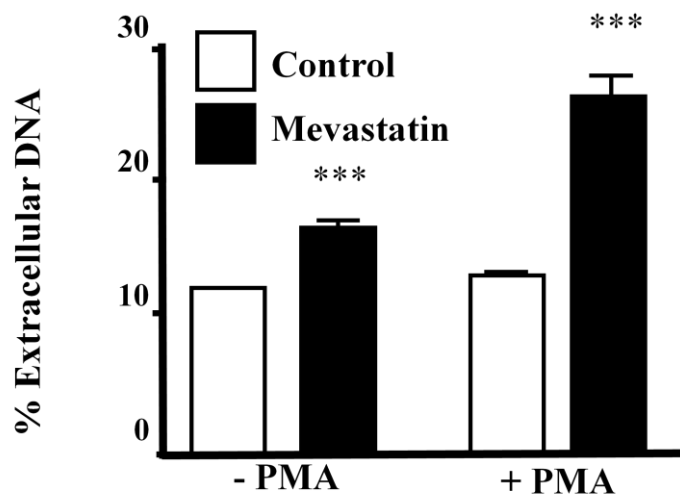


Figure 3.8 Mevastatin enhances PMA-induced NET formation. Quantification of NET-production by primary human neutrophils \pm stimulation with PMA and treatment with mevastatin or vehicle control. *** $P < 0.005$ by one-way ANOVA with Tukey's post test comparing control versus statin-treated group.

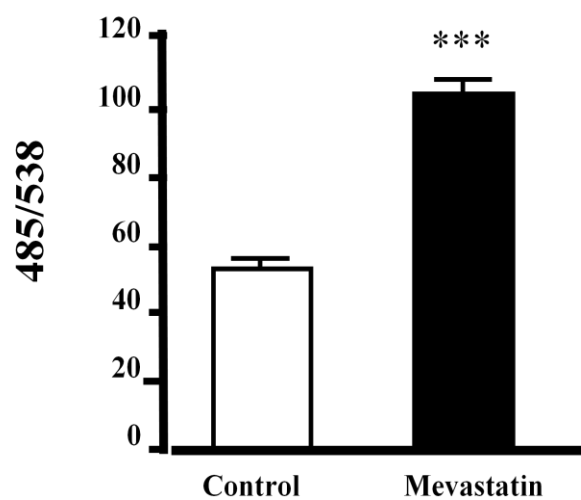


Figure 3.9 Mevastatin enhances NET entrapment of *S. aureus*. Entrapment of fluorescently labeled *S. aureus* by PMA-stimulated human neutrophils treated with mevastatin or vehicle control. *** $P < 0.005$ by two-tailed Student's t-test comparing control versus statin-treated group.

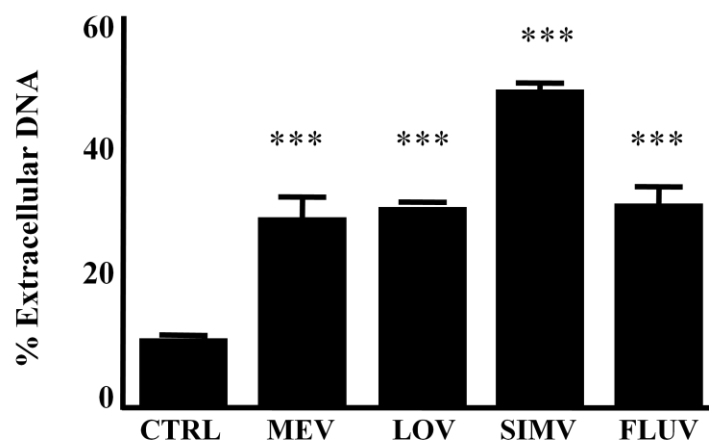


Figure 3.10 Multiple statins enhance NET production. Quantification of NET-production by primary human neutrophils \pm stimulation with PMA and treatment with different statins (MEV = mevastatin, LOV = lovastatin, SIMV = simvastatin, FLUV = fluvastatin) or vehicle control (CTRL). *** $P < 0.005$ by one-way ANOVA with Dunnet's post test versus control group.

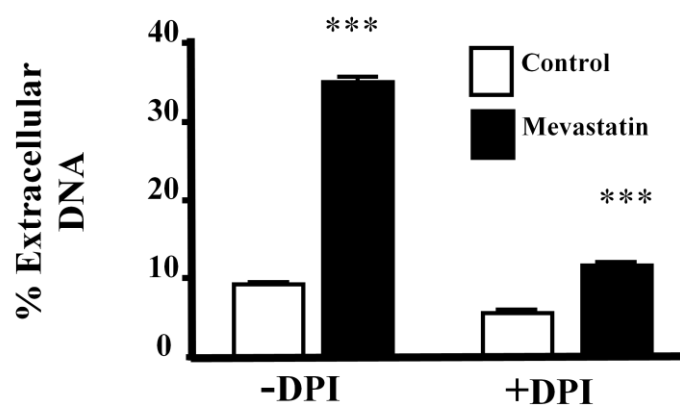


Figure 3.11 Statin-induced NET production is dependent on reactive oxygen species production. NET production of PMA-stimulated human neutrophils treated with 10 $\mu\text{g/ml}$ DPI or vehicle control to inhibit the NADPH-oxidase-dependent ROS production. *** $P < 0.005$ by one-way ANOVA with Tukey's post test comparing control versus statin-treated group.

level of ROS signal. Another biochemical marker of NET formation identified in response to LPS or ROS is deimination of arginine residues in histones to citrullines, a post-translational modification catalyzed by peptidyl arginine deaminase 4 (PAD-4) that facilitates chromatin decondensation (Wang et al., 2009). However, we found that in contrast to control neutrophils, treatment with an inhibitor of PAD-4-mediated histone citrullination failed to block the increased NET production associated with mevastatin or simvastatin treatment (Figure 3.12 and 3.13).

Statin-Induction of Macrophage Extracellular Traps (METs)

Statin enhancement of *S. aureus* killing was not restricted to human neutrophils, but rather was also observed in macrophages/monocytes (Figure 3.1). Similar to our findings with neutrophils, statin treatment of RAW 264.7 macrophages reduced phagocytic uptake of *S. aureus* (Figure 3.14). Unlike neutrophils, eosinophils or mast cells (von Kockritz-Blickwede et al., 2008), release of nuclear DNA to form extracellular traps has never been reported for macrophages. However, we found a subset of macrophages capable upon PMA stimulation to elaborate extracellular DNA to form NET-like structures we now refer to as macrophage extracellular traps (METs). Like NETs, RAW 264.7 METs could be readily visualized with DNA stains such as DAPI and/or in combination with antibodies against histone-DNA-complexes, and were increased by 2.2-fold in statin-treated cells vs. controls (Figure 3.15). Using mammalian cell viability staining, we found that formation of METs, as previously reported for neutrophils and mast cells, is associated with death of the cell (Figure 3.16). Using the BacLight Live/Dead viability assay, we found that the great majority of *S. aureus*

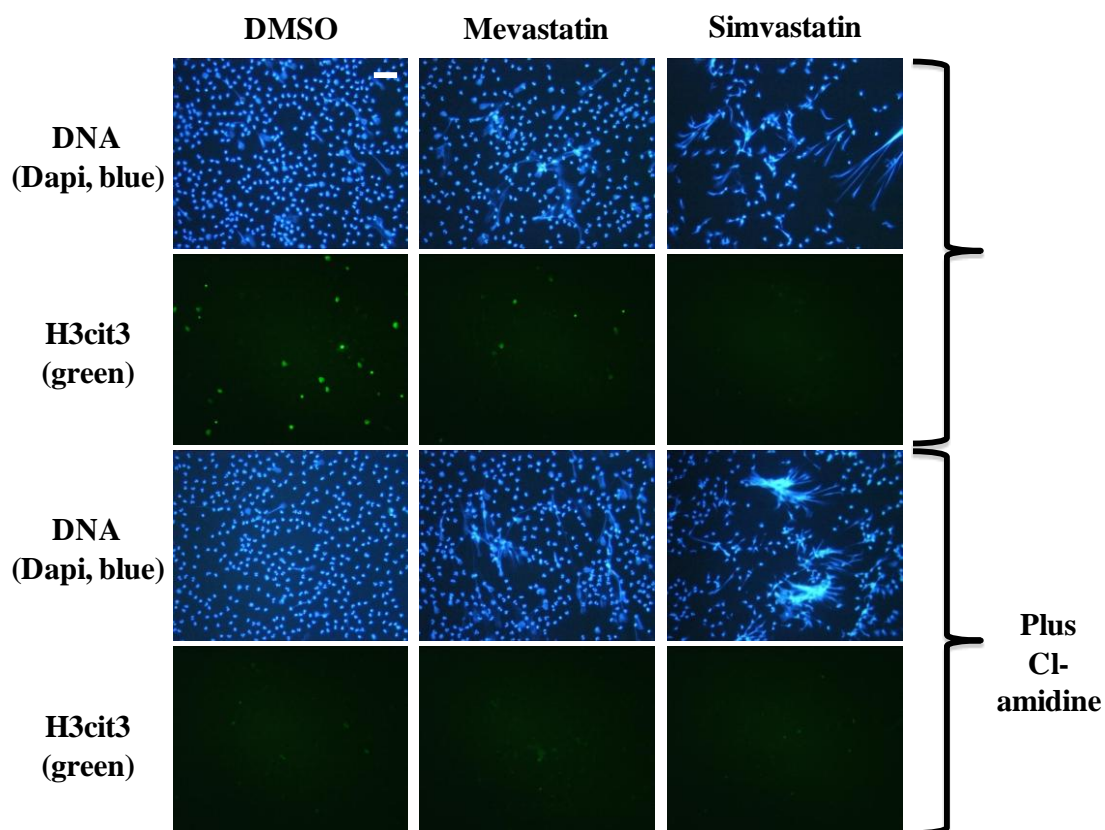


Figure 3.12 Statin-induced NETs are not dependent on histone citrullination.

Representative fluorescent images of human neutrophils stimulated with mevastatin/simvastatin or vehicle control and PMA to induce NETs. Cl-amidine (200 μ M) was used to block the PAD-4 mediated histone citrullination. Net-formation was visualized in blue (Dapi) and histone citrullination (H4Cit3) was visualized by Alexa green-immunostaining. Bars represent 30 μ m.

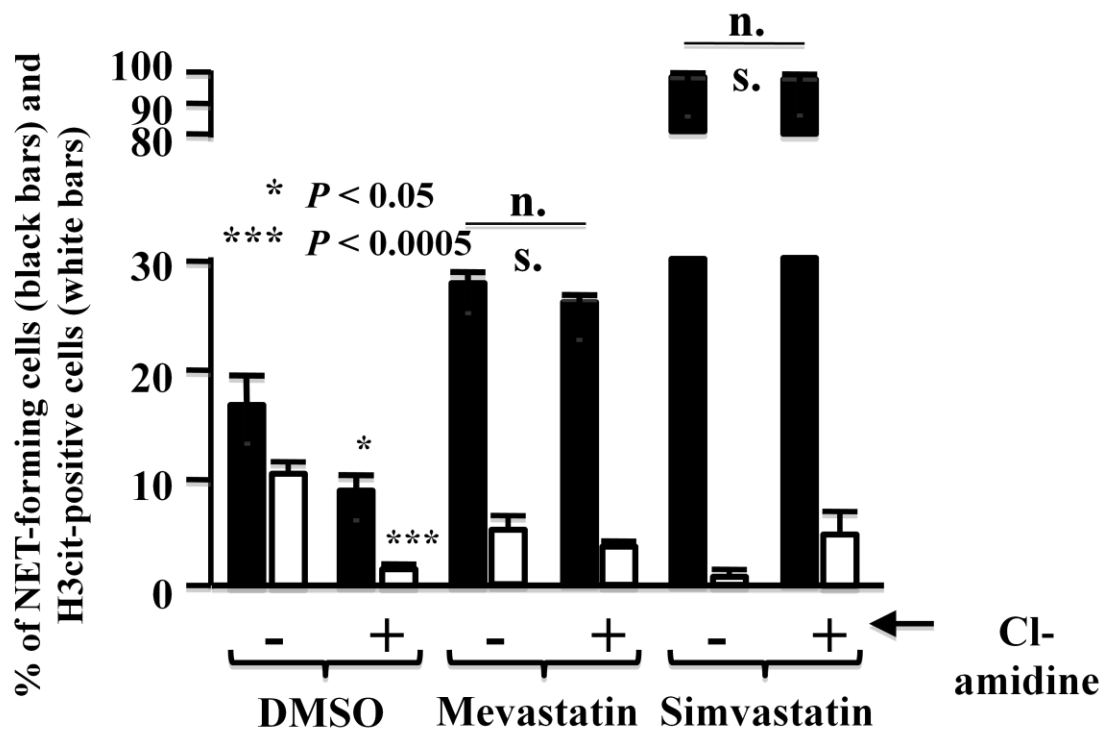


Figure 3.13 Statin-induced NETs are not dependent on histone citrullination.

Quantification of results from Figure 3.12 by direct visualization and enumeration of % of NET-forming neutrophils (black bars) or H4Cit3-positive neutrophils (white bars), average of 4 high-power fields (HPF) counted containing approximately 125 cells.

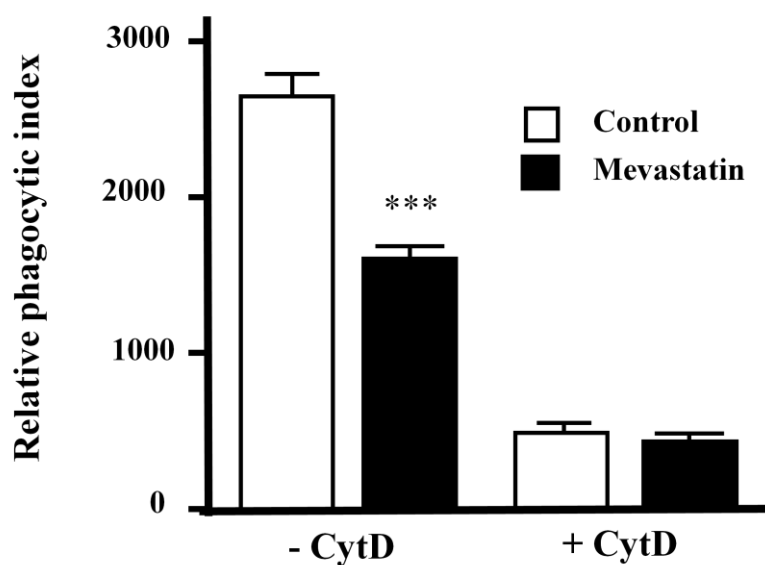


Figure 3.14 Mevastatin inhibits macrophage phagocytosis of *Staphylococcus aureus*. Mean fluorescence intensity was used as parameter for phagocytosis of RAW 264.7 cells after infection with FITC-labelled *S. aureus* Wood strain bioparticles measured by flow cytometry. As control, 10 $\mu\text{g/ml}$ cytochalasin D was added to the samples 10 min prior to infection to prevent phagocytosis. *** $p < 0.005$ by one-way ANOVA with Tukey's post test comparing control versus statin-treated group.

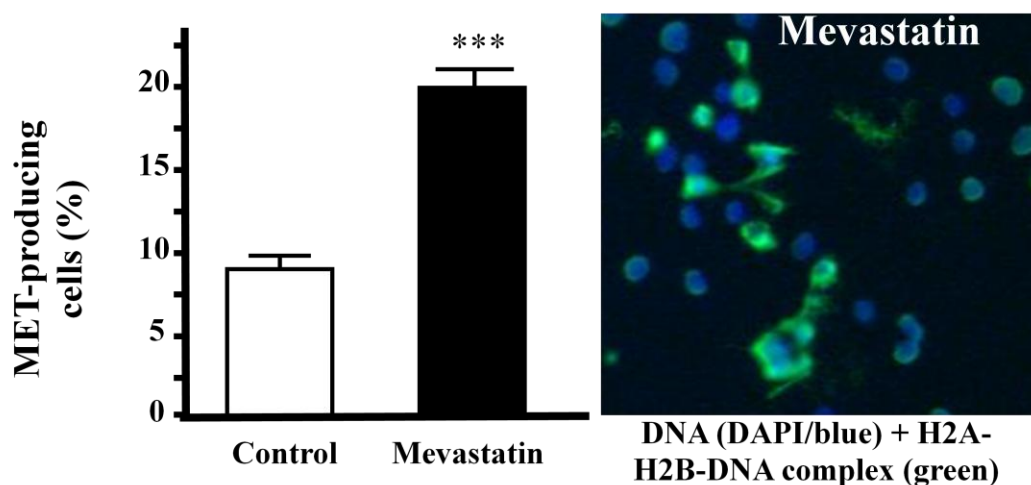


Figure 3.15 Mevastatin induces RAW 264.7 cells to produce METs. Quantification of MET production by RAW 264.7 cells after overnight treatment with mevastatin or vehicle control and subsequent stimulation with PMA. Experiment was performed at 3 independent occasions with similar results, representative experiment shown \pm standard deviation. *** $P < 0.005$ by t-test; representative fluorescent images of RAW 264.7 cells MET formation visualized using blue (Dapi) and Alexa green-immunostaining for H2A-H2B-DNA-complexes.

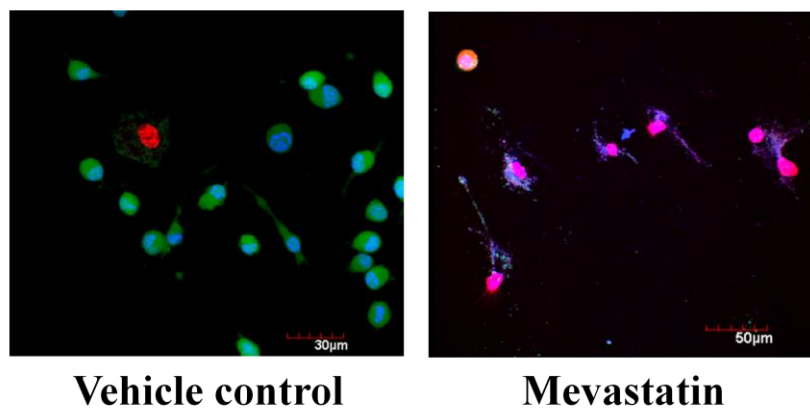


Figure 3.16 Mevastatin-induced METs are associated with dead cells. Live/Dead viability/cytotoxicity staining to determine viability of MET-producing cells after overnight treatment with mevastatin and subsequent stimulation with PMA.

entrapped within METs were nonviable as indicated by the red staining (Figure 3.17). This suggests that similar to NETs, METs can subserve an innate immune function. The murine cathelicidin peptide CRAMP was visualized within MET structures induced by statin treatment in RAW 264.7 cells (Figure 3.18). Similarly, we found that statin treatment could elicit production of DNA-based extracellular traps from thioglycolate-elicited murine peritoneal macrophages (Figure 3.19).

Statin Therapy *In Vivo* Enhances Phagocyte Bactericidal Activity

To extend our observations to statin treatment of the whole animal, thioglycolate-stimulated peritoneal cells were extracted and purified from mice that had been pre-fed with standard chow supplemented with or without simvastatin. In both groups of mice, the composition of the peritoneal cell population was 60-70% neutrophils and 10-20% mononuclear cells (Figure 3.20). In good correlation to the *in vitro* data, the peritoneal cells from simvastatin-treated mice showed increased production of extracellular traps (Figure 3.21) as well as enhanced killing of *S. aureus* compared to peritoneal cells isolated from control mice (Figure 3.22).

Protective Effect of Statin Therapy Against *Staphylococcus aureus* Pneumonia

To determine the effects of statin therapy on resistance to infection, we used an established intranasal inoculation model of *S. aureus* pneumonia (Bubeck Wardenburg et al., 2007; Bubeck Wardenburg and Schneewind, 2008). In comparison to mice fed standard chow, mice fed standard chow supplemented with simvastatin showed reduced bacterial levels in the lung 48 h post-challenge (Figure 3.23). Histopathological changes

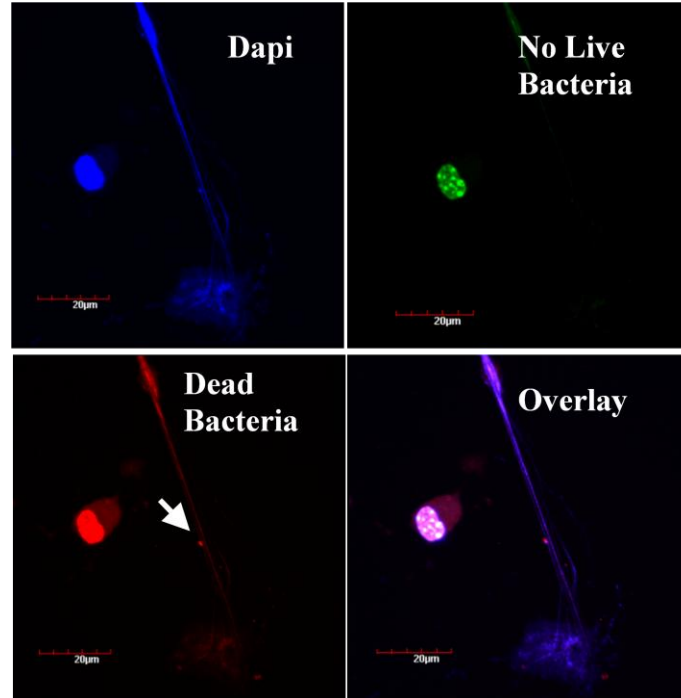


Figure 3.17 METs trap and kill *S. aureus*. Live/Dead BacLight™ Bacterial Viability staining to determine viability of *S. aureus* entrapped in the METs produced by RAW cells. Arrow points to dead *S. aureus* entrapped in MET.

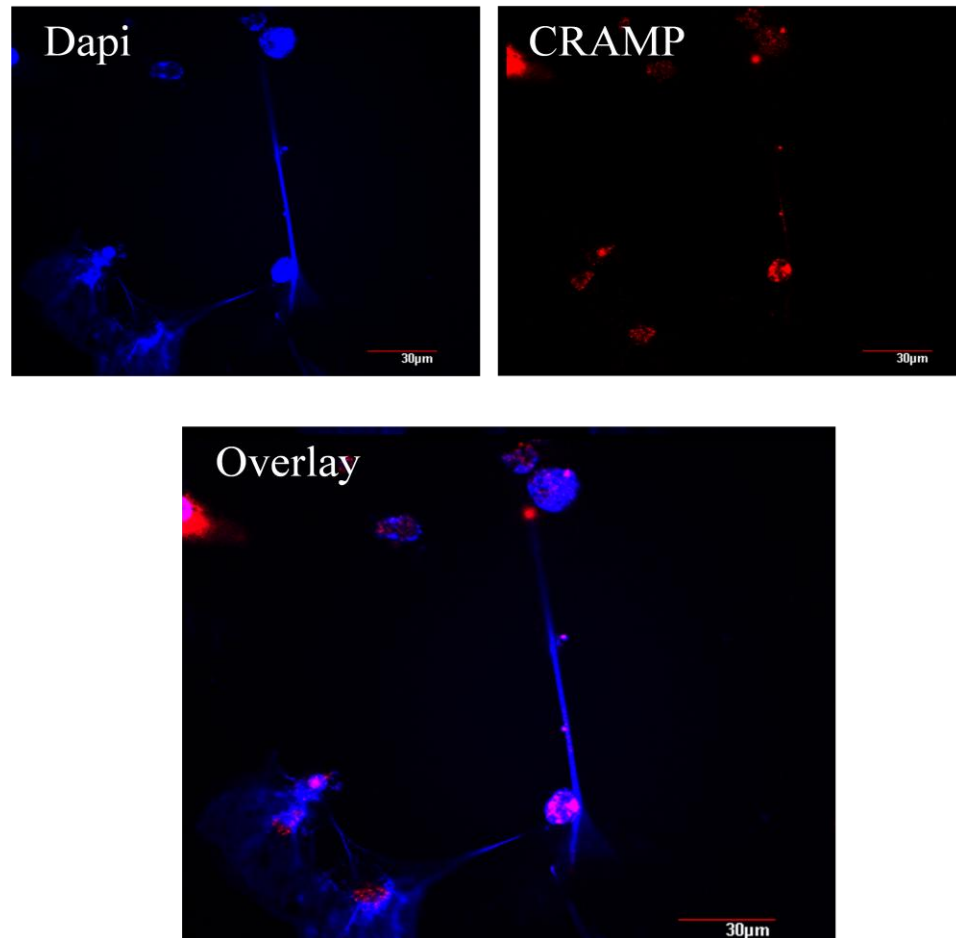


Figure 3.18 Simvastatin enhances MET production. Representative fluorescent images of RAW 264.7 cells stimulated with simvastatin or vehicle control and PMA to induce METs. MET-formation was visualized in blue (Dapi) and CRAMP-expression (rabbit anti mouse CRAMP, left panel) compared was visualized by Alexa red-immunostaining.

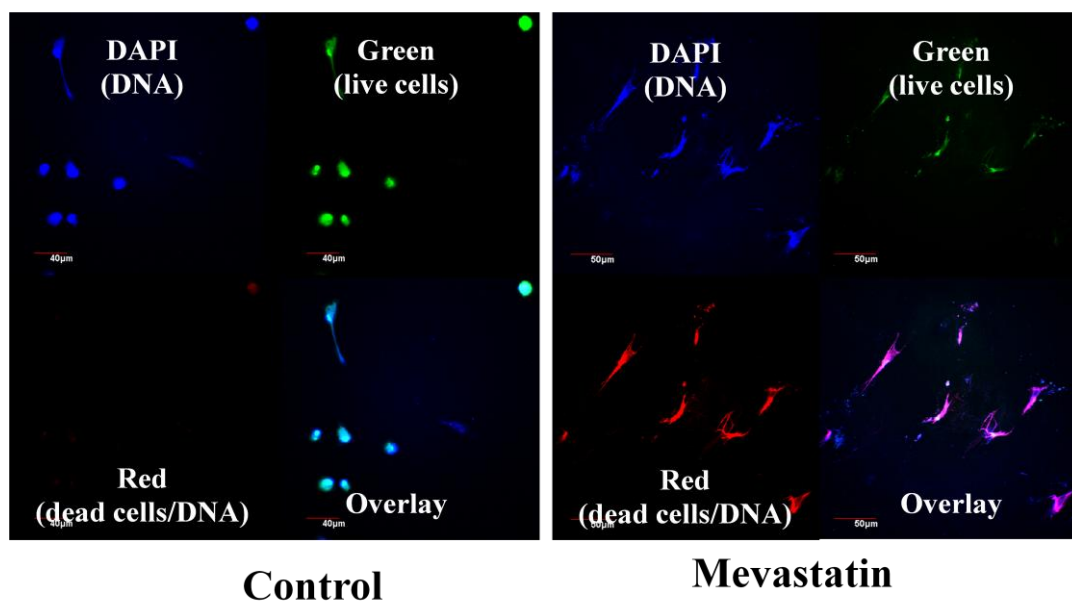


Figure 3.19 Mevastatin enhances MET production by primary murine peritoneal macrophages. Representative fluorescent images of murine peritoneal macrophage extracellular traps, stained with Live/Dead viability/cytotoxicity kit for mammalian cells to determine viability of trap-forming cells after overnight treatment with mevastatin and subsequent stimulation with PMA. Note that all trap-forming macrophages are dead as shown by the red dye.

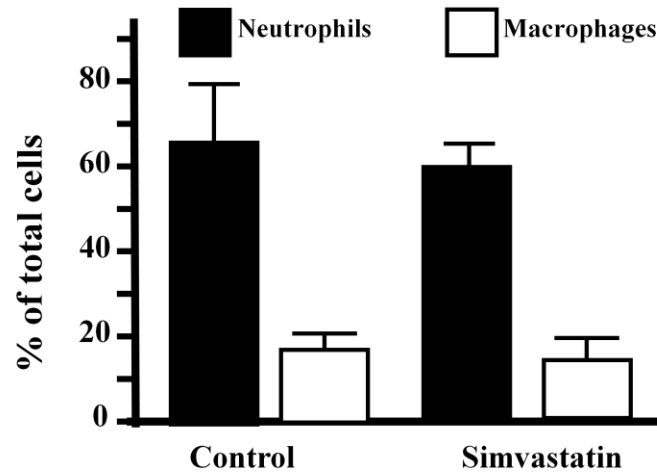


Figure 3.20 Control and simvastatin-treated mice have similar peritoneal neutrophil and macrophage contents. Cell content (percentage of total cells) of thioglycolate-induced peritoneal cells extracted from mice fed with standard chow or standard chow + simvastatin.

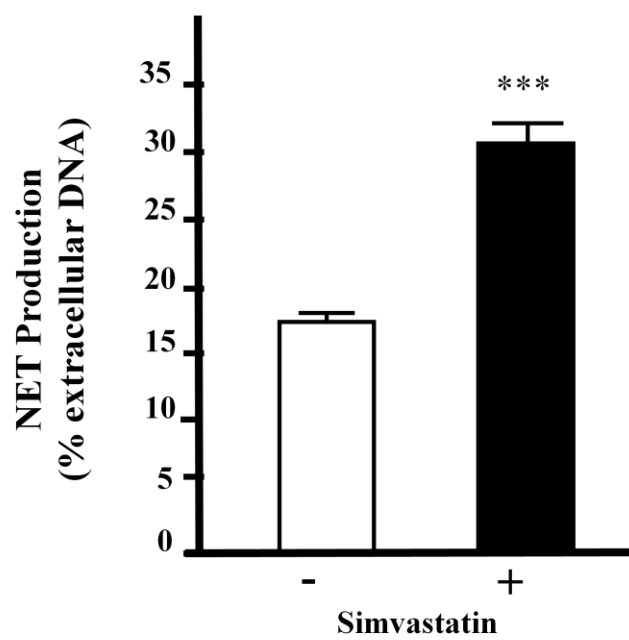


Figure 3.21 Peritoneal cells from simvastatin-treated mice have enhanced ET production. Quantification of NET production by peritoneal cells. *** $P < 0.005$ by two-tailed Student's t-test comparing control versus statin-treated group.

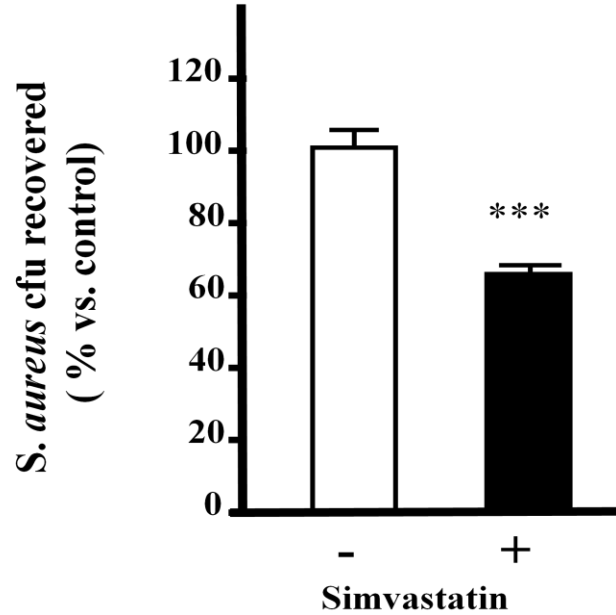


Figure 3.22 Peritoneal cells from simvastatin-treated mice have enhanced anti-staphylococcal activity. *Ex vivo* killing of *S. aureus* strain Newman by thioglycolate-induced peritoneal cells extracted from mice fed with standard chow or standard chow + simvastatin. *** $P < 0.005$ by two-tailed Student's *t*-test comparing control versus statin-treated group.

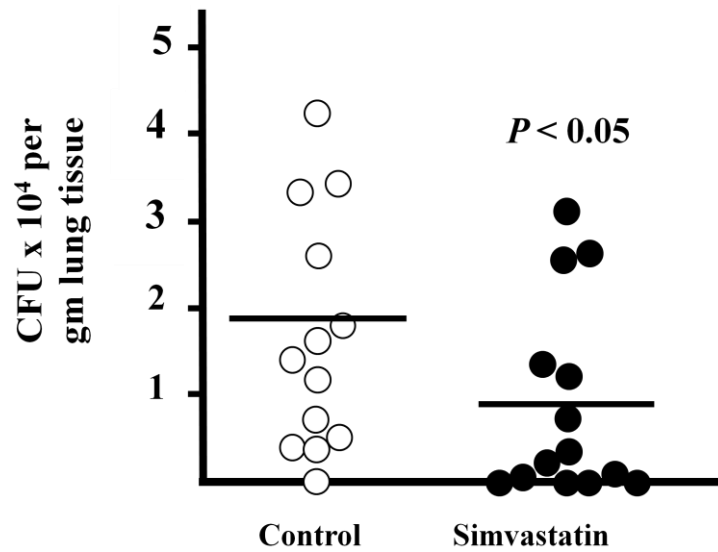


Figure 3.23 Simvastatin-treated mice have enhanced clearance of *S. aureus* pneumonia. Recovered bacteria from lungs of mice pre-fed for with standard chow or standard chow supplemented with simvastatin and infected intranasally with 2×10^8 CFU *S. aureus* strain Newman for 48 h. Data shown are pooled from 2 independent experiments with each $n = 10$ or $n = 7$ mice, respectively. * $P < 0.05$ by two-tailed Mann Whitney test.

characteristic of severe bacterial pneumonia were diminished in simvastatin-treated mice. Whereas the majority of bronchi and alveolar spaces were obliterated by inflammatory exudates, immune cell infiltrates, and staphylococci in control mice (Figure 3.24, top), the simvastatin-treated group exhibited smaller, discrete areas of inflammation, surrounded by large unaffected areas of lung tissue (Figure 3.24, bottom). Recruitment of a mixed population of neutrophils and macrophages was evident in the inflammatory lesions of both groups. To determine if the effect of statin treatment to enhance extracellular trap production was operating *in vivo*, we also examined lung sections for extracellular traps. In correlation with our *in vitro* data, significantly more (~7-fold) more extracellular traps, enriched in the cathelicidin peptide CRAMP were found in the simvastatin-treated group (Figure 3.25A and 3.25B). Many of the extracellular traps from simvastatin-treated mice were quite large and extended into the alveolar space (Figure 3.25A), whereas traps from control mice were small and localized to the alveolar wall. While the more severe degree of inflammation and pneumonia in control mice vs. simvastatin-treated mice precludes a meaningful analysis by total levels, immunofluorescence for CRAMP expression on a per cell basis (CRAMP/DAPI ratio, calculated from the data shown Figure 3.26) was significantly greater in simvastatin-treated animals (1.01 ± 0.12) than controls (0.87 ± 0.16 , $P < 0.001$).

Inhibition of Sterol Production Stimulates the Formation of Phagocyte Extracellular Traps

We next sought to determine the relationship between the mevalonate pathway for cholesterol biosynthesis, which statins target medically, and the observed effect of these

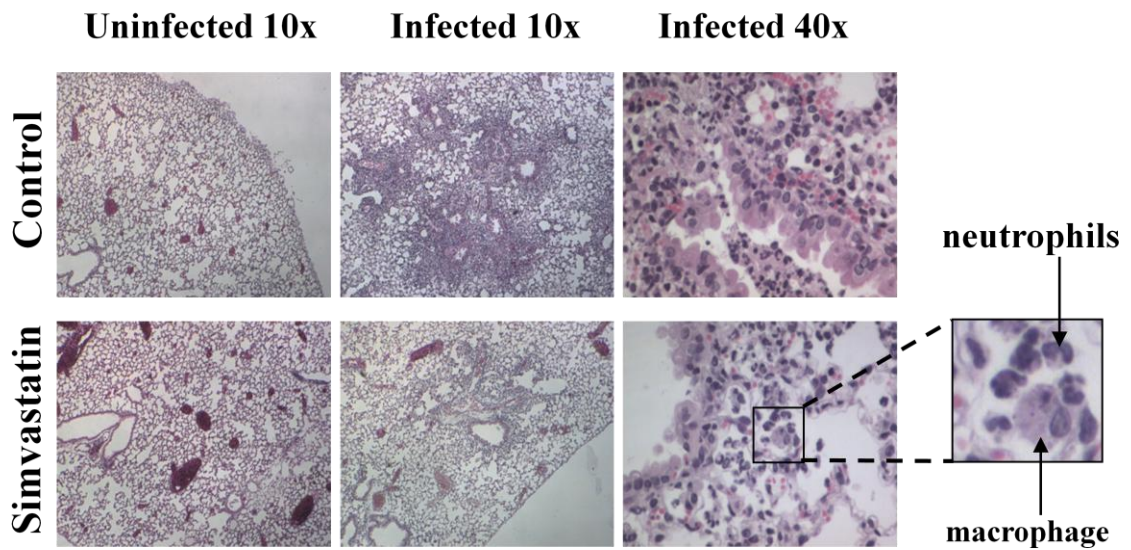


Figure 3.24 Reduced pulmonary inflammation in simvastatin-treated mice after *S. aureus* pneumonia challenge. Representative light micrograph (HE-stained) of lung tissue sections of infected mice pre-fed for with standard chow (control, upper panel) or standard chow supplemented with simvastatin (lower panel).

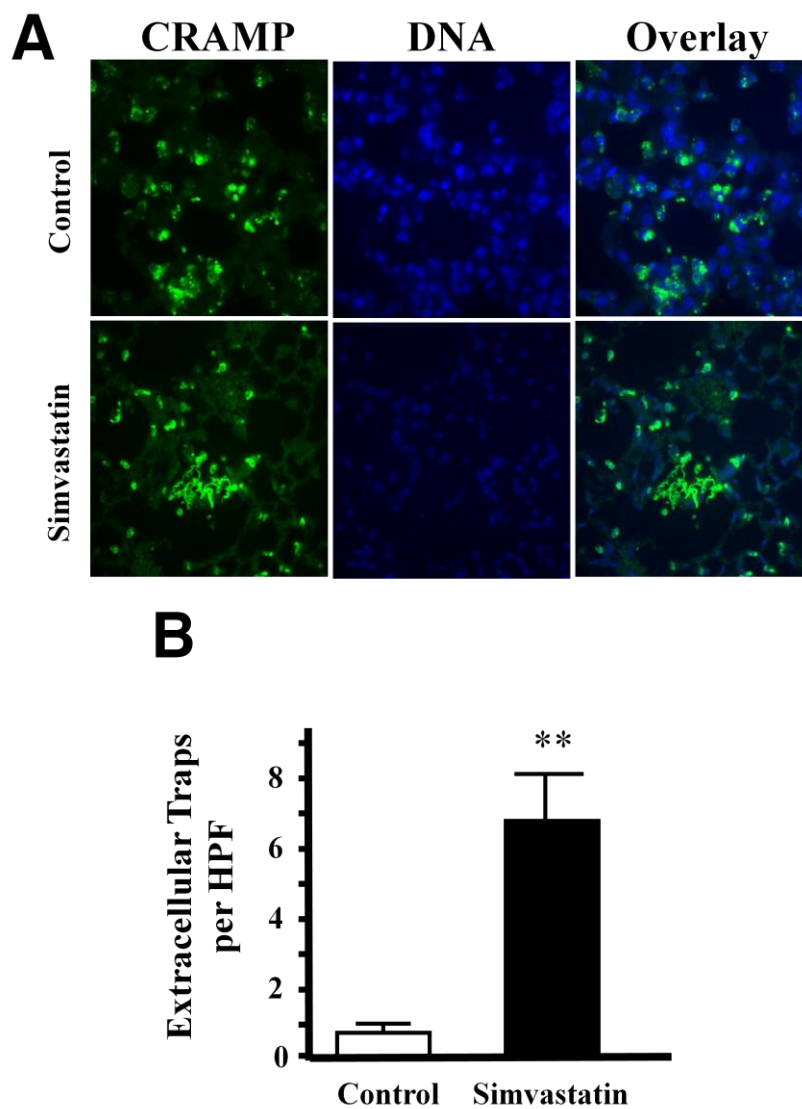


Figure 3.25 Enhanced ET production in simvastatin-treated mice after *S. aureus* pneumonia challenge. (A) Representative fluorescent images of extracellular trap formation (visualized by Alexa 488 (green)-labelled CRAMP production and counterstained with Dapi) in paraffin-embedded lung sections of mice pre-fed for with standard chow or standard chow supplemented with simvastatin and intranasally infected with 2×10^8 cfu of *S. aureus* strain Newman for 48 h. (B) Quantification of *in vivo* extracellular trap production visualized in (A). Data are shown as average of 6 high-power field (HPF) obtained with a 10x/0.3 UPlanFCN objective. ** $P < 0.01$ by two-tailed Student's t-test.

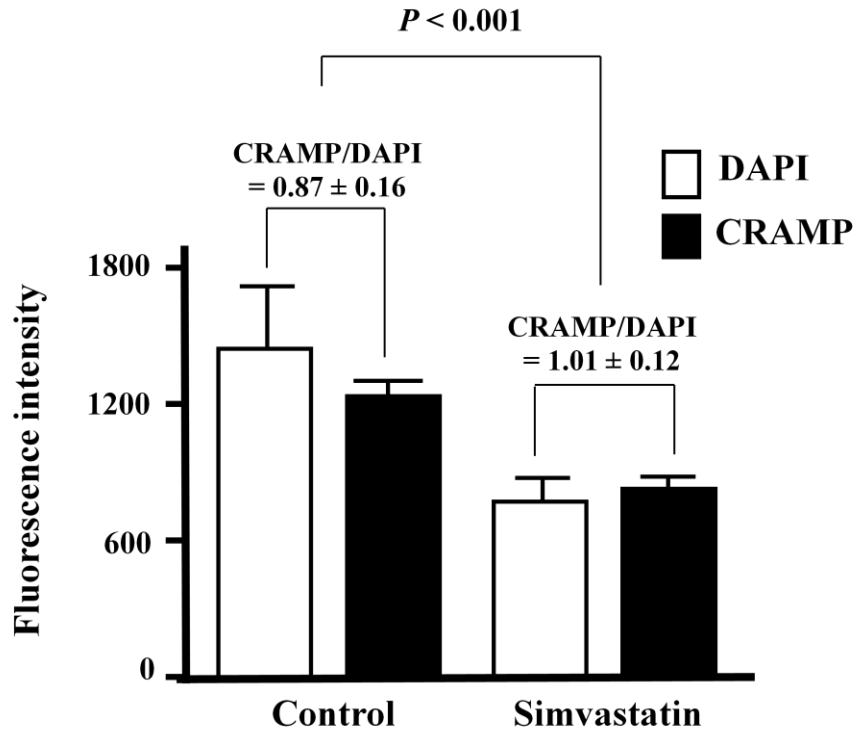


Figure 3.26 Enhanced CRAMP production in simvastatin-treated mice.

Quantification of CRAMP expression compared to total cell amount (DAPI staining) in lung tissue of mice fed with standard chow or standard chow supplemented with simvastatin and infected for 48 with *S. aureus* strain Newman. Mean fluorescence intensity was quantified using Image J 1.41 software.

drugs to boost phagocyte antibacterial function. Effective inhibition of HMG-CoA reductase by siRNA knockdown of *Hmgcr* in thioglycolate-induced macrophages (Figure 3.27A) was sufficient to enhance both their killing of *S. aureus* (Figure 3.27B) and their production of NETs (Figure 3.28), suggesting that statin treatment was not exerting an off-target effect. Moreover, addition of an excess of the downstream HMG-CoA reductase product mevalonate (1 mM) almost completely blocked the bactericidal enhancing properties of statin therapy on macrophages (Figure 3.29).

The mevalonate pathway is involved in a number of cellular processes including protein prenylation and cholesterol synthesis (Figure 3.30). A critical juncture is the processing of farnesyl pyrophosphate (FPP), which can be used in protein prenylation pathways, such as farnesylation or geranylgeranylation, or committed to the cholesterol synthetic pathway by conversion to squalene. To pinpoint which of these branch pathways mediates NET induction by statins, we performed NET quantitation assays following treatment with inhibitors against farnesyl transferase (FTI-277), geranylgeranyl transferase (GGTI-298) and squalene synthase (YM-53601). We found that YM-53601 induced NETs to a level similar to mevastatin treatment (Figure 3.31 and 3.32), whereas GGTI-298 only resulted in a very small increase, and FTI-277 had no effect (Figure 3.31). As observed with statins, treatment of neutrophils with YM-53601 resulted in enhanced clearance of *S. aureus* (Figure 3.33 and 3.34). These data suggest the effects of statins in boosting NET production and bacterial killing are mediated by intermediates of the sterol synthetic pathway.

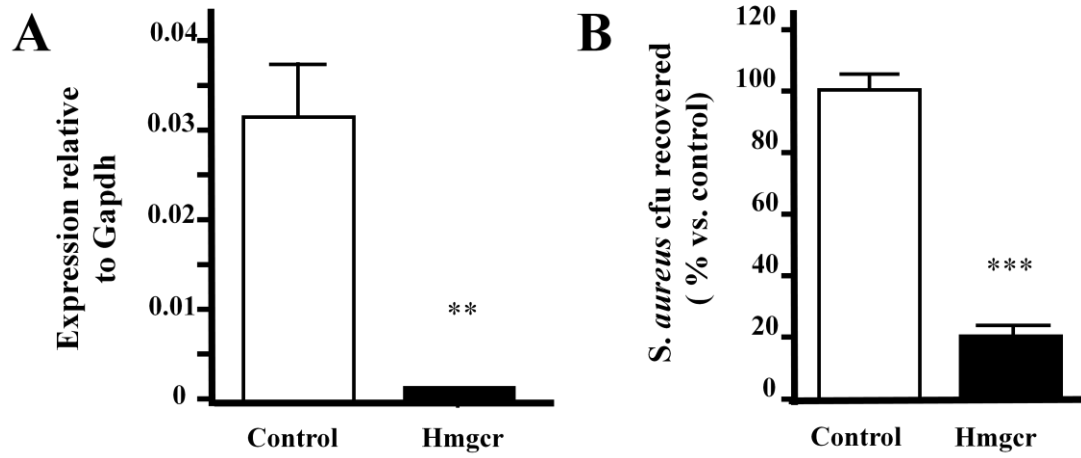


Figure 3.27 siRNA knockdown of HMG-CoA reductase enhances the anti-staphylococcal activity of macrophages. (A) Hmger transcript expression in thioglycolate-induced macrophages transfected with Hmger siRNA. (B) Killing of *S. aureus* by murine thioglycolate-induced peritoneal macrophages following transfection with Hmger or control siRNA.

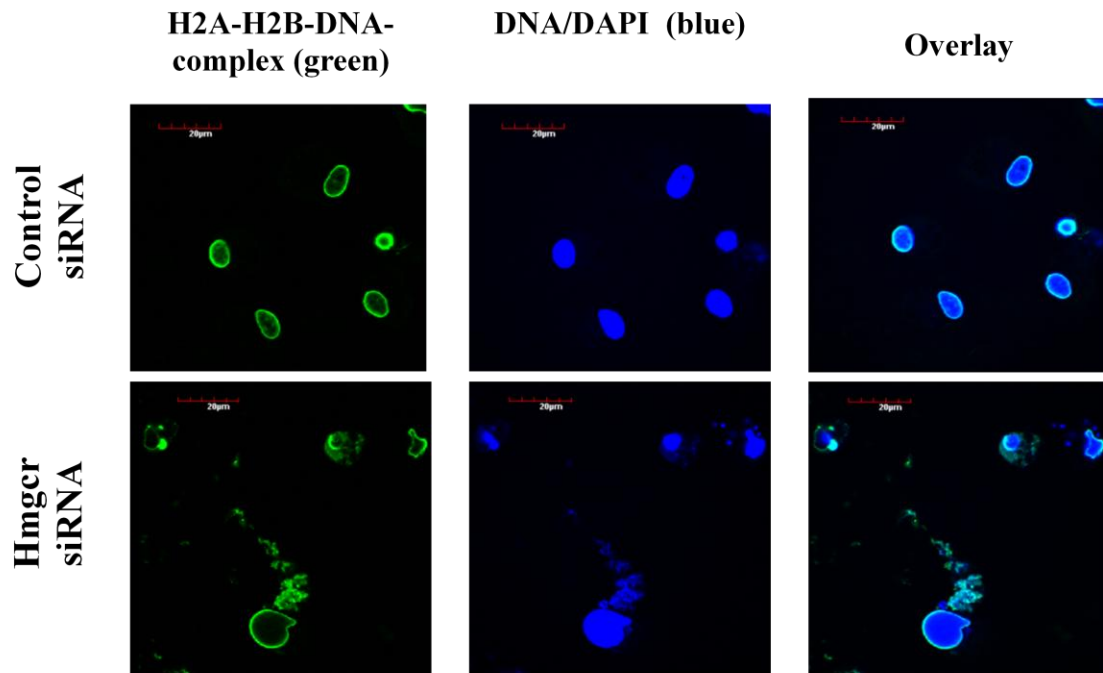


Figure 3.28 siRNA knockdown of HMG-CoA reductase enhances MET production. Representative fluorescent images of murine thioglycolate-induced peritoneal macrophages following transfection with Hmgcr or control siRNA forming extracellular traps. MET-formation was visualized in blue (Dapi) and H2A-H2B-DNA-complexes were visualized by Alexa green-immunostaining.

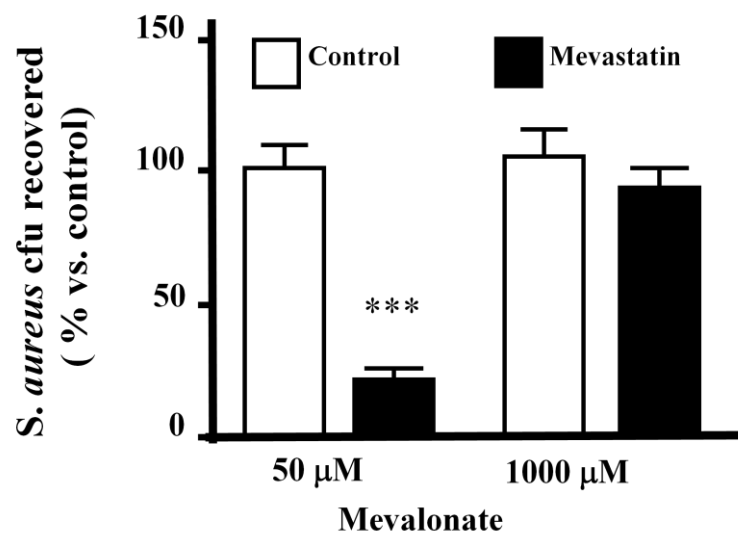


Figure 3.29 Mevalonate reverses statin-induced killing enhancement. Killing of *S. aureus* by RAW 264.7 cells following treatment with mevastatin or vehicle control \pm mevalonate. *** $P < 0.005$ by one-way ANOVA with Tukey's post test comparing control versus statin-treated group.

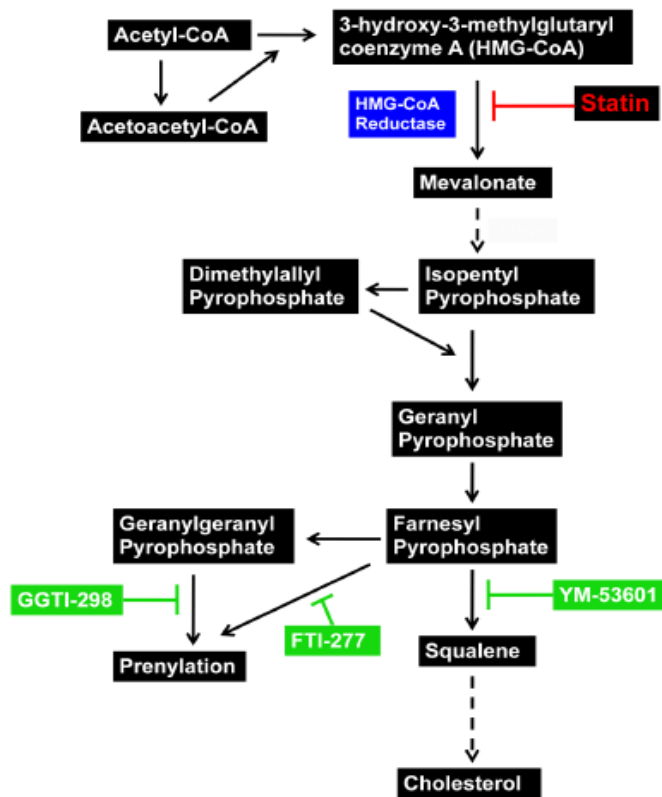


Figure 3.30 The mevalonate pathway. Diagram of the mevalonate pathway.

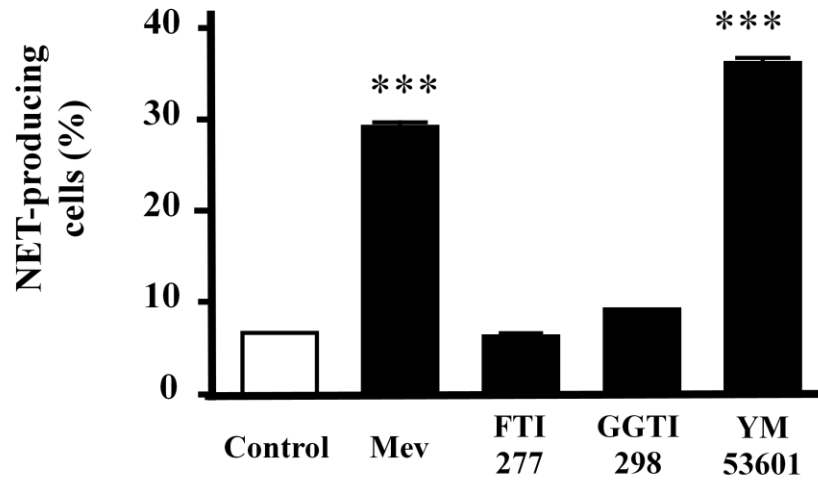


Figure 3.31 Inhibitor of sterol synthesis enhances NET production. Neutrophil extracellular trap (NET) production following treatment of primary human neutrophils with inhibitors of the mevalonate pathway. *** $P < 0.005$ by one-way ANOVA with Dunnet post test versus control group.

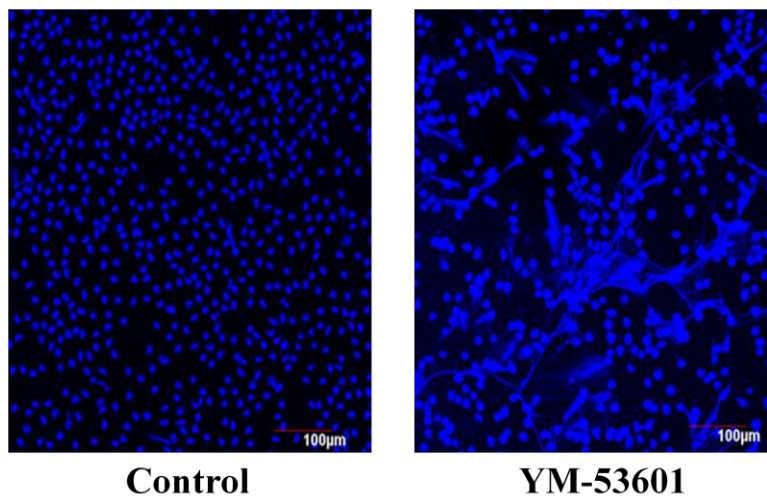


Figure 3.32 YM-53601 enhances NET production. Representative fluorescent images of NET formation by human primary neutrophils in response to YM-53601 or vehicle control treatment visualized by DAPI staining.

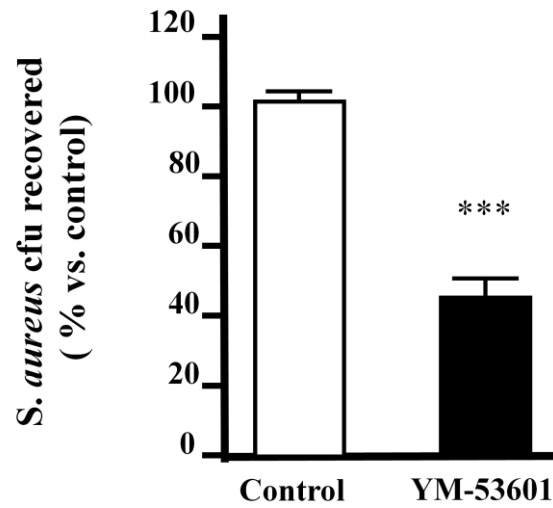


Figure 3.33 YM-53601 enhances neutrophil clearance of *S. aureus*. Killing of *S. aureus* by human neutrophils following treatment with YM-53601 or vehicle control. *** $P < 0.005$ by two-tailed Student's t-test comparing control versus statin-treated group.

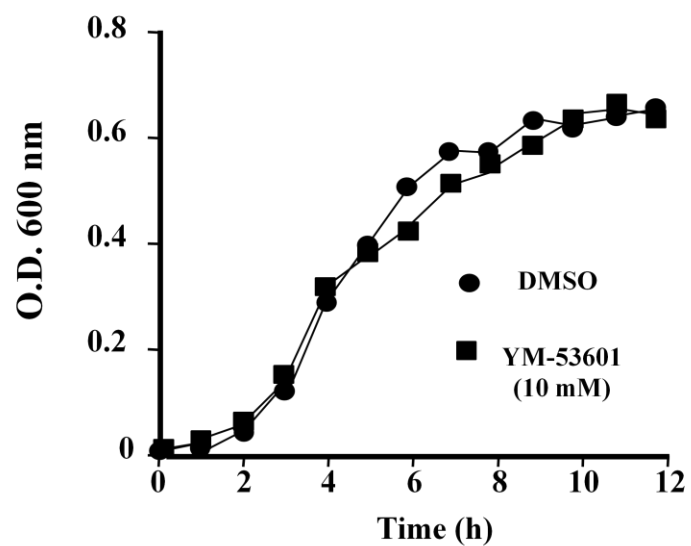


Figure 3.34 YM-53601 does not inhibit *S. aureus* growth. Growth of *S. aureus* strain Newman in RPMI containing YM-53601 (10 μ M) or DMSO vehicle control.

DISCUSSION

Our results demonstrate that statin treatment to inhibit the sterol pathway fundamentally alters the innate immune behavior of phagocytic cells. In response to the leading bacterial pathogen *S. aureus*, statins enhance formation of phagocyte ETs and promote bacterial killing. Thus we conclude that reports of protective effects of statins against severe bacterial infection may not be limited to modulation of cytokine responses, but may also involve alterations in phagocytic cell function. Our findings corroborate human clinical data and the therapeutic benefits of statins observed in mice challenged systemically with *S. aureus* or *Salmonella typhimurium* (Catron et al., 2004; Chaudhry et al., 2008); however, one report has described reduced clearance of *Klebsiella pneumoniae* from the lung in mice receiving statin treatment (Fessler et al., 2005). Further studies of the relationship between the sterol synthesis pathways and innate immune function will be important to understand potential infectious disease ramifications for millions of individuals receiving chronic statin therapy.

EXPERIMENTAL PROCEDURES

Animals

Male 10-12 week old CD1 or C57Bl/6 mice (Charles River Laboratories, CA) were used in this study. Mice were maintained under standard conditions according to institutional guidelines. All experiments were approved by the UCSD Animal Care Committee.

Bacterial Strains

The bacterial strains used in this study were *Staphylococcus aureus* strain Newman, methicillin-resistant *S. aureus* (MRSA) strain Sanger 252, *Streptococcus agalactiae* strain COH1, and *Salmonella typhimurium* strain ATCC13311. All bacteria were grown in Todd-Hewitt broth (THB) at 37°C to mid-log phase, collected by centrifugation at 9000 rcf for 10 min, washed once with PBS and diluted to the required concentration. For growth curve analysis, midlog phase *S. aureus* Newman was diluted 1:100 in RPMI medium in the presence or absence of 50µM mevastatin (Sigma), 10µM YM-53601 or vehicle control (DMSO), incubated at 37°C + 5% CO₂ and bacterial density (optical density at 600 nm) was measured hourly.

Cell Culture

Human neutrophils were purified from healthy volunteers using the PolymorphPrep™ system (Axis-Shield, Fresenius) per manufacturer's recommendations. Neutrophils were cultured at 37°C + 5% CO₂ in serum-free, antibiotic-free RPMI at 10⁶ cells/mL (500 µl per well in 24-well plate) in the presence of 50µM mevastatin (Sigma), 10 µM simvastatin (Sigma), 10µM GGTI-298 (Sigma), 10µM FTI-277 (Sigma), or 10µM YM-53601 with appropriate concentrations of vehicle control. After 1 h, cells were stimulated with 156ng/mL phorbol myristic acetate (PMA, Sigma) for an additional 1 h. RAW 264.7 murine and U937 human monocyte-macrophage cells were cultured at a density of 10⁶ cells/mL in DMEM and RPMI 1640, respectively, supplemented with 10% heat-inactivated FBS and penicillin/streptomycin (Invitrogen).

After 24 h, cells were further incubated in medium without FBS and stimulated overnight with 50 μ M statin or vehicle control in the presence of 50 μ M mevalonolactone (Sigma) to prevent apoptosis and maintain physiologic relevance.

***In Vitro* Bactericidal Assays**

Human as well as murine cells were infected with bacteria at a MOI = 1. After centrifugation for 10 min at 1,500 rpm, infected neutrophils were incubated for 20 min at 37°C in 5% CO₂. RAW 264.7 and U937 cells were incubated for 4 and 8 h, respectively. After incubation, Triton X-100 (0.06% final concentration) was added to lyse infected cells. Lysates were diluted and plated on THA plates for enumeration of surviving CFU. Percent killing by statin-treated leukocytes was determined by dividing the number of CFU recovered from statin-treated neutrophils by the number of CFU from vehicle-treated neutrophils.

Phagocytosis

For determination of phagocytosis by flow cytometry, Alexa-Fluor 488-labelled *S. aureus* wood strain bioparticles (Invitrogen) were added to cells at MOI 1. After 20 min of incubation at 37°C, PMNs were washed twice with cold HBSS (Invitrogen) and mean fluorescence intensity (phagocytosis) was measured using a FACSCaliburTM flow cytometer (BD Biosciences). For infection of RAW cells, *S. aureus* bioparticles were added at MOI = 1 and centrifuged for 10 min at 1500 rpm. After an additional 10 min of incubation at 37°C, cells were washed twice with cold PBS, detached with trypsin and washed again with cold PBS. Again, mean fluorescence intensity (phagocytosis) was

measured using flow cytometry.

Oxidative Burst

Cells were incubated with 10 μ M 2',7' dichlorofluorescein (Sigma) for 30 min at 37°C. After incubation, cells were washed twice with HBSS, and fluorescence intensity was measured using a FACSCaliburTM flow cytometer (Beckton Dickinson).

Entrapment Assay

Cells were seeded at a density of 5x10⁵ cells/mL in RPMI and treated with mevastatin or vehicle control as described above to induce formation of extracellular traps. Then, cells were infected with FITC-labelled *S. aureus* strain Newman (carboxyfluorescein, Invitrogen, 30 min at 4°C) at a MOI = 20 bacteria per cell. After centrifugation for 10 min at 1,500 rpm and an additional 5 min of incubation at 37°C, cells were gently washed twice with HBSS to remove unbound bacteria. To release bacteria from extracellular traps, PMNs were incubated in the presence of 50U/mL of DNase I for 15 min at 37°C. 100 μ L of supernatant was transferred to a 96-well plate. Relative number of bacteria in the supernatant was determined by reading the absorption/emission at 485nm/538nm in a SpectraMAX® Gemini EM fluorescence reader (Molecular Devices).

Induction and Quantification of Extracellular Traps

To induce extracellular traps (ETs) from human PMNs, PMNs were cultured in serum-free, antibiotic-free RPMI at 10⁶ cells/mL in the presence of 50 μ M mevastatin

(Sigma), 10 μ M Simvastatin (Sigma), 50 μ M Lovastatin (Sigma), 50 μ M Fluvastatin (Cayman), 10 μ M GGTI-298 (Sigma), 10 μ M FTI-277 (Sigma), or 10 μ M YM-53601 with appropriate concentrations of vehicle control. After 1 h, cells were stimulated with 156ng/mL phorbol myristic acetate (PMA, Sigma) for an additional 1h. RAW 264.7 cells were cultured at a density of 10⁵ cells/mL in DMEM and RPMI 1640, respectively, supplemented with 10% heat-inactivated FBS and penicillin/streptomycin. After 4 h, cells were stimulated overnight in medium without FCS supplemented with 50 μ M statin or vehicle control and in the presence of 50 μ M mevalonolactone (Sigma). To induce METs, 156 ng/mL PMA was added 2 h prior to analysis. An established method for NET quantification (Fuchs et al., 2007) was adapted for our purposes. 500mU/mL micrococcal nuclease (Sigma) was added to neutrophils to release ETs. After 15 min of incubation at 37°C, 0.5mM EDTA was added to stop nuclease activity, and supernatants were collected. Total genomic DNA was isolated using DNAzol supplemented with polyacryl-carrier (Molecular Research Center, Inc.) per manufacturer's instructions. DNA was quantified using the PicoGreen dsDNA quantification kit (Invitrogen). Fluorescence was measured using a SpectraMAX® Gemini EM fluorescence reader. The percentage extracellular DNA was determined by dividing the amount of ET DNA by the total DNA. For peritoneal cells, 156 ng/mL PMA was added 1 h prior to analysis. ET production was assessed using a modified version on the above ET protocol. Briefly, 500mU/mL micrococcal nuclease (Sigma) was added to neutrophils to release ETs. After 15 min of incubation at 37°C, 0.5mM EDTA was added to stop nuclease activity, and supernatants were collected. The supernatant was centrifuged for 5 minutes at 3000 rcf to remove non-adherent cells. Total genomic DNA was isolated using DNAzol supplemented with

polyacryl-carrier (Molecular Research Center, Inc.) per manufacturer's instructions. DNA was quantified using the PicoGreen dsDNA quantification kit (Invitrogen). Fluorescence was measured using a SpectraMAX® Gemini EM fluorescence reader. The percentage extracellular DNA was determined by dividing the amount of ET DNA by the total DNA.

Fluorescence Microscopy

For visualization of extracellular traps, cells were seeded on poly-L-lysine coated glass-slides (neutrophils) or on glass-bottom microtiter plates (macrophages), cell-type specifically treated with statins or vehicle control as described above, infected with *S. aureus* strain Newman at a MOI of 1, centrifuged at 800 rpm for 10 min and further incubated for 20 min. The Live/Dead viability/cytotoxicity kit for mammalian cells (Invitrogen) was used without fixation of cells to visualize NETs or METs and to determine cell viability of trap-forming cells by fluorescence microscopy. Hoechst-33342-trihydrochloride (final concentration 1 μ M) was added to the sample 5 min prior to microscopic analysis to stain DNA (blue). The Live/Dead BacLight™ Bacterial Viability Kit (Invitrogen) was used to determine viability of *S. aureus* entrapped in the METs by fluorescence microscopy. Infected cells were stained as recommended by the manufacturer, washed 3 times with PBS, fixed with 1% paraformaldehyde for 5 min, washed again and mounted onto glass slides using Prolong Gold (Invitrogen), a mounting medium that contains the DNA-staining dye Dapi (blue). For immunofluorescence staining of cathelicidin peptides (murine CRAMP or human LL-37), infected cells were fixed with 4% paraformaldehyde, washed with PBS, blocked and permeabilized in the presence of PBS + 3% BSA + 2% goat serum + 0.2% TritonX100 for 45 min. Then the

samples were washed again and incubated with polyclonal rabbit anti-CRAMP/LL-37 (1:300-diluted)(Dorschner et al., 2001) or with mouse monoclonal anti H2A-H2B-DNA complex (#PL2-6 mouse IgG2b stock:2.65 mg/ml 1:3000 diluted) in the presence of PBS+2% BSA overnight at 4°C (Losman et al., 1992). A universal rabbit IgG (Dako) or mouse IgG2b (Thermo Scientific) served as isotype negative control. After washing x 3 with PBS, samples were incubated with secondary Alexa 488 or Alexa 568-labelled goat anti rabbit/mouse IgG antibodies (1:500; Molecular Probes) for 45 min at room temperature and embedded using Prolong Gold-Dapi.

Paraffin-embedded lung tissue samples were deparafinized by immersing successively in 3 changes of xylene for 10 min each and rehydrated by immersing in decreasing concentrations of ethanol (100%, 95%, 70%, each twice for 5 min). After washing with PBS, slides were heated in a microwave for 10 min in the presence of citrate puffer (Dako) for antigen retrieval. After cooling down for 20 min, the slides were washed with PBS, and immuno-stained as described above. Mounted samples were examined using an inverted confocal laser-scanning 2-photon microscope Olympus Fluoview FV1000 with FluoviewTM Spectral Scanning technology (Olympus). Images were obtained using a 20x/0.7 or 60x/1.42 PlanApo objectives. Alternatively images were recorded using an Olympus Spinning Disc Confocal IX81 microscope with a Xenon DG5 illumination source driven by driven by SlideBook software (Intelligent Imaging Innovations). In this case, images were obtained using a 10x/0.3 UPlanFCN, 20x/0.45 LUCPlanFLN or 40x/1.0 oil UPlanApo objective. Mean fluorescence intensities of images were quantified using Image J 1.41 software.

FACS Analysis

For determination of neutrophil and macrophage content in peritoneal lavage from thioglycolate-treated mice, cells were incubated for 5 min with anti-CD32 antibodies to block Fc receptors, stained with phycoerythrin (PE)-conjugated anti-F4/80 (Serotec), fluorescein isothiocyanate (FITC) conjugated anti-Ly6G (Gr1) (BDPharmingen) or with their respective isotype control antibodies and incubated for 30 min at 4°C. Labelled cells were analyzed by flow cytometry in a FACScalibur (Becton Dickinson).

***Ex Vivo* Killing Assays**

CD1 mice were fed with chow +/- 500mg/kg simvastatin ad libitum. After 5 days of feeding, mice were injected with 3mL 3% thioglycolate solution (BD Biosciences), and peritoneal cells isolated by lavage with PBS 4 h later. Cells were treated with 1x RBC lysis buffer (eBioscience) to lyse erythrocytes, resuspended in RPMI medium, and seeded in tissue culture plates at a density of 10^6 cells/mL. Cells were immediately infected with *S. aureus* strain Newman using a multiplicity of infection (MOI) = 1 and then incubated at 37°C, 5% CO₂ for 30 min. After incubation, Triton X-100 (0.06% final concentration) was added to lyse neutrophils and lysates were plated on THA for enumeration of surviving CFU.

***Staphylococcus aureus* Pneumonia Model**

Outbred CD1 mice were fed with either pulverized chow or pulverized chow supplemented with 500mg/kg simvastatin (generic, pharmaceutical-grade from Dr. Reddy's Laboratories Ltd.) ad libitum. After 5 d of feeding, mice were anesthetized with

ketamine/xylazine intraperitoneally, then a sub-lethal dose of *S. aureus* strain Newman ($1-2 \times 10^8$ CFU in 30 μ l PBS) was introduced intranasally to induce pneumonia as previously described (Bubeck Wardenburg et al., 2007; Bubeck Wardenburg and Schneewind, 2008). Mice were sacrificed after 2 d, and left lung tissue was infused and preserved in 10% buffered formalin (Fisher Scientific) for histological analysis. Right lung tissue was homogenized for 1 min using zirconia beads (1 mm, Biospec) in a Mini-BeadbeaterTM (BioSpec Products) and plated onto THB agar (THA) plates for overnight incubation and enumeration of colony-forming units (CFU).

Lung Histology

Formalin-fixed lung tissue was embedded in paraffin, and then cut into 3- μ m-thick sections. Tissue sections were stained with hematoxylin and eosin (H&E) and then examined microscopically for pathological alterations using a Zeiss Axiolab microscope (Zeiss 10x/0.25 Achromplan, 20x/0.5 Plan-Neofluor or 40x/0.65 Achromplan objective) with an attached Sony Digital Photo 3CCD-Camera DKC-5000 at calibrated magnifications.

siRNA Transfection

To harvest thioglycolate-induced peritoneal macrophages, 12-wk old C57 Bl/6 mice were injected intraperitoneally with 3 mL of 3% thioglycolate. After 3-6 d, macrophages were harvested by peritoneal lavage with PBS and cultured in RPMI + with 10% FBS + penicillin/streptomycin. For killing assays, macrophages were cultured at 1.4×10^6 cells/mL. For extracellular trap visualization, macrophages were cultured at 5×10^5 cells/mL. Mouse Hmgcr and control SMARTpool[®] siRNAs (Dharmacon) were

transfected into macrophages using DeliverX™ transfection reagent (Panomics) per manufacturer's instructions. The next day, *S. aureus* (MOI = 1) were added and brought in close proximity to macrophages by centrifugation. After 8 h of co-incubation, macrophages were lysed with 0.06% Triton X-100 and plated onto THA plates for CFU enumeration. Percent killing by Hmgcr siRNA-transfected macrophages was determined by dividing the number of CFU recovered from Hmgcr siRNA-transfected macrophages by the number of CFU from control siRNA-transfected macrophages.

Quantitative RT-PCR

Total RNA was extracted from cells using Trizol Reagent (Invitrogen). cDNA was prepared using Superscript® III First-Strand Synthesis Supermix for qRT-PCR (Invitrogen). cDNAs were amplified in SYBR® GreenER™ qPCR SuperMix (Invitrogen) using an ABI Prism 7100 Sequence Detection System (Applied Biosystems). The primer sequences are as follows: Gapdh F- AATGTGTCCGTCGTGGATCT; Gapdh R- CATCGAAGGTGGAAGAGTGG; Hmgcr F- TCGTCATTCATTCCTCGACAAA; Hmgcr R- GATTGCCATTCCACGAGCTAT. Hmgcr primers sequences were taken from Primer Bank (<http://pga.mgh.harvard.edu/primerbank/index.html>, Primer Bank ID# 18043195a2) (Wang et al., Spandidos et al.). Cycling conditions for PCR amplifications were 15 s at 95°C, 25 s annealing at 57°C, and 45 s at 72°C. Data are expressed as relative mRNA expression levels normalized to the housekeeping gene Gapdh.

Statistics

Data were analyzed by using GraphPad Prism 4.0 (GraphPad software). Each

experiment was performed at least three times at independent occasions, and within each experiment all samples were processed in triplicate. Differences were analyzed using a Student's T-test, a one-way ANOVA or a two-tailed Mann Whitney test; P values < 0.05 were considered significant.

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Chapter IV
Conclusions and Discussion

INTRODUCTION

Sepsis is a complex, systemic inflammatory illness that occurs as a result of severe infections. It is a leading cause of admission to intensive care units and results in more than 210,000 deaths annually in the United States alone (Hotchkiss, 2009). This disease is characterized by a combination of hyper- and hypo-inflammatory responses. In its initial stages, the immune system becomes hyperactivated. Overproduction of cytokines and chemokines, also known as the cytokine storm, can lead to tissue damage, and, in extreme cases, septic shock. As the disease progresses, the anti-inflammatory response begins to dominate, leading to immunosuppression. In its compromised state, the immune system is less able to clear infections, and patients become susceptible to overwhelming infections.

A number of clinical and experimental reports have shown a correlation between reduced risk of sepsis and statin therapy (Almog, 2007; Ando, 2000; Kruger, 2006; Liappis, 2001; Martin, 2007; Merx, 2005; Merx, 2004; Pahan, 1997). The attenuation of sepsis may, in part, be attributable to the immunomodulatory effects of statins. Statin treatment reduces induction nitric oxide, pro-inflammatory cytokines and vascular leakage in experimental models of sepsis (Ando, 2000; Jacobson, 2005; Pahan, 1997). Although anti-inflammatory agents may blunt hyper-inflammatory responses, they are not sufficient to improve outcomes in septic patients and may actually lead to increased risk of secondary infection. Indeed, these results have been observed in clinical trials using corticosteroids to treat sepsis (Hotchkiss, 2003). Thus, the protective effects of statin therapy must extend beyond their anti-inflammatory effects.

We have found that statins enhance innate immune clearance of bacteria both *in*

vitro and *in vivo*, which provides an explanation as to why, unlike other anti-inflammatory agents, they attenuate sepsis. We provide the first evidence that statin therapy enhances the bactericidal activity of leukocytes. Furthermore, we demonstrated that this effect is, at least in part, mediated by the increased propensity of statin-treated leukocytes to produce extracellular traps (ETs) in response to inflammatory stimuli. Similar to other known modes of ET production, this effect is dependent on the production of reactive oxygen species (ROS) (Fuchs, 2007). We have also used statin therapy to find that macrophages produce an analogous, previously-undescribed ET. Finally, we found that the effect of statins on ET release was mediated by their ability to inhibit the sterol synthetic pathway.

Collectively, our findings add to our growing understanding of the effect of statin therapy on innate immune functions. These results also have several important therapeutic implications. Finally, our discoveries provide valuable insight the cell biological aspects of ET formation. The following discussion will review our findings in the context of current research and evaluate their possible future applications.

STATINS IN THE CONTEXT OF INNATE IMMUNE FUNCTION

The effect of statin therapy on innate immune clearance of pathogens must be considered in the context of its other immunomodulatory effects. As mentioned above, statins are thought to attenuate the inflammatory response. Thus, the surprising finding that statin therapy enhances the clearance of *S. aureus* and other bacteria has important implications for our understanding of innate immune function.

***In Vitro* Bacterial Clearance**

At present, *in vitro* bacterial clearance by leukocytes is known to be influenced by phagolysosomal killing, degranulation and ET-based killing. We and others have demonstrated that statin therapy inhibits phagocytosis, oxidative burst and degranulation (Bokoch, 1992; Fujimoto, 2009; Loike, 2004). Furthermore, Benati et al. showed that phagolysosomal killing of Group B *Streptococcus* by HL-60 neutrophils was profoundly inhibited by statin therapy (Benati, 2009). Because ETs are a relatively new discovery, their contribution to innate immune defense against pathogens is still being elucidated. Our results may give an indication of their bactericidal potency. Indeed, despite downregulation of other antimicrobial factors, we observed enhanced bacterial clearance. Part of this may be due to the release of histones during ET formation. While ETs generally contain very similar antimicrobial components relative to those used in phagolysosomal killing and degranulation, they also contain histones. As demonstrated by Brinkmann et al., blocking antibodies against histone H2A significantly reduces the ability of NETs to kill *S. aureus* (Brinkmann, 2004). Thus, the exposure of bacteria to histones may be one reason for the enhanced clearance of *S. aureus* by statin-treated cells. In addition, the ability of ETs to trap and kill bacteria, combined with their relatively large volume, gives them ability to kill many bacteria simultaneously. It is conceivable that, at high bacteria to neutrophil ratios, this property of ETs would give them a significant bactericidal advantage over phagocytosis, where bacteria must be individually cleared.

***In Vivo* Bacterial Clearance**

In vivo bacterial clearance is influenced by a wide array of events that occur during infection. In addition to enhancing the bactericidal activity of leukocytes, statin therapy also affects other facets of host pathogen defense. First, statin therapy limits the internalization of bacteria. Not only does it reduce phagocytosis, but it has also been shown to limit the susceptibility of endothelial cells to invasion by *S. aureus* and *Streptococcus pneumonia* (Horn, 2008; Rosch 2010). This would increase the number of extracellular bacteria and place greater importance on ET-mediated killing. In this sense, statin therapy shifts bacterial clearance from primarily intracellular to extracellular killing, similar to what is seen in vitro. In addition to their bactericidal activity, ETs also function to trap bacteria. This would likely limit bacterial dissemination, leading to greater localization of infections.

The anti-inflammatory effects of statin therapy has important implications for the formation of ETs. During an infection, cytokines, chemokines and bacterial components play a role in recruiting leukocytes to the site of infection. Statin therapy has also been shown to reduce neutrophil infiltration into a site of inflammation, which would reduce the number of cells at the site of infection that are available to produce ETs (Cowled, 2007). Therefore, it is somewhat surprising that, given these effects, statin therapy also enhances bacterial clearance. This may suggest that ETs are especially effective in clearance of bacteria. It is also possible that statin therapy simultaneously enhances other innate immune mechanisms, which, in collaboration with ETs, leads to enhanced bacterial clearance. In any case, further studies will be necessary to fully understand the full impact of statin therapy on bacterial clearance.

Statins have also been shown to inhibit production interleukin-8 (IL-8) and

inducible nitric oxide synthase (iNOS)- both of which have been shown to stimulate ETs release (Mantuano, 2007; Pahan, 1997; Rezaie-Majd, 2002). As such, one might think that, together, these two factors would significantly reduce the number of ETs produced in statin-treated cells. However, since we observed enhanced ET production in statin-treated mice, the ET-promoting effect of statin therapy is probably more than sufficient to compensate for these inhibitory factors.

The Effect of Statin Therapy on Clearance of Other Pathogens

Our findings corroborate human clinical data relating to sepsis as well as a report from Chaudry et al. showing the enhanced bacterial clearance in statin-treated mice challenged systemically with *S. aureus* or *Salmonella typhimurium* (Chaudhry, 2008). However, a report from Scott Worthen's group described reduced clearance of *Klebsiella pneumoniae* from the lungs in mice receiving lovastatin treatment (Fessler, 2005). One possible explanation for the discrepancy is that statin has differing effects on the clearance of different classes of pathogens. Should this prove to be correct, it would also suggest that the same is true for ETs. It is becoming increasingly clear that ETs do not affect all pathogens equally. On one hand, different classes of pathogens are killed by different ET components. For example, histone H2A does not kill *Candida albicans*, although it has potent anti-bacterial and anti-parasitic effects (Brinkmann, 2004; Guimaraes-Costa, 2009; Urban, 2009). On the other hand, numerous pathogenic bacteria have developed virulence mechanisms, such as DNases, that protect them from ET entrapment and clearance (Beiter, 2006; Buchanan, 2006). Thus, it is possible that effect of statin therapy on clearance of *K. pneumoniae* was the result of an inherent resistance

of this bacterium to ET-mediated clearance.

Statins and Antigen Presentation

The effects of statin therapy on innate immune function may have important implications for the development of adaptive immunity. Antigen presentation is one of the key functions of the innate immune system. After internalization of pathogens, certain phagocytic cells, such as dendritic cells, macrophages and neutrophils, have the ability to process their antigens and present them to T-cells via major histocompatibility complexes (MHCs), thus stimulating the adaptive immune response. The downregulation of phagocytosis by statin therapy would likely limit this process. In addition, ETosis likely inhibits the ability of cells to signal to T-cells, as it results in the death and lysis of cells. However, this has not yet been definitively proven. Corroborating these hypotheses, several groups have reported that statin therapy inhibits antigen presentation via inhibition of MHC II and CD1d activity (Khan, 2009; Kwak, 2000). Therefore, while statin therapy enhances innate immune clearance of bacteria, it may also have detrimental effects on the development of adaptive immunity.

THERAPEUTIC IMPLICATIONS

Statins are amongst the most widely-used chronic medications in the world. At present, it is generally use primarily for the treatment of hyperlipidemia. However, our findings suggest that statin therapy may have broader applications in the context of non-metabolic diseases. Furthermore, our understanding of statin therapy may allow us to

develop novel, more effective strategies for combating infectious diseases. Finally, the effect of statin therapy on ET production may give us insight into the potential negative consequences of statin usage.

Statin Therapy as an Innate Immune Enhancer

The finding that statins enhance innate immune clearance of bacteria has important implications for the development of novel therapeutic compounds. Antibiotic-resistant bacteria are becoming an increasingly important problem in both community and hospital settings. Methicillin-resistant *S. aureus* (MRSA) may account for >60% of hospital *S. aureus*, and it is estimated to have caused >18,000 deaths in the US alone in 2005 (Klebens, 2006; Klebens, 2007). In addition to *S. aureus*, other bacteria, such as *Neisseria gonorrhoeae* and *Acinetobacter baumannii*, are beginning to develop resistance to multiple antibiotic treatments (Livermore, 2009). Given the speed at which antibiotic resistance evolves, development of alternative treatment strategies is imperative. One possible treatment strategy would be boost innate immune clearance of bacteria. As we have demonstrated, statins could potentially be used in this context. However, as previously mentioned, the immunomodulatory effects of statin therapy may be detrimental to the clearance of certain types of organisms. As such, more research is needed to determine the spectrum of pathogens against which statin therapy would be effective.

The Development of Novel Therapeutic Strategies

Our understanding of statin therapy may also allow us to develop novel,

pathogen-specific therapies. Staphyloxanthin, a squalene derivative, protects *S. aureus* from oxidative damage in the lysosome (Liu, 2005). Liu et al showed that certain bisphosphonate (BPH) compounds can inhibit *S. aureus* squalene synthase, leading to disruption of staphyloxanthin production (Liu, 2008). Unpublished data from Eric Oldfield's laboratory at the University of Illinois Champagne-Urbana, suggest that some BPH inhibitors of *S. aureus* squalene synthase may also inhibit human squalene synthase. Furthermore, we that inhibition of human squalene synthase with YM-53601 enhanced ET production and bacterial clearance by human neutrophils. Thus, we reasoned that a dual-specificity squalene synthase inhibitor could potentially be used as a dual anti-staphylococcal antibiotic and innate immune booster. In support of this theory, we found that BPH-1102A, a novel BPH compound, has the unique ability to inhibit staphyloxanthin while enhancing ET production in PMA-activated primary human neutrophils (Figure 4.1 and 4.2). This example illustrates one of many possible applications of statin therapy to development novel therapeutics.

Statins and Autoimmunity

The finding that statin therapy enhances extracellular trap production may also have important implications for autoimmune disease, particularly systemic lupus erythematosus (SLE) and small vessel vasculitis (SVV). SLE is an autoimmune disorder that is usually characterized by the presence of high titers of anti-nuclear antibodies (ANAs). In their initial description of NETs, Brinkmann et al. suggested that they play a role in the pathogenesis of SLE, as the release of chromatin into circulation would elicit an immune response from ANAs (Brinkmann, 2004). We would thus expect that statin

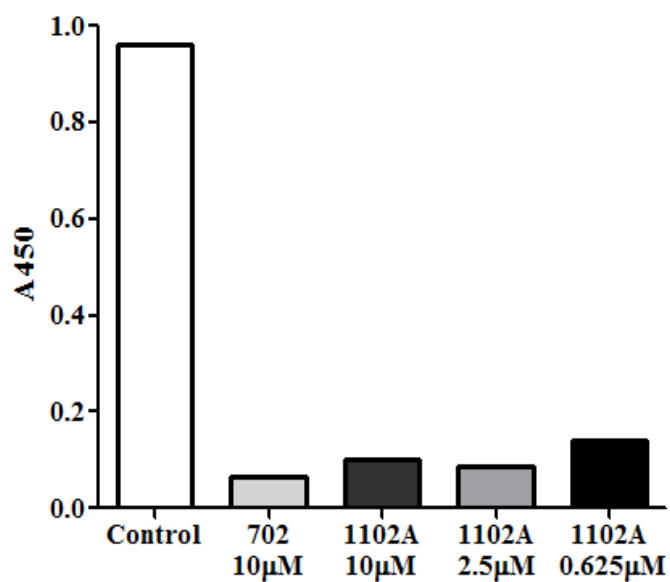


Figure 4.1 BPH-1102A inhibits *S. aureus* staphyloxanthin production. Quantification of staphyloxanthin production by *S. aureus* Newman strain after treatment with BPH-702 – a known staphyloxanthin inhibitor, BPH-1102A or vehicle control.

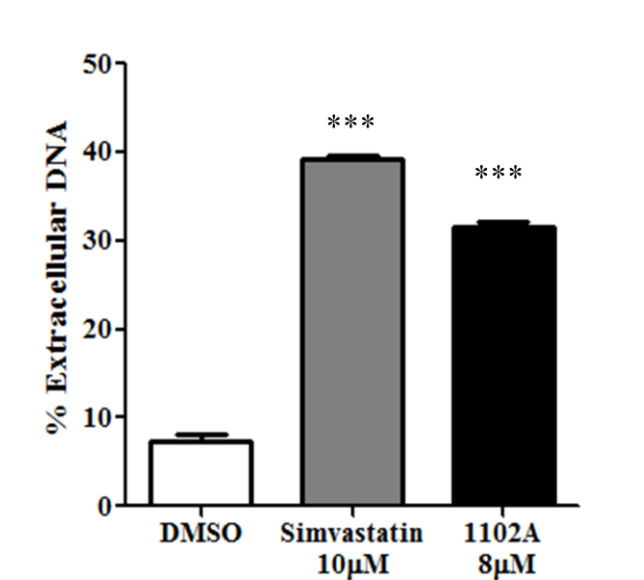


Figure 4.2 BPH-1102A enhances NET production. Quantification of NET-production by primary human neutrophils after stimulation with PMA and treatment with simvastatin, BPH-1102A or vehicle control. *** $P < 0.005$ by one-way ANOVA with Dunnet's post test comparing to vehicle control group.

therapy affects the pathogenesis of SLE. To date, no large-scale studies have linked statin usage to the development of SLE. Furthermore, several studies have tested statin therapy as a possible treatment for SLE, none of which reported worsening of SLE symptoms in response to statin therapy (Costenbader, 2007; de Kruif, 2009; Ferreira, 2007; Graham, 2008). However, there have been numerous case reports documenting the development of drug-induced SLE following statin therapy (Noel, 2007). It is possible that there are numerous factors that influence the effect of statin therapy on the development of SLE. For example, as we showed, statins require a strong inflammatory activator in order to induce ETs. It is possible that statin-induced ETs contribute in some way to the development or pathogenesis of SLE, but only in certain situations, such as during bacterial infections. As such, the relationship between ET production and SLE must be investigated in greater detail.

SVV is an autoimmune disease that has been linked to the production of antineutrophil cytoplasm autoantibodies (ANCA). Kessenbrock et al. demonstrated that ANCA stimulate neutrophils to produce NETs, suggesting a possible positive feedback loop, with ANCA stimulating NET release and neutrophil cytoplasmic components released during NETosis stimulating a further immune reaction (Kessenbrock, 2009). Currently, there is no definitive evidence linking the development of SVV to statins. However, given the effect of statins on NET production, this is an area that warrants further investigation.

STATINS AND THE CELL BIOLOGY OF ET PRODUCTION

At present, the molecular mechanisms underlying ET production are not well

understood. One of the key factors limiting progress is the pathway redundancy of ET stimuli. Indeed, the majority of known ET inducers are inflammatory activators (von Kockritz-Blickwede, 2009). The discovery that statins enhance ET production is the first to implicate a relationship between the sterol synthetic pathway and ET production. By understanding how inhibition of sterol synthesis leads to ET production, we can gain valuable insight into the mechanistic underpinnings of ET release. Furthermore, the ET-stimulating properties of statins makes them potential tools with which new aspects of ET production can be probed.

Sterol Mediators of Statin Therapy

In order to fully understand the mechanism by which statin therapy induces ETs, the sterol mediator whose inhibition leads to ET production must be identified. Preliminary data suggests cholesterol or a cholesterol derivative, at least in part, mediates statin-induced ET formation. We found that inhibition of 24-dehydrocholesterol reductase (Dhcr24), the enzyme responsible for the final step in cholesterol synthesis, with triparanol is sufficient to induce NET production (Figure 4.3). To confirm that cholesterol inhibition leads to statin-induced NET production, we used methyl- β -cyclodextrin (M β CD), a sugar that binds cholesterol. When added to cells, M β CD removes cholesterol from membranes (Figure 4.4). However, M β CD complexed with cholesterol, which is also known as water-soluble cholesterol, can deliver cholesterol to membranes. We found that water-soluble cholesterol was sufficient to inhibit triparanol-induced NETs (Figure 4.3). Collectively, these data demonstrate that inhibition of cholesterol, or a downstream derivative of cholesterol induces NETs.

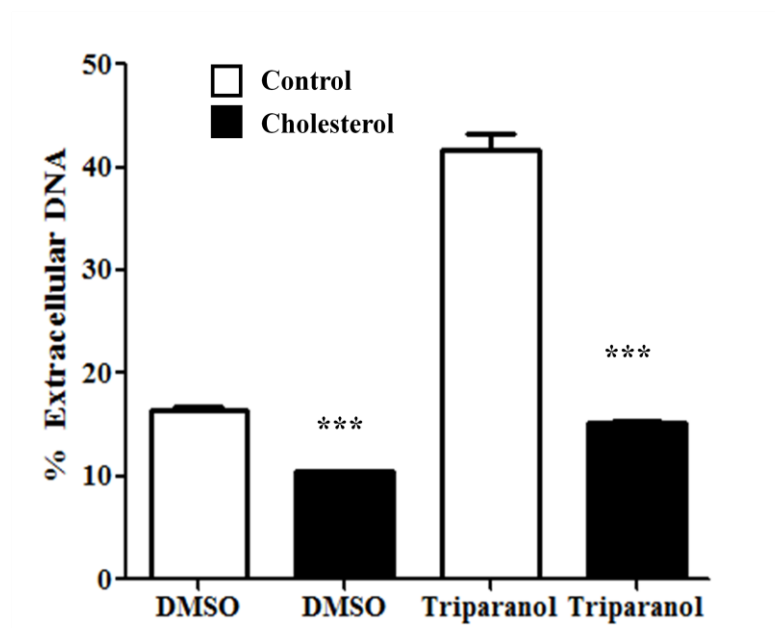


Figure 4.3 Cholesterol suppresses triparanol-induced NET production. NET production of PMA-stimulated human neutrophils treated with triparanol or vehicle control and 10 $\mu\text{g/ml}$ water soluble cholesterol or vehicle control. *** $P < 0.005$ by one-way ANOVA with Tukey's post test comparing control versus cholesterol-treated group.

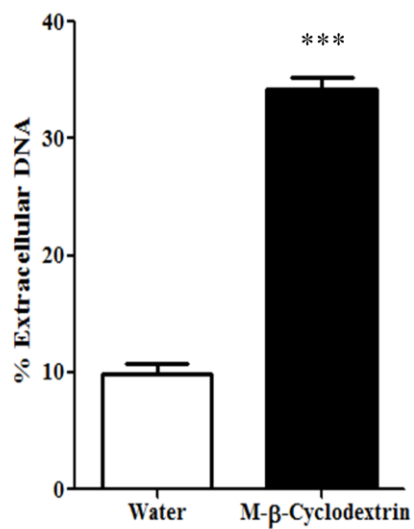


Figure 4.4 Methyl-beta-cyclodextrin enhances NET production. Quantification of NET-production by primary human neutrophils after treatment with methyl-beta-cyclodextrin. *** $P < 0.005$ by two-tailed Student's t-test comparing control versus statin-treated group.

However, it has been reported that granulocytes do not produce cholesterol (Shechter, 1980; Yachnin, 1984). In particular, Shechter et al. showed that treatment of primary human granulocytes with [3H]-labeled squalene resulted in production of [3H]-labeled lanosterol, but not cholesterol (Shechter, 1980). It is possible that these findings may have resulted from the use of unactivated neutrophils. As demonstrated by the Lipid MAPs mass consortium, macrophage production of cholesterol is strongly induced following stimulation. Consistent with this, we found that mevastatin induced ET production only in PMA-activated neutrophils. Thus, further studies must be conducted to determine whether or not neutrophils are able to produce cholesterol during inflammation.

The Relationship to ROS

Statin enhancement of ET production is dependent on the production of ROS, as evidenced by the inhibitory effect of DPI treatment of statin-induced NET production. One possible explanation for the relationship between ROS generation and sterol synthesis is that sterols act as antioxidants. As previously mentioned, Liu et al. demonstrated that *Staphylococcus aureus* produces staphyloxanthin, a carotenoid derived from squalene, to protect itself from the ROS it encounters in leukocyte phagolysosomes (Liu, 2005). If sterols have an analogous mechanism in leukocytes, then inhibition of sterol synthesis would render leukocytes more sensitive to ROS-mediated ET induction.

Work from the Lipid MAPS consortium has shown an increase in sterol synthesis in macrophages following stimulation with lipopolysaccharide. Thus, if sterols act as antioxidants, sterol synthesis following an inflammatory stimulus would prevent ET

production. In this sense, one might hypothesize that sterol production is counterbalanced with ROS during inflammation. In response to an inflammatory stimulus ROS and sterol production are both increased. Initially, the increase in sterols is able to prevent ROS-induced ET production. However, ROS production eventually overwhelms the antioxidant effect of sterols, leading to ET production. Should this hypothesis prove true, it would have important implications not only for infectious disease, but also for metabolic syndromes with inflammatory components, such as atherosclerosis and type II diabetes mellitus.

ETosis vs. Mitochondrial Release

In our studies, we did not specifically examine the effect of statin therapy on mitochondria-derived ETs. Similar to ETosis, mitochondrial release is dependent on the production of ROS. As statin therapy appears to increase leukocyte sensitivity to ROS, we would expect that it would also lead to greater production of mitochondria-derived ETs. In macrophages, Mammalian Live/Dead staining showed that statin-induced METs are associated with dead cells, suggesting that they are produced as a result of ETosis. However, it is unclear whether or not phorbol myristate acetate (PMA), the inflammatory stimulus used in our methods, is sufficient to induce mitochondrial release. Indeed, work from Yousefi et al. showed that neutrophils and eosinophils must be primed with GM-CSF, $\text{Ifn-}\gamma$ or $\text{Ifn-}\alpha$ before they release mitochondria in response to an inflammatory stimulus (Yousefi, 2009). As such, further studies are required to determine if statin therapy also enhances release of mitochondria-derived ETs in neutrophils. Furthermore, it must also be determined whether or not macrophages also have the ability to release their

mitochondria.

Histone Hypercitrullination

Histone hypercitrullination is thought to play a critical role in ET formation. This event is mediated by the enzyme PAD4, and leads to unwinding of DNA from histones (Wang, 2009). We are the first to report the elaboration of ETs independent of histone hypercitrullination. Not only did we not observe enhanced hypercitrullination of histones during statin-induced ETs, but cl-amidine, a PAD4 inhibitor, did not inhibit statin-induced NET production. Our results suggest that there are multiple mechanisms by which DNA can be unwound from histones during NET release. Thus, by understanding the mechanism by which statin therapy circumvents the need for histone hypercitrullination in ET formation, we may be able to find new pathways through which ETs can be generated.

ETs in Other Cell Types

We are the first to demonstrate the generation of macrophage extracellular traps (METs). Interestingly, Fuchs et al. reported that peripheral blood mononuclear cells did not release ETs (Fuchs, 2007). Our ability to visualize these structures was likely predicated upon two key factors. First, we did not use serum in our assays. As we have demonstrated, human, mouse and fetal calf serums contain heat-stable nucleases that are capable of degrading ETs. As METs are generated much more slowly than NETs, nucleases have more time to degrade them and confound their visualization. In addition to using serum-free conditions, our use of statins also facilitated our ability to visualize

METs. Because statin treatment strongly increased the frequency of MET generation, we were able to identify them even in conditions where they were not produced by untreated macrophages. Thus, the combination of culture conditions and stimulus allowed us to identify an ET that had eluded others. It is therefore likely that similar conditions could allow us to identify new types of ETs made by other cell types.

CONCLUDING REMARKS

We have demonstrated a novel effect of statin therapy on the enhancement of bacterial clearance through the production of ETs. This is particularly important in that it resolves the paradoxical findings that statin therapy simultaneously attenuates sepsis while reducing markers of inflammation. The fact that bacterial clearance is enhanced despite downregulation of “classical” effectors of innate immunity gives important clues both about the bactericidal potency of ETs and the potential strategies with which bacterial infections can be cleared. However, statin therapy may not be beneficial for every type of infection, as evidenced by its effect on clearance of *K. pneumoniae*. Furthermore, statins may have detrimental effects on other aspects of immunity, such as stimulation of T-cells. Thus, more work is necessary to determine the breadth of situations in which statins may be beneficial.

Our discoveries also have important implications for the therapeutic value of statin therapy. On one hand, its innate immune boosting qualities may allow it to circumvent many of the current problems associated with antibiotic-resistant bacteria. On the other hand, our understanding of statin therapy may help us develop more effective

therapies for infection, as evidenced by our studies with BPH-1102A. Despite their wide-ranging therapeutic benefits, our finding that statin therapy enhances ET formation may also implicate possible links between statins and autoimmune diseases, particularly those involving ANAs and ANCAs. However, as these relationships are currently speculative, more research is necessary to determine the extent to which they are accurate.

In addition to their potential therapeutic implications, statins represent new tools with which ET formation and leukocyte function can be probed. We are the first to report a link between ET formation and the sterol synthetic pathway, and we have used this information to implicate the inhibition of cholesterol synthesis in ET production. Not only is this important for understanding the basic biology of ET release, but it also provides new insights into neutrophil biology, countering previous reports suggesting that granulocytes do not synthesize cholesterol. Sterol inhibition may act to sensitize leukocytes to ROS-induced ETs. However, unlike other known forms of ET production, this process is not dependent on histone hypercitrullination, suggesting a possible alternative pathway for generate ETs. Finally, using a unique combination of cell culture conditions and statin treatment, we were able to demonstrate the existence of METs, a previously-undescribed type of ET. Thus, by capitalizing on the ET-inducing effects of statin therapy, we were able gain important insight into the basic biology ET and leukocyte biology.

In sum, our results provide a novel mechanistic link between metabolism and innate immune function. Not only do our findings have important ramifications ranging from basic cell biology to clinical medicine, but the breadth of these implications exemplifies the complex and integrated relationship between innate immunity and other

biological systems. Indeed, this work impacts and was influenced by many “distinct” fields of biology, including, but not limited to, lipid biology, regulation of cell death and host-pathogen interactions. Thus, as we continue to learn about the innate immune system, we must consider biological questions not only from a purely immunological standpoint, but also in the context of the multitude of interacting systems that innervate to create, modify and regulate immune responses.

EXPERIMENTAL PROCEDURES

Cell Culture

Human neutrophils were purified from healthy volunteers using the PolymorphPrep™ system (Axis-Shield, Fresenius) per manufacturer’s recommendations. Neutrophils were cultured at 37°C + 5% CO₂ in serum-free, antibiotic-free RPMI at 10⁶ cells/mL (500 µl per well in 24-well plate).

Pigment Assay

For assay of pigment inhibition, *S. aureus* strain Newman was grown to mid-logarithmic phase (5 x 10⁷ CFU/ml) in THB. The bacteria were then added at a final concentration of 2.5 x 10⁴ CFU/ml to 1 ml of THB containing the test compounds in 5 ml polystyrene tubes (B-D Falcon). The tubes were incubated in a shaking incubator at 37°C for 24 h, and the bacteria were then pelleted and washed once in phosphate-buffered saline. The pigment was extracted from the washed pellets by addition of 100 µl of methanol and incubation at 42°C for 1 h. The supernatants containing the extracted

staphyloxanthin pigment were assayed at OD_{450nm} in a Perkin-Elmer MBA 2000 spectrophotometer (Norwalk, CA). The BPH-702 control compound was a kind gift of Eric Oldfield (University of Illinois, Urbana, IL).

Induction and Quantification of Extracellular Traps

To induce extracellular traps (ETs) from human PMNs, PMNs were cultured in serum-free, antibiotic-free RPMI at 10⁶ cells/mL in the presence of 15uM BPH-1102A, 10μM triparanol (Sigma), 20uM methyl-β-cyclodextrin (Sigma) and/or 10μg/mL water-soluble cholesterol (Sigma) with appropriate concentrations of vehicle control. After 1 h, some cells were stimulated with 156ng/mL phorbol myristic acetate (Sigma) for an additional 1h. An established method for NET quantification (Fuchs et al., 2007) was adapted for our purposes. 500mU/mL micrococcal nuclease (Sigma) was added to neutrophils to release ETs. After 15 min of incubation at 37°C, 0.5mM EDTA was added to stop nuclease activity, and supernatants were collected. Total genomic DNA was isolated using DNAzol supplemented with polyacryl-carrier (Molecular Research Center, Inc.) per manufacturer's instructions. DNA was quantified using the PicoGreen dsDNA quantification kit (Invitrogen). Fluorescence was measured using a SpectraMAX® Gemini EM fluorescence reader. The percentage extracellular DNA was determined by dividing the amount of ET DNA by the total DNA.

Statistics

Data were analyzed by using GraphPad Prism 4.0 (GraphPad software). Each

experiment was performed at least three times at independent occasions, and within each experiment all samples were processed in triplicate. Differences were analyzed using a Student's T-test or one-way ANOVA; P values < 0.05 were considered significant.

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