SIMVASTATIN AND ML 141 INHIBIT ENDOTHELIAL HOST CELL PROCESSES TO LIMIT THE INVASION OF STREPTOCOCCUS PYOGENES

A THESIS

SUBMITTED TO THE GRADUATE SCHOOL

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE

MASTER OF SCIENCE

BY

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BALL STATE UNIVERSITY

MUNCIE, INDIANA

JULY 2014
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BALL STATE UNIVERSITY
MUNCIE, INDIANA
JULY 2014
ABSTRACT

THESIS: Simvastatin and ML 141 Inhibit Endothelial Host Cell Processes to Limit the Invasion of *Streptococcus pyogenes*

STUDENT: Lindy M. Caffo

DEGREE: Master of Science

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*Streptococcus pyogenes* is a human pathogen that can manipulate host cell machinery in human umbilical vein endothelial cells (HUVEC), exploiting host cell endocytosis to become an intracellular pathogen. It is postulated that deep tissue infection is initiated by bacterial migration across the endothelial cell barrier. Deep tissue invasive *S. pyogenes* infection has been associated with persistent infections that potentially lead to sepsis and necrotizing fasciitis. Simvastatin, a common cholesterol-lowering drug, inhibits localization of Rho GTPase isoforms that regulate host cell endocytosis. We performed bioassays to examine how simvastatin may influence *S. pyogenes* invasion in HUVEC. Simvastatin treatment decreased *S. pyogenes* invasion 87±12%.

Host cell actin stress fibers are typically disassembled in response to *S. pyogenes* infection; however, simvastatin treatment decreased actin stress fiber disassembly by 12% compared to control. Simvastatin decreased HUVEC binding to fibronectin by 47±1% compared to treatments lacking simvastatin treatment. *S. pyogenes* uses fibronectin for invasion of host cells. Because these cellular processes are regulated by CDC42, we addressed if specific inhibition of CDC42 would limit *S. pyogenes* invasion. CDC42 activation is inhibited with a newly-characterized target inhibitor, ML 141. Compared to control groups, ML 141 reduced *S. pyogenes* HUVEC invasion by 85±3% and host cell actin depolymerization by 9%. Overall, simvastatin and ML
141 treatments inhibit *S. pyogenes* invasion of HUVEC. Additional human pathogens, including *Staphylococcus aureus* and *Neisseria gonorrhoeae*, use the fibronectin-binding mechanism to invade host cells; our work may provide a future defense strategy against multiple invasive pathogens.
ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

3-hydroxy-3-methylglutaryl-coenzyme (HMG-CoA)

4-[3-(4-methoxyphenyl)-5-phenyl-3,4-dihydropyrazol-2-yl]benzenesulfonamide (ML 141)

Bovine serum albumin (BSA)

Cell division cycle (CDC) 42

Colony forming units (CFU)

Dimethyl sulfoxide (DMSO)

Fetal bovine serum (FBS)

Guanosine diphosphate (GDP)

Guanosine triphosphatases (GTPases)

Guanosine triphosphate (GTP)

Human umbilical vein endothelial cells (HUVEC)

Inhibitory concentration (IC)_{50}

Lipoteichoic acid (LTA)

Multiplicity of infection (MOI)

Phosphate buffered saline (PBS)

Phosphatidylinositol 3-kinase (PI3K)
Room temperature (RT)

*Staphylococcus aureus* (*S. aureus*)

*Streptococcus pyogenes* (*S. pyogenes*)

Todd Hewitt broth (THB)

Transmission electron microscopy (TEM)
INTRODUCTION

*Streptococcus pyogenes* is a Gram-positive bacterium that colonizes human tissue (1). Invasion by group A *S. pyogenes* has been characterized in epithelial tissue as a causative agent in mild infections of the skin and upper respiratory tract (2). Recently, it has been hypothesized that transient bacteremia is associated with *S. pyogenes* spread to deep tissue from colonies originating in the oropharynx (3). Though deep tissue infection represents a small number of confirmed cases (4), *S. pyogenes* with access to deep tissue may evade antibiotic treatment and persist (1). Once *S. pyogenes* populations are established within host cells, antibiotics associated with *S. pyogenes* clearance, including β-lactams and penicillin, do not reach bactericidal concentrations. Deep tissue invasive infection by *S. pyogenes* has been associated with a wide range of severe health issues, including sepsis and necrotizing fasciitis, with greater than 50% mortality rates. The ability of *S. pyogenes* to migrate through tissue barriers, establish populations in intracellular niches, and potentially evade antibiotic treatment are aspects of pathogenicity that await the development of effective therapeutic treatments.

Host cell invasion by *S. pyogenes* begins with adherence of bacteria to the host cell (1). *S. pyogenes* adhesion to epithelial and endothelial cells is attributed to the wide range of bacterial adhesins and corresponding host cell ligands (5). *S. pyogenes* have at least 11 known surface proteins associated with host cell adhesion (6). The most widely understood and characterized adhesion proteins that bind fibronectin, a host glycoprotein, are prtF1/SfbI and M1 (7). The *S. pyogenes* strain 90-226 examined in our work has the M1 protein but lacks the allelic variant of SfbI, prtF1 (8). In the absence of prtF1 on *S. pyogenes* surface, M1 protein is characterized as the major invasin. More than 150 M protein serotypes are encoded by *emm* genes (9). Serotypes M1 and M3 are isolated from clinical specimens in more than 50% of *S. pyogenes* severe invasive
infection cases (3). Understanding the roles of adhesins and invasins from clinical isolates that promote host cell invasion is important to limit disease progression to deep tissue.

Host cell invasion by *S. pyogenes* has only recently been characterized in endothelial cells (10). Endothelial cell invasion represents a tissue barrier that may permit bacterial access to otherwise sterile locations (11). The M1 protein on 90-226 *S. pyogenes* surface initiates a phagocytic-like uptake into endothelial cells (10). Host cell binding to the integrin ligand, fibronectin, has been identified as a main facilitator of *S. pyogenes* internalization (8). The M1 protein facilitates a complex formation of bacterial-bound fibronectin creating a bridge with the host cell integrin α5β1 (11). Fibronectin possesses a tripeptide RGD sequence that has an affinity for binding aggregated α5β1 (12). Disassembly of actin stress fibers enables actin monomers to reorganize at the plasma membrane and engulf *S. pyogenes*-fibronectin-α5β1 complexes through zipper-like uptake (1). Internalization is accompanied by the formation of membrane protrusions by F-actin that tightly engulf *S. pyogenes* (10). Once internalized, Ochel et al. demonstrated that some *S. pyogenes*-containing vacuoles are targeted for degradation by the late-endosome, with some *S. pyogenes* populations escaping the phagolysomes. Persisting *S. pyogenes* reside and replicate in the intact cytosol, potentially protected from host defense mechanisms.

Molecular pathways associated with endocytosis may serve as important targets to limit *S. pyogenes* invasion. *S. pyogenes* M1 protein-mediated invasion is mediated through the phosphatidylinositol 3-kinase (PI3K) pathway (13). PI3K drives signaling specific to internalization that follows adherence by *S. pyogenes* to the host cell. The M1 protein on *S. pyogenes* induces cytoskeletal changes that are dependent on the PI3K pathway. M1 activates the
lipid kinase signaling cascade when fibronectin binds to α5β1. The p110 subunit of PI3K is recruited to the plasma membrane for activation that is dependent on the p85 regulatory subunit and adaptor molecules. Purushothaman et al. found the constitutively active p110 subunit enhances invasion of the 90-226 M1 strain. Binding of effector molecules results in a cascade that leads to actin polymerization, facilitating S. pyogenes invasion. Additionally, guanosine triphosphate (GTP)-loaded cell division cycle (CDC)42, a small guanosine triphosphatase (GTPase) of the Rho family, regulates actin dynamics and promotes the disassembly of actin stress fibers (14). Host CDC42 may be a central regulator of mechanisms exploited by S. pyogenes to invade endothelial cells. Potentially, small-molecule inhibition of host cell processes regulated by CDC42 may limit bacterial progression into host cells and deep tissue.

In this work we have explored two different small-molecule inhibitors of CDC42. Simvastatin, a common cholesterol-lowering drug, non-specifically inhibits CDC42 by decreasing synthesis of isoprenoid intermediates of the cholesterol biosynthesis pathway (15). Statins lower cholesterol and diminish isoprenoid intermediates by inhibiting the rate-limiting enzyme of cholesterol biosynthesis at the 3-hydroxy-3-methylglutaryl-coenzyme (HMG-CoA) reductase step (16, 17). Isoprenoid intermediates localize host cell proteins possessing a CaaX, prenylated cysteine residue, aliphatic acid, and recognition amino acid (x), domain to cell membranes (18). With simvastatin treatment, the reduction of isoprenoid intermediates delocalizes CDC42, a CaaX domain containing host protein, to the cytosol. Alternatively, without simvastatin treatment, CDC42 remains membrane bound.

ML 141, 4-[3-(4-methoxyphenyl)-5-phenyl-3,4-dihydropyrazol-2-yl]benzenesulfonamide, is a newly characterized small-molecule that inhibits CDC42 activation
by limiting GTP-loading (19). Hong et al. found that ML 141 is highly selective for CDC42 nucleotide binding activity (20). Eight GTPases in the Rho family were screened for GTP-binding and their results indicate only CDC42 activity was limited by ML 141. Additionally, ML 141 is a potent inhibitor, as it was observed that ML 141 inhibited CDC42 with an inhibitory concentration (IC)\textsubscript{50} of 2-4 µmol/L. High concentrations of GTP were not able to compete with ML 141 to restore CDC42 activity, suggesting ML 141 is a non-competitive inhibitor. Evidence also suggests that when ML 141 binds, CDC42 undergoes a conformational change. Due to the conformational change in CDC42, neither GTP nor guanosine diphosphate (GDP) are able to bind to CDC42. Inhibition is reversible as indicated by the restoration of CDC42 activity when ML 141 is removed (19). Our research explored treatment with simvastatin and ML 141 to limit bacterial invasion of HUVEC through inhibition of CDC42.

Severe infections and potential evasion of antibiotics are among the hallmarks of \textit{S. pyogenes} pathogenesis (1). Therefore, the purpose of this study was to determine if simvastatin and ML 141 could serve as non-antibiotic-based strategies that would be protective to the host cell by limiting host mechanisms that internalize \textit{S. pyogenes}. 
HYPOTHESIS

Simvastatin and ML 141 limit host CDC42 localization and activity to inhibit endothelial cell endocytic uptake hijacked by *Streptococcus pyogenes* for invasion.
MATERIALS AND METHODS

**Endothelial cell cultures, bacterial strains, and growth conditions.** Human umbilical vein endothelial cells (HUVEC, EMD Millipore, Billerica, MA, SCCE001) were cultured in EndoGRO-LS complete media (EMD Millipore, SCME001) and maintained in 75 cm² vented cap flasks (Thermo-Fisher Scientific, Pittsburgh, PA, 10-126-37) at 37°C/5% CO₂.

*Streptococcus pyogenes* (90-226, M1+ and M1-, kindly provided by Dr. P. Cleary, University of Minnesota, and described, 21) were cultured in Todd Hewitt broth (THB, Thermo-Fisher Scientific, DF0492-17-6) and grown at 37°C with no shaking.

**Invasion assay.** For multiplicity of infection (MOI) determination, two days prior to infection, *S. pyogenes* were cultured in 5 ml THB 18-24 h from a single colony grown on THB/blood (Cleveland Scientific, Bath, OH, SBCIT-100) agar (Moorhead & Company, Rocklin, CA, gelidium), and 10 µl were subcultured 18 h prior to infection. HUVEC were seeded in 24-well plates (Sigma-Aldrich, St. Louis, MO, CLS3524) that had been coated with Attachment Factor (Life Technologies, Carlsbad, CA, S006100) for 30 min at 37°C/5% CO₂. HUVEC were plated at a density of 1.5 x 10⁵ cells/well. For infection, *S. pyogenes* were harvested by centrifugation, 37°C/3 min/10,000 RPM, washed, and resuspended in fresh 0.85% saline (NaCl, Thermo-Fisher Scientific, S-271-500). Bacteria were diluted to 4.8x10⁸ colony forming units (CFU)/ml and used to infect HUVEC at an MOI of 3, 30, or 300 for 2 h at 37°C/5% CO₂. CFU of 90-226 *S. pyogenes* had been determined from a growth curve, fitting an OD600 of 0.5 to 4.8x10⁸ CFU/ml. After infection, each well was washed three times with sterile 1X phosphate buffered saline (PBS, Life Technologies, 20012043) to remove unattached *S. pyogenes*. HUVEC were then incubated at 37°C, 5% CO₂ for 45 min in 10% fetal bovine serum (FBS, Atlanta...
Biologicals, Flowery Branch, GA, S111S0)/ PBS containing 500 µg/ml gentamicin (Sigma-Aldrich, G1272). Following the incubation with gentamicin to remove extracellular bacteria, HUVEC were washed three times with PBS. Sterile distilled ice-cold water (Life Technologies, 15230-162) was added to each well for 5 min at room temperature (RT) to permeabilize the host cells. Serial dilutions of this bacteria-containing water were performed in saline, plated on THB/blood agar, and incubated for 18-20 h at 37°C. The following day, colonies were counted to determine CFU/ml recovered from invasion.

**Compound treatment.** HUVEC were pretreated with simvastatin (0.001, 0.01, 0.1, and 1.0 µmol/L; EMD Millipore, 567021) with ML 141 (10 µmol/L), or with dimethyl sulfoxide (DMSO, Thermo-Fisher Scientific, BP231-1), a vehicle control for simvastatin and ML 141, at the same volume. HUVEC infections with *S. pyogenes* were at an MOI 30. Invasion was carried out as described above for assessment in the presence of compound treatment.

**Adhesion assay.** To compare adherent bacteria to intracellular bacteria, HUVEC were pretreated with DMSO or with simvastatin and infected with M1+ or M1- bacteria as above. CFU of 90-226 isogenic mutant, *M1-*, had been determined from a growth curve, fitting an OD600 of 0.98 to 4.8x10⁸ CFU/ml. Following incubation with bacteria, HUVEC were washed with PBS. To assess *S. pyogenes* adhesion, HUVEC were not treated with gentamicin to maintain extracellular bacteria association. HUVEC were lysed and serial dilutions plated on THB/blood agar as above.

**Transmission electron microscopy (TEM).** HUVEC were plated on 12 mm cover glass (Thermo-Fisher Scientific, 12-545-82) in a 24-well plate and invasion proceeded as described above without the addition of gentamicin. Following 2 h infection with *S. pyogenes* and PBS washes, HUVEC were washed for 10 min and fixed (1 h, RT) in 2.5% glutaraldehyde (Electron
Microscopy Sciences, Hatfield, PA, 16000)/ 2.0% paraformaldehyde (Electron Microscopy Sciences, 15700)/ 2 mmol/L calcium/ 1mmol/L magnesium. HUVEC were washed two times with PBS and post-fixated for 1 h with 1.0% osmium tetroxide (Electron Microscopy Sciences, 19100) and 3.0% reduced potassium ferrocyanide (Electron Microscopy Sciences, 26603-01) in 0.1 mol/L PBS. HUVEC were dehydrated in an ethanol series from 50% to 100% and infiltrated overnight in a 2:1, then 1:1 ethanol-propylene oxide resin (Electron Microscopy Sciences, 20401). HUVEC were embedded in EMBed812 (Electron Microscopy Sciences, 13940). Specimens were baked at 50°C for 24 h and 60°C for an additional 24 h to polymerize the plastic. Cover glass was removed by snap-freezing in liquid nitrogen leaving the HUVEC layer embedded in the plastic. Ultra-fine sections were collected on Pioloform slot grids and counterstained for 1.5 h in 1.0% aqueous uranyl acetate (Sigma-Aldrich, 6159-44-0) and Reynold’s lead citrate (Sigma-Aldrich, 6107-83-1). Imaging was conducted at 120KV using a JEOL JEM-1400 equipped with a Gatan Ultrascan 1000XP camera.

**Bactericidal activity.** The procedure outlined above for our invasion assays was followed, to determine the effects of simvastatin and ML 141 on *S. pyogenes*. In 24-well plates, simvastatin at 1.0 µmol/L, ML 141 at 10 µmol/L, or DMSO, at the same volume, was added to 4.8x10^8 CFU/ml *S. pyogenes* in 500 µl warmed EndoGRO-LS complete media. Plates were incubated for 2 h at 37°C/ 5% CO₂, the addition of Attachment Factor, plating of HUVEC and gentamicin treatment were excluded. Bacteria were serially plated on THB/blood agar for 18-20 h incubation. Colony counts were performed to assess the effects of simvastatin or ML 141 on planktonic growth.
**Bacteria incubation with simvastatin prior to invasion assay.** *S. pyogenes* were harvested by centrifugation, 37°C/ 3 min/ 10,000 RPM and washed with saline. *S. pyogenes* were diluted to 4.8x10⁸ CFU/ml and were resuspended and incubated with DMSO or simvastatin in 500 μl EndoGRO-LS complete media (2 h, 37°C, 5% CO₂). *S. pyogenes* were pelleted 37°C/ 3 min/ 10,000 RPM and simvastatin and DMSO-treated media removed. *S. pyogenes* were resuspended in fresh saline and HUVEC were infected with bacteria at an MOI of 30 for 2 h at 37°C/ 5% CO₂. Invasion was assessed as described above.

**Flow cytometry.** For fibronectin binding analysis, HUVEC were plated on 35 mm culture dishes (Thermo-Fisher Scientific, 08-772A) at 3x10⁵ cells/ml and pretreated with simvastatin or DMSO as described above. Following 24 h pretreatment, HUVEC were lifted using cell scrapers (Thermo-Fisher Scientific, 8100240), and 100 μl counted for normalization using the Accuri C6 flow cytometer (BD, Franklin Lakes, NJ). 96-well non-tissue treated culture plates (Sarstedt, Newton, NC, 82-1581-001) were coated with 20 μg/ml fibronectin (Sigma-Aldrich, F2006), washed extensively with PBS, and blocked with 2% bovine serum albumin (BSA, Thermo-Fisher Scientific, BP-1600-100) in PBS (30 min, RT). A volume of 200 μl HUVEC was incubated on fibronectin-coated plates for 2 h at 37°C/ 5% CO₂. HUVEC were washed with PBS and adherent HUVEC removed using trypsin (Life Technologies, 25200056). HUVEC were washed in FACS buffer (2% BSA/ 0.1% sodium azide, Sigma-Aldrich, S8032/ PBS), fixed in FACS buffer/ 4% paraformaldehyde and counted by flow cytometry.

**ML 141 washout assay.** HUVEC plating and infection with *S. pyogenes* were set up as described above, with the addition of one ML 141 treatment group. Following incubation, DMSO and ML 141 treatments were replenished and ML 141 washout was replaced with
warmed EndoGRO-LS complete media for 1 h (37°C, 5% CO₂). HUVEC were infected at an MOI of 30 for 2 h (37°C, 5% CO₂). Gentamicin removed extracellular bacteria (45 min, 37°C, 5% CO₂) and intracellular bacteria were released from HUVEC by the addition of water. Lysates were serially diluted in saline and plated on THB/blood agar for colony counts.

**Immunofluorescence.** For actin assessment, HUVEC were seeded at 6X10⁵ cells/ml in 35 mm plates that had been coated with Attachment Factor. HUVEC were pretreated with simvastatin, with ML 141, or with DMSO and treated with gentamicin as described above. HUVEC were washed three times with PBS and fixed with 4% paraformaldehyde for 30 min, RT. HUVEC were gently washed four times with PBS, permeabilized and blocked with 1% BSA/ 0.1% Triton (Sigma-Aldrich, T8787). HUVEC were gently washed four times with PBS, probed for actin with Alexa Fluor 488 phalloidin (1:40, Life Technologies, A12379) in PBS (30 min, RT) and washed three more times with PBS. Actin stress fiber assessment was conducted using a Zeiss Axiovert200 microscope equipped with a plan-apochromat 40X/ 1.2 numerical aperture water immersion lens. A total of 200 HUVEC were assessed for actin stress fiber disassembly from randomly selected fields-of-view. Images were collected using a LSM 5 Pascal scan head.

**ML 141 synthesis.** 4-[3-(4-methoxyphenyl)-5-phenyl-3,4-dihydropyrazol-2-yl]benzenesulfonamide (ML 141) was prepared following standard synthetic procedures (22), in the Department of Chemistry, Ball State University by Dr. R. Sammelson.

**Statistical analyses.** Data were analyzed by Student’s t-test when comparing two experimental groups or by one-way ANOVA followed by Student-Newman-Keuls post-hoc analysis when three or more experimental groups were examined. For actin assessment, values were computed
by $\chi^2$ test of association. A $P$-value of ≤0.05 was used as the threshold for statistical significance (Sigma Stat, Systat, Point Richmond, CA).
**RESULTS**

*Endothelial cell invasion by S. pyogenes was limited by simvastatin.* We first conducted a preliminary study to establish a baseline for optimal *S. pyogenes* invasion in the absence of simvastatin. HUVEC were infected with *S. pyogenes* at a range of MOI (3, 30, and 300). Intracellular *S. pyogenes* were plated on THB/blood agar. We found an increase of *S. pyogenes* invasion at an MOI of 30, therefore an MOI of 30 served as our optimal MOI for HUVEC infection with *S. pyogenes* (Fig. 1). To assess inhibition of invasion with simvastatin, HUVEC were pretreated with DMSO or with simvastatin (0.001, 0.01, 0.1, and 1.0 µmol/L, 24 h, 37°C, 5% CO₂). HUVEC were infected with *S. pyogenes* at an MOI of 30 (2 h, 37°C, 5% CO₂). Gentamicin was added to remove extracellular bacteria (45 min, 37°C, 5% CO₂) and water was used to lyse the HUVEC. At 1.0 µmol/L, invasion by *S. pyogenes* in simvastatin-treated HUVEC was 87±12% less than DMSO (Fig. 2, A). At 0.1 µmol/L, invasion of HUVEC was reduced by 82±10%. Simvastatin treatment at 0.01 µmol/L did not display inhibitory effects (Fig. 2, B).

*Simvastatin does not limit bacterial adherence to endothelial cells.* To assess whether the inhibition of invasion was due to inhibition of adhesion, HUVEC were pretreated with DMSO or with simvastatin (1.0 µmol/L, 24 h, 37°C, 5% CO₂). HUVEC were infected with *S. pyogenes* at an MOI of 30 and incubated for 2 h (37°C, 5% CO₂). Lysates were plated on THB/blood agar. Simvastatin treatment did not limit bacterial adhesion to HUVEC. Simvastatin had no detectable inhibition of adhesion regardless of whether the infecting strain was M+ or M- (Fig. 2, C and D).

*Endothelial cell damage in response to S. pyogenes infection was mitigated with simvastatin treatment.* By TEM analysis, cellular structures in response to treatment and infection were examined (Fig. 3). With simvastatin treatment, HUVEC organelles were intact. In the DMSO
control, *S. pyogenes* were present extracellularly and intracellularly and was potentially undergoing bacterial replication. The infected DMSO control also indicated the bacteria are undergoing endocytosis.

*Simvastatin treatment was not effective as a bactericide and invasion was not limited by S. pyogenes exposure to the compound.* To determine if incubating bacteria with simvastatin had an effect on bacterial viability and virulence, *S. pyogenes* incubated with DMSO or with simvastatin (1.0 µmol/L, 2 h) and plated on THB/blood agar 18-20 h at 37°C. Colony counts of viable bacteria from simvastatin treatment displayed no significant difference between treatment groups (Fig. 4, A). Additionally, to assess if bacterial incubation with simvastatin inhibited the ability of bacteria to invade, *S. pyogenes* were incubated with simvastatin (1.0 µmol/L, 2 h), pelleted by centrifugation, and resuspended in saline. HUVEC were infected with bacteria that had been incubated with simvastatin or DMSO for 2 h. HUVEC invasion by *S. pyogenes*, incubated with 1.0 µmol/L simvastatin prior to infection, was not limited when compared to the DMSO control (Fig. 4, B).

*Simvastatin inhibited endothelial cell binding to fibronectin.* To examine whether simvastatin inhibits invasion by decreasing host cell binding to fibronectin, HUVEC were pretreated 24 h with simvastatin (1.0 µmol/L) or with DMSO. HUVEC were lifted from tissue culture plates by cell scrapers and transferred to fibronectin-coated plates for 2 h at 37°C/5% CO₂. HUVEC were washed extensively with PBS to remove non-adherent cells. HUVEC were lifted using trypsin, washed, and analyzed by flow cytometry. Simvastatin treatment reduced HUVEC binding to fibronectin from 12±1% to 6±1% (Fig. 5).
**Inhibition of invasion by ML 141 was reversible.** HUVEC were pretreated with DMSO or with ML 141 (10 µmol/L, 24 h, 37°C, 5% CO₂). HUVEC were infected at an MOI of 30 and incubated for 2 h (37°C, 5% CO₂). Gentamicin removed extracellular bacteria (45 min, 37°C, 5% CO₂) and water was used to lyse the HUVEC. At 10 µmol/L, invasion by *S. pyogenes* in ML 141-treated HUVEC was 87±3% less than DMSO (Fig. 6, A). To determine whether ML 141 is a reversible inhibitor of *S. pyogenes* invasion, HUVEC were pretreated with DMSO or ML 141 (10 µmol/L) for 1 h and washed with PBS. DMSO and ML 141 treatments were replenished except the ML 141 washout group was replaced with warmed media (1 h, 37°C, 5% CO₂). HUVEC were infected at an MOI of 30 for 2 h (37°C, 5% CO₂). Gentamicin removed extracellular bacteria (45 min, 37°C, 5% CO₂) and intracellular bacteria were released from HUVEC by the addition of water. Lysates were serially diluted and plated on THB/blood agar for colony counts. We found that *S. pyogenes* invasion of HUVEC was restored in the washout samples (Fig. 6, B).

**Bactericidal activity by ML 141 was not detected.** To assess whether the apparent inhibition of invasion by ML 141 was due to bactericidal activity of ML 141, *S. pyogenes* were treated with 10 µmol/L ML 141 or DMSO (2 h, 37°C, 5% CO₂). *S. pyogenes* were plated on THB/blood agar to determine the number of viable bacteria in response to ML 141 treatment. There was no observed bactericidal activity with ML 141 treatment compared to DMSO control (Fig. 6, C).

**Compounds that limit *S. pyogenes* invasion maintained actin stress fibers in endothelial cells.** HUVEC were treated with DMSO, simvastatin (1.0 µmol/L), or ML 141 (10 µmol/L) 24 h prior to infection. HUVEC were infected with *S. pyogenes* at an MOI of 30 in all treatment groups. Addition of equal volumes of saline served as uninfected controls. HUVEC were treated with
gentamicin to remove extracellular bacteria (45 min, 37°C, 5% CO₂) and fixed with 4% paraformaldehyde. HUVEC were permeabilized by detergent and blocked by BSA addition. Alexa Fluor 488 phalloidin was used for detection of actin. In response to *S. pyogenes* infection, treatment by simvastatin (1.0 µmol/L, Fig. 7, A) and ML 141 (10 µmol/L, Fig. 7, B) reduced actin disassembly as compared to DMSO controls. Our work suggests compounds that limit *S. pyogenes* invasion through actin dynamics and fibronectin binding is associated with inhibition of host CDC42 localization and activity (Fig. 8).
DISCUSSION

In this study, we examined the effects of therapeutic inhibitors, simvastatin and ML 141, on invasion by *S. pyogenes*. We have identified an M1 serotype of *S. pyogenes*, 90-226, that efficiently adhered and invaded confluent monolayers of HUVEC. Additionally, we found that simvastatin and ML 141 limit *S. pyogenes* invasion but not adherence to HUVEC. Limiting *S. pyogenes* invasion of endothelial cells may slow severe invasive disease progression to sterile regions of the body and increase efficiency of antibiotic treatment to clear bacteria.

*S. pyogenes* invasion is associated with a multi-step process initiated with bacterial adherence to the host cell (1). *S. pyogenes* surface adhesins are characterized by their specificity to host cell binding (11). Weak, non-specific adherence to the surface of the host cells is associated with lipoteichoic acids (LTA) and pili structures (23). LTA are glycerol phosphate polymers in chains that covalently bind to the host plasma membrane, whereas pili are filamentous structures that retract to closer associate *S. pyogenes* with the host cell (11). Firm adherence is attributed to adhesion proteins on the streptococcal surface and their association with proteins of the host extracellular matrix (24). We have demonstrated that adherence of both an M1+ strain and of an M1- isogenic mutant was maintained in the presence of simvastatin. Our results indicate that non-specific adherent factors, including pili and LTA, maintain functional association with HUVEC in the presence of simvastatin treatment.

Following non-specific adherence to the host cell, anchored *S. pyogenes* surface adhesion proteins, including the M1 protein, establish intimate host cell contact necessary for invasion of cells. M1 proteins bind to fibronectin and create a complex with the host cell integrin α5β1 (25). We found that simvastatin decreased binding of the host cell to fibronectin, suggesting this is a step of invasion that is inhibited by simvastatin. Further supporting this notion, Cue et al.
demonstrated the M1- isogenic mutant binds fibronectin with low affinity (8), implying the important role of the M1 protein for invasion. Taken together, our data indicate simvastatin inhibits invasion through decreased binding of the host cell to fibronectin.

*S. pyogenes* undergo a tight association with the host cell membrane during internalization that has been associated with polymerization of actin at the port of entry (11). This mechanism of invasion is consistent with the zipper model (26). CDC42 was found to be a central regulator of actin polymerization (14). The role of CDC42 in actin polymerization suggests that an inhibitor with specificity to this singular small Rho family GTPase would limit *S. pyogenes* invasion. To evaluate an inhibitor molecule that has specificity to CDC42 (19), we examined ML 141. Our data indicate that ML 141 inhibits HUVEC invasion. We observed that inhibition by ML 141 is reversible, which is consistent with mechanism assays previously described that identified ML 141 binding to GTP is reversible (20). Furthermore, inhibition of actin depolymerization by ML 141 during invasion suggests we are limiting bacterial uptake through a mechanism that is dependent on CDC42.

Simvastatin and ML 141 treatment potentially protect the host cell. Intracellular *S. pyogenes* persist within host cells presumably avoiding antibiotic clearance and host defense mechanisms. By protecting host cells through methods that limit internalization, we would alleviate concerns associated with spread to deep tissue that have severe health implications. These results are consistent with a previous investigation in which *Staphylococcus aureus* invasion of host cells was limited by simvastatin (15) and ML 141 treatments of HUVEC (Cordero et al., unpublished data). *S. aureus* is an invasive human pathogen (27) associated with severe health implications, including septic shock, associated with spread to deep tissue (28). The mechanisms used by *S. pyogenes* and *S. aureus* to compromise host tissue are fundamentally
similar. Our work suggests that identification of compounds that limit internalization by fibronectin association may be applicable to other pathogens that use this mode of cell entry.
REFERENCES

Figure 1 A multiplicity of infection (MOI) of 30 is optimal for Streptococcus pyogenes invasion of endothelial cells. Human umbilical vein endothelial cells (HUVEC) were infected at an MOI of 3, 30, or 300 for 2 h (37°C, 5% CO₂). Gentamicin removed extracellular bacteria (45 min, 37°C, 5% CO₂) and intracellular bacteria were released from HUVEC by the addition of water. Medium was serially diluted and plated on Todd Hewitt broth (THB)/blood agar for colony counts. Data presented as colony forming units (CFU)/ml ± SEM with n=3/treatment (* more than MOI 3, † more than MOI 300, P≤0.05 by one-way ANOVA followed by Student-Newman-Keuls post-hoc analysis).
Figure 2 Invasion by Streptococcus pyogenes is inhibited when endothelial cells are treated with simvastatin at: **Panel A.** 1.0 µmol/L and **Panel B.** 0.1 µmol/L. Human umbilical vein endothelial cells (HUVEC) were pretreated with simvastatin (0.001, 0.01, 0.1, 1.0 µmol/L) or with vehicle control, dimethyl sulfoxide (DMSO) 24 h prior to infection. HUVEC were infected at a multiplicity of infection (MOI) of 30 (2 h, 37°C, 5% CO₂). Gentamicin removed extracellular bacteria (45 min, 37°C, 5% CO₂) and water was used to lyse the HUVEC. Lysates plated on Todd Hewitt broth (THB)/blood agar were used to determine viable bacteria recovered from
HUVEC invasion. Data presented as percent control ± SEM with n=3/treatment (A, * less than DMSO, $P \leq 0.05$ by Student’s $t$-test; B, * less than DMSO, † less than 0.001, ‡ less than 0.01, $P \leq 0.05$ by one-way ANOVA followed by Student-Newman-Keuls post-hoc analysis). **Panel C.**

*M1+ and Panel D. M1- S. pyogenes adhere to HUVEC treated with simvastatin.* HUVEC were pretreated with simvastatin (1.0 µmol/L) or with DMSO 24 h prior to M1+ or M1- *S. pyogenes* infection. HUVEC were infected at MOI of 30 for 2 h (37°C, 5% CO$_2$). Intracellular and extracellular bacteria were released from HUVEC by the addition of water. Medium containing lysates was serially diluted and plated on THB/blood agar for colony counts (C and D, data presented as percent control ± SEM displayed no significant differences between treatment groups with n=3/treatment, $P>0.05$ by Student’s $t$-test).
Figure 3 *Simvastatin treatment mitigates host cell damage in response to Streptococcus pyogenes infection*. Human umbilical vein endothelial cells (HUVEC) were pretreated with simvastatin (1.0 μmol/L) or vehicle control, dimethyl sulfoxide 24 h prior to *S. pyogenes* infection. HUVEC were infected at a multiplicity of infection of 30 for 2 h (37°C, 5% CO₂). The scale bar is 5 μm. Black arrow indicates an extracellular bacterium and black arrowhead indicates an invading bacterium potentially undergoing replication. White arrowhead indicates intact mitochondria. White N denotes the nucleus of HUVEC.
Figure 4 Simvastatin is nonbactericidal. **Panel A.** Bacteria were incubated with dimethyl sulfoxide (DMSO) or simvastatin (1.0 µmol/L, 2 h, 37°C, 5% CO₂). *Streptococcus pyogenes* were plated on Todd Hewitt broth/blood agar (18-20 h, 37°C). Data presented as colony forming units (CFU)/ml ± SEM with n=3/treatment (*P*>0.05 by Student’s *t*-test). **Panel B.** *S. pyogenes* incubation with simvastatin does not limit bacterial invasion of endothelial cells. Bacteria were incubated with DMSO or simvastatin (1.0 µmol/L) for 2 h prior to infection to mimic exposure of *S. pyogenes* to simvastatin treatment during invasion assays. *S. pyogenes* were pelleted and simvastatin and DMSO-treated media were removed. Human umbilical vein endothelial cells
(HUVEC) were infected at a multiplicity of infection of 30 for 2 h at 37°C/5% CO₂. HUVEC were treated with gentamicin (45 min, 37°C, 5% CO₂) to remove extracellular bacteria. HUVEC were lysed with water and plated for colony counts to determine if incubation of \textit{S. pyogenes} with simvastatin limits endothelial cell invasion. Data presented as percent control ± SEM ($P>0.05$ by Student’s \textit{t}-test).
**Figure 5** *Human umbilical vein endothelial cells (HUVEC) have decreased binding to fibronectin in response to simvastatin treatment.* HUVEC were pretreated with dimethyl sulfoxide or with simvastatin (1.0 µmol/L) for 24 h. HUVEC were washed extensively with PBS, lifted from tissue culture plates, and transferred to plates coated with fibronectin for 2 h. Following incubation on fibronectin-coated plates, HUVEC were recovered with trypsin. Recovered HUVEC were counted using an Accuri flow cytometer. Data presented as percent adherent cells ± SEM with n=5/treatment (* P≤ 0.05 by Student’s t-test).
Figure 6 Invasion by Streptococcus pyogenes is inhibited when endothelial cells are treated with ML 141. Panel A. Human umbilical vein endothelial cells (HUVEC) were pretreated with dimethyl sulfoxide (DMSO) or ML 141 (10 µmol/L) 24 h prior to infection. HUVEC were infected at a multiplicity of infection (MOI) of 30 and incubated (2 h, 37°C, 5% CO₂). Gentamicin removed extracellular bacteria (45 min, 37°C, 5% CO₂) and water was used to lyse the HUVEC. Data presented as percent control ± SEM with n=3/treatment (* less than DMSO, \( P \leq 0.05 \) by Student’s \( t \)-test). Panel B. ML 141 is a reversible inhibitor of S. pyogenes invasion. HUVEC were pretreated with DMSO or ML 141 (10 µmol/L) for 1 h and washed with phosphate buffered saline. DMSO and ML 141 treatments were replenished and ML 141
washout was replaced with warmed media for 1 h (37°C, 5% CO₂). HUVEC were infected at an MOI of 30 for 2 h (37°C, 5% CO₂). Gentamicin removed extracellular bacteria (45 min, 37°C, 5% CO₂) and intracellular bacteria were released from HUVEC by the addition of water. Lysates were serially diluted and plated on Todd Hewitt broth (THB)/blood agar for colony counts. Data presented as internalized bacteria ± SEM with n=4/treatment (* less than control, † less than ML 141 washout, P≤0.05 by Student’s t-test when data were transformed by log(10)). Panel C. ML 141 is not bactericidal. S. pyogenes were treated with ML 141 (10 µmol/L) or DMSO for 2 h. Viable bacteria were recovered and assessed by colony counts on THB/blood agar. Data presented as colony forming units (CFU)/ml ± SEM (P>0.05, Student’s t-test).
Figure 7 Human umbilical vein endothelial cell (HUVEC) actin stress fiber disassembly in response to Streptococcus pyogenes infection is reduced by simvastatin and ML 141 treatment.

Panel A. HUVEC were pretreated 24 h with dimethyl sulfoxide (DMSO) or simvastatin (1.0 µmol/L). HUVEC were infected with S. pyogenes for 2 h or incubated with an equal volume of saline for uninfected controls. HUVEC were treated with gentamicin (45 min, 37°C, 5% CO₂), fixed, blocked, and probed with Alexa Fluor 488 phalloidin for actin. Quantification for percent actin disassembly was determined by evaluation of 200 HUVEC taken from randomly selected fields-of-view. The scale bar is 50 µm. Data presented as percent actin stress fiber disassembly (*less than infected control, $P \leq 0.05$ by $\chi^2$ test of association). Panel B. Preparation of HUVEC for imaging and quantification was carried out as described in Figure 7, Panel A, but HUVEC were treated with DMSO or ML 141 (10 µmol/L, * less than infected control, $P \leq 0.05$).
Figure 8. Simvastatin and ML 141 inhibit localization and activity of host CDC42 to limit Streptococcus pyogenes invasion of endothelial cells. Schematic demonstrates proposed mechanism of human umbilical vein endothelial cell invasion by Streptococcus pyogenes and the method of inhibition with simvastatin and ML141 treatments. To initiate invasion, S. pyogenes M1 protein binds to the host ligand, fibronectin, and creates a complex with the α5β1 integrin expressed on the endothelial cell surface. The role of CDC42 in bacterial invasion is attributed to
actin stress fiber disassembly that forms filapodial protrusions to engulf infecting bacteria. Simvastatin depletes isoprenoid intermediates that serve as membrane anchors of CDC42 and other Rho GTPases for localization to cell membranes. ML141 inhibits CDC42 activity by eliciting a conformational change of CDC42 to limit guanine nucleotide binding.
APPENDIX I:

Preliminary studies of Streptococcus pyogenes ATCC 19615

Prior to our work with 90-226 M+ and M-, we had begun our exploration of S. pyogenes invasion and inhibition with simvastatin and ML 141 with another strain. ATCC 19615 is a strain of group A S. pyogenes that has not been well characterized in published work and the protein expression on the surface of ATCC 19615 is not as well described as those of 90-226. The culture methods were performed in 5 ml tryptic soy broth two days prior to invasion assays with agitated overnight incubation (37°C, 150 RPM). After 24 h, 10 µl was subcultured and incubated as described above. HUVEC plating and compound treatments were consistent with methods described above. ATCC 19615 S. pyogenes at an MOI of 300 were used for infection. From colony counts plated on tryptic soy agar, we had observed that S. pyogenes ATCC 19615 invasion and adhesion was inhibited with simvastatin and ML 141 treatment. Due to high variability in the number of recovered bacteria, we were not consistently reporting a statistical difference from our control groups. We did find the concentrations of simvastatin and ML 141 used in the invasion and adhesion assays were not bactericidal. Following approximately three months of laboratory culturing, we had diminished colony counts from lysed HUVEC in both compound treatment and control groups. Early work by CG Becker, published in 1964, indicated that we may have lost invasiveness due to continuous laboratory culturing (29). S. pyogenes expression of surface proteins that promote invasion can relapse with agitated incubation. As per suggestion of Dr. Patrick Cleary, we altered our culture methods to those that have been described in the methods of this document. Additionally, we received the 90-226 M+ and isogenic mutant strains that have been characterized as more stable laboratory strains of S. pyogenes expressing the M1 protein (8).