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Host cell invasion by *Staphylococcus aureus* stimulates the shedding of microvesicles

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ABSTRACT

During severe sepsis, microvesicles that are positive for tissue factor (TF) are at increased levels within blood and in pulmonary lavage. These microvesicles potentially disperse TF, the major initiator of the coagulation cascade, throughout multiple organ systems, initiating fibrin deposition and resultant ischemia. The source of these microvesicles has remained incompletely defined. Although TF^+ microvesicles are shed from cells that express nascent TF transcript in response to injury, recent findings revealed that circulating, full-length TF protein is detectable prior to these nascent transcripts. This finding suggested that the protein is released from constitutive sources as an acute response. We examined whether *Staphylococcus aureus*, the Gram-positive bacteria that is emerging as one of the most common etiologic agents in sepsis, is capable of stimulating the release of TF^+ microvesicles from a pulmonary cell line that constitutively expresses TF protein. We found that host cell invasion stimulated an acute release of TF^+ microvesicles and that these microvesicles mediated the transfer of the protein to TF-negative endothelial cells. We also found that transfer was inhibited by cholesterol-lowering simvastatin. Taken together, our findings reveal that *S. aureus* pathogenesis extends to the acute release of TF^+ microvesicles and that inhibiting dispersal by this mechanism may provide a therapeutic target.

1. Introduction

From 1999 to 2005, the estimated number of sepsis-associated deaths in the US exceeded one million [1]. Etiology has shifted from predominantly Gram-negative to Gram-positive bacteria with *Staphylococcus aureus* emerging as one of the most commonly identified pathogens [2]. The severe coagulopathy that characterizes sepsis has been associated with an increase in TF^+ microvesicles within blood [3] and within pulmonary lavage [4]. These microvesicles that are characteristically 1–4 μm in size are shed from cellular membranes continuously, yet their numbers increase in response to specific inflammatory mediators. Their increased shedding potentially disseminates TF, the major initiator of coagulation, leading to fibrin deposition and ischemia throughout multiple organ systems [3–5].

The shedding of TF^+ microvesicles in response to pathogen invasion was reported initially in Ebola virus infection and subsequently has been identified during infection by a subset of clinically important pathogens associated with severe coagulopathy [6]. The primary source for the initiating TF has been attributed to the stimulation of nascent transcript [4,6,7]. However, recent findings indicated that these inducible sources are insufficient to account for the total level of circulating TF protein detected acutely in response to injury [7]. More recently, full-length TF protein was detected in lavage prior to the detection of TF transcript in healthy volunteers instilled with lipoteichoic acid, a cell wall component of Gram-positive bacteria [8], raising the possibility that full-length protein is released prior to the induction of new transcript. In support of this concept, subsets of pulmonary cells are known to constitutively express full-length TF protein [9–12]. These findings led to the hypothesis that an acute source of TF^+ microvesicles during infection may include constitutively expressed TF protein within pulmonary cells. We therefore explored whether H441 cells, a lung-derived cell line that we found to constitutively express TF, would generate TF^+ microvesicles and whether the pathogenesis of *S. aureus* extends to the stimulation of their acute release.

2. Materials and methods

2.1. S. aureus infection

Bacterial cultures were pelleted (3 min, 10,000 rpm, 37 °C) and washed once in saline. For the majority of studies, the strain used was ATCC 29213 (American Type Culture Collection, Manassas, VA). This strain induces sepsis *in vivo* [13] and is invasive

Abbreviations: TF, tissue factor; HUVEC, human umbilical vein endothelial cells; ATCC, American Type Culture Collection.

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[14,15]. To investigate the role of invasion, minimally invasive ATCC 700699 also was examined.

2.2. TF detection

H441 cells (ATCC) were seeded at 3×10^4 into 35 mm dishes. On day 2 of plating, cells were infected and suspended using cell scrapers. To detect intracellular TF, surface proteins were fixed in FACS buffer (2% BSA/0.1% sodium azide/PBS) containing 4% paraformaldehyde, permeabilized in 0.5% Tween-20/PBS (15 min on ice), and incubated with TF-PE (BD, Franklin Lakes, NJ; 30 min, on ice). To detect surface TF, cells were stained with TF-PE prior to fixing and were not permeabilized. The isotype control (BD) confirmed specificity of antibody staining.

2.3. Microvesicle isolation

H441 cells were seeded at 2×10^5 or U-87 MG cells (ATCC) at 2×10^6 into 100 mm culture dishes. Preliminary data had indicated higher density plating led to diminished TF expression in the H441 cells. Culture dishes for the U-87 MG cells were coated with Attachment Factor (Life Technologies, Carlsbad, CA). On day 2, supernatants were harvested following infection at an MOI of 300. Low speed centrifugation cleared the supernatant (10 min, 500×*g*, 4 °C). The microvesicle fraction was isolated from the cleared supernatant by higher speed centrifugation (30 min, 17,000×*g*, 4 °C). To remove bacteria that may have been isolated by this centrifugation, pellets were incubated (30 min, 5% CO_2, 37 °C) with 50 μg/ml gentamicin (Life Technologies) /20 μg/ml lysostaphin (Sigma–Aldrich). Centrifugations were repeated and the resultant pellet harvested. The nomenclature of shed vesicles has yet to be standardized [16,17]. Using the same centrifugation strategy, the resultant pellet has been termed either the microvesicle fraction [18] or the microparticle fraction [19,20]. For the current study, the resultant pellet is referred to as the microvesicle fraction. The pellet was resuspended in FACS buffer, incubated with TF-PE, fixed in FACS buffer containing 0.74% formaldehyde, and immediately analyzed using an Accuri C6 flow cytometer (BD) by gating on particles within the 1–4 μm range.

2.4. Microvesicle transfer assay

Human umbilical vein endothelial cells (HUVEC; Life Technologies) were seeded at 5×10^4 into 35 mm culture dishes coated with Attachment Factor. The next day, cells were incubated with simvastatin (EMD Millipore, Germany) or with dimethyl sulfoxide (18–20 h). Microvesicles were harvested from H441 or from U-87 MG cells infected at an MOI of 400, resuspended in 1.2 ml PBS. Endocytosis in HUVEC was halted (10 min, 4 °C), freshly harvested microvesicles added (500 μl from the H441 or 100 μl from the U-87 MG cells), allowed to adhere (20 min, 4 °C), and endocytosis initiated (1 h, 37 °C).

2.5. Statistical analysis

Differences between groups were considered statistically significant at $P \leqslant 0.05$ by Student's *t*-test or one-way ANOVA followed by Student–Newman–Keuls post-hoc analysis (Sigma Stat, Systat, Point Richmond, CA, USA).

3. Results

3.1. Host cell invasion mobilizes TF

Previous work had shown that constitutively expressed TF protein localizes within intracellular pools and at the cell surface and can be mobilized for release within shed microvesicles [21–23]. We explored whether host cell invasion by *S. aureus* is capable of stimulating this mobilization. In response to increasing MOI, intracellular pools diminished, suggesting that the intracellular source of TF had been mobilized (Fig. 1, Panel A). Cell surface TF decreased as well (Fig. 1, Panel B), indicating that host cell invasion stimulates the mobilization of both intracellular and cell surface TF.

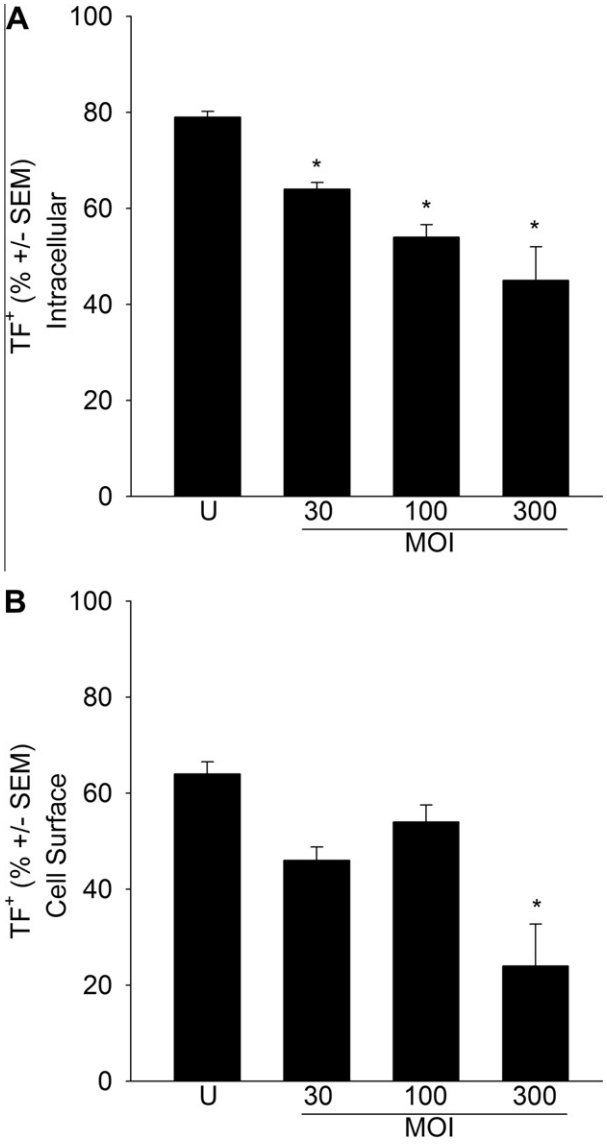

Fig. 1. Levels of intracellular and cell surface tissue factor (TF) decrease with increasing multiplicities of infection (MOI). H441 were incubated with *Staphylococcus aureus* (1 h) at increasing MOI, harvested, and TF detected by flow cytometric analysis. Panel A to detect intracellular TF, cell surface proteins were fixed prior to permeabilizing and staining with TF-PE. Panel B for the detection of cell surface TF, cells were stained with TF-PE prior to fixing and were not permeabilized. Data are representative of replicate experiments (*$P \leqslant 0.05$ by one-way ANOVA followed by Student–Newman–Keuls post-hoc analysis; $n = 3$/group).

3.2. Particles within the microvesicle fractions are TF+

To evaluate whether the decrease in intracellular and cell surface TF that had been observed in response to infection corresponded to an accumulation of shed TF+ microvesicles, supernatants from uninfected and infected H441 cells were subjected to centrifugation and

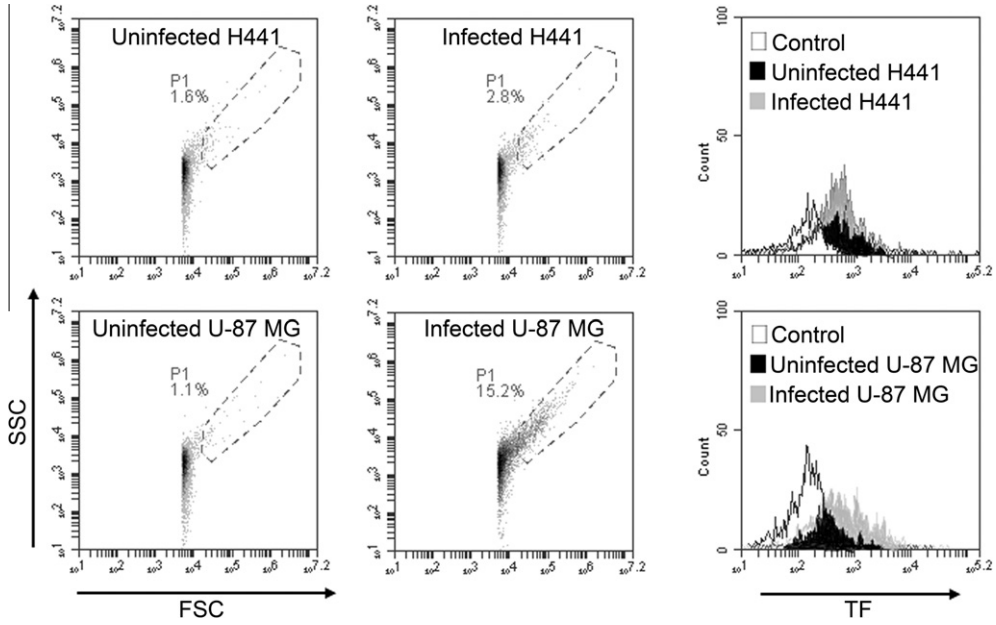

Fig. 2. Particles within the microvesicle fraction increase in response to invasion and are positive for tissue factor (TF^+). H441 or U-87 MG cells were incubated with *Staphylococcus aureus* (1 h). Following infection, supernatants were harvested, microvesicle fractions isolated by centrifugation, pellets stained with TF-PE and analyzed by flow cytometry, gating for 1–4 μm particles. Data are representative of replicate experiments.

the microvesicle fractions examined by flow cytometry. U-87 MG cells, a glioblastoma-derived cell line with well-characterized TF^+ microvesicle release [24], was examined as a comparator. In both cell types, the microvesicle population increased in response to infection and microvesicles were TF^+ (Fig. 2). TF^+ microvesicles also were detected within the fraction isolated from uninfected cells, supporting the concept that the shedding of microvesicles from cell membranes appears to be a normal physiologic process [17] that is accelerated in a variety of pathogenic states [25]. These data indicate that the host cell response to invasion by *S. aureus* includes an increased release of TF^+ microvesicles. The release corresponds to the mobilization of TF, suggesting that in this lung-derived cell line, TF mobilization results in release as TF^+ microvesicles.

3.3. Viable, invasive bacteria are required for TF release

To begin to explore underlying mechanisms of TF release, we examined whether active bacterial invasion is required or whether stimulation of endocytosis by heat-killed bacteria is sufficient. TF release was detectable only in response to viable, invasive bacteria, not merely endocytic uptake (Fig. 3, Panel A). To explore further the potential role of invasiveness, the response to a minimally invasive strain was examined. TF release again was detectable only in response to invasive ATCC 29213, not in response to minimally invasive ATCC 700699 (Fig. 3, Panel C), further supporting the concept that active bacterial invasion is required for the release of TF by the host cell.

3.4. Simvastatin limits the transfer of TF from TF^+ microvesicles to TF-negative endothelial cells

Microvesicles function as transporters, transferring biomolecules such as DNA, RNA, and protein, from donor to acceptor cells [17]. We therefore examined whether the TF released within shed microvesicles could be transferred to TF-negative acceptor cells. In response to incubation with the microvesicle fraction from H441 cells, intracellular TF became detectable within HUVEC (Fig. 4, Panel A). Transfer of microvesicles from U-87 MG cells was more robust (Fig. 4, Panel B). Pretreatment of HUVEC with actinomycin D, an inhibitor of transcription, failed to attenuate the level of TF detected in HUVEC, indicating that full-length TF protein had been transferred rather than TF transcription induced (data not shown).

Previous work had indicated that simvastatin inhibits endocytic uptake that is dependent on the precise localization of small-GTPases within cellular membranes [14,15,26,27]. Although incompletely understood, transfer of biomolecules to the acceptor cell relies in part on endocytic uptake [17]. We therefore examined whether pretreatment at the acceptor cell could limit the transfer of TF from shed microvesicles. The more robust TF transfer by U-87 MG-derived microvesicles was examined. Simvastatin pretreatment of TF-negative HUVEC inhibited transfer (Fig. 4, Panel C). Taken together, these findings indicate that the mobilization and release of TF in the form of microvesicles can lead to the transfer of TF to endothelial cells and that this transfer is inhibited partially by simvastatin.

4. Discussion

Until recently, the biological relevance of host cell invasion by *S. aureus* had been challenged [28]. Historically, *S. aureus* had been considered an extracellular pathogen, and invasion appeared to be an *in vitro* artifact. However, an emerging concept is that invasion by *S. aureus* contributes significantly to pathogenesis by stimulating pro-inflammatory responses, enabling the evasion of antibiotics and immune cell surveillance, and establishing intracellular bacterial reservoirs of chronic infection [28–34]. Our findings would suggest that pathogenesis extends to the release of TF^+ microvesicles. An alternative possibility is that the acute release of TF may provide a mechanism for walling-off infection [35]. Although this possibility remains to be examined directly, inhibitors of coagulation have been found to limit damage to pulmonary tissue rather than promote the spread of infection [36], suggesting that the observed acute release of TF would contribute to pathogenesis.

Controversy has surrounded the nomenclature and characterization of shed microvesicles [16], yet their importance in the dis-

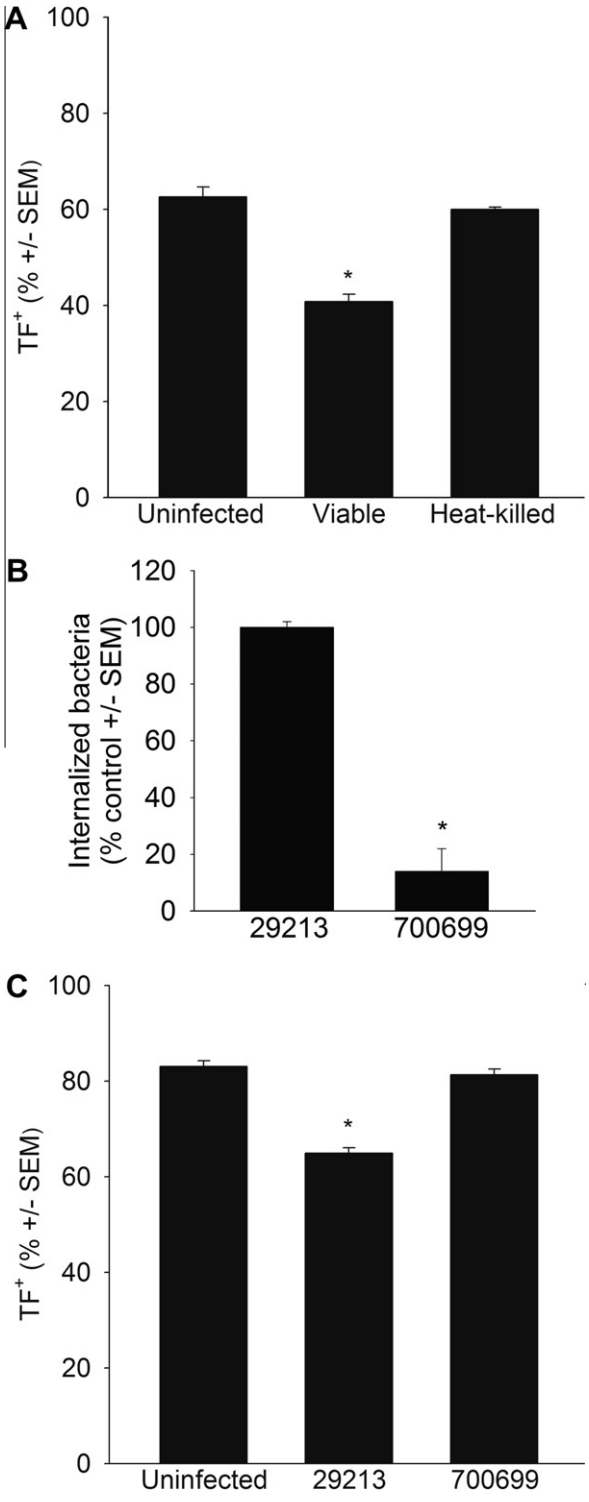

Fig. 3. Viable, invasive bacteria are required for tissue factor (TF) release from H441 cells. Panel A bacteria were heat-killed (80 °C, 20 min), incubated with H441 cells (1 h), and TF detected by flow cytometric analysis. TF release was not detectable in response to heat-killed bacteria. Panel B H441 cells were infected with American Type Culture Collection (ATCC) 700699 or with ATCC 29213 (1 h), extracellular bacteria removed using the bactericides gentamicin and lysostaphin, permeabilized using the detergent saponin, and serial dilutions of the medium containing bacteria plated on tryptic soy agar for colony counts (16 h). ATCC 700699 was found to be minimally invasive compared to ATCC 29213. Panel C H441 cells were incubated with the minimally invasive strain ATCC 700699 or with ATCC 29213 and TF detected using flow cytometry. TF release from H441 cells was not detectable in response to the minimally invasive strain ($^{*}P \leqslant 0.05$ by one-way ANOVA followed by Student–Newman–Keuls post-hoc analysis, Panels A and C; or by Student's *t*-test, Panel B; $n = 3$/group. Data are representative of replicate experiments).

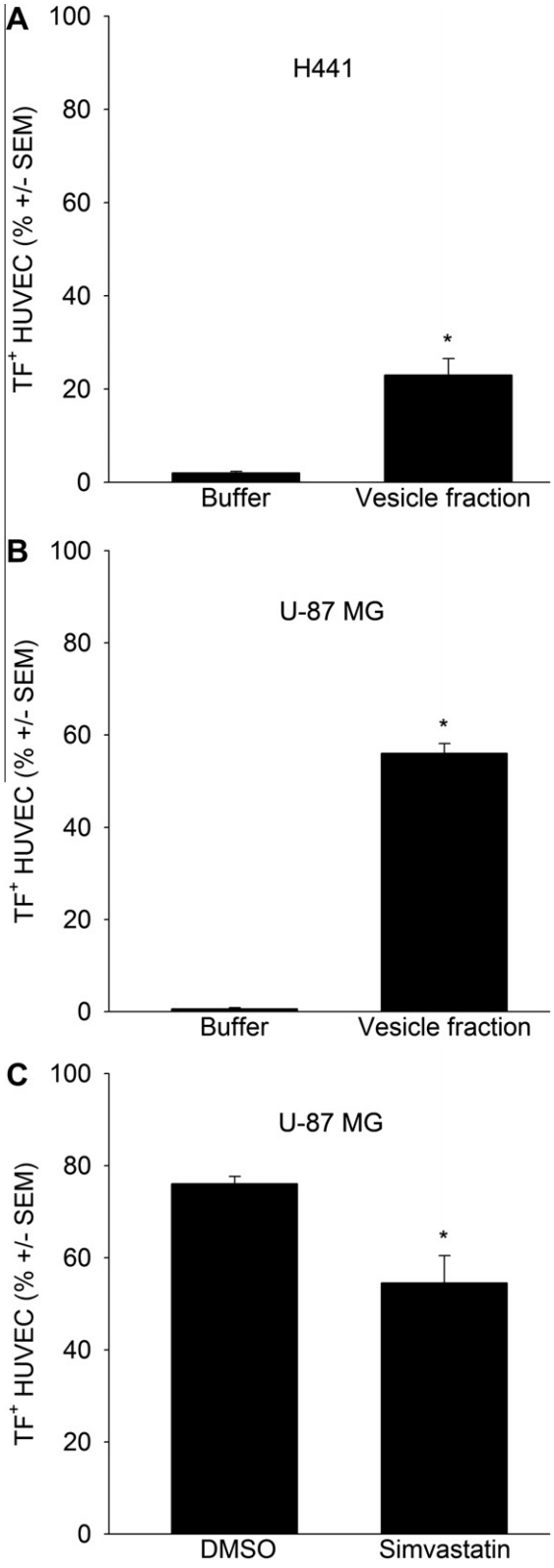

Fig. 4. Simvastatin limits transfer of tissue factor (TF) from TF^{+} microvesicles to TF-negative endothelial cells. Panel A TF-negative human umbilical vein endothelial cells (HUVEC) were incubated with TF^{+} microvesicles (1 h) harvested from infected H441 or Panel B from infected U-87 MG. Panel C HUVEC were pretreated with simvastatin (10 μM) or with dimethyl sulfoxide (DMSO) as the vehicle control (18–20 h) prior to incubation (1 h) with the microvesicle fraction from infected U-87 MG. Intracellular and cell surface TF was detected by flow cytometric analysis ($^{*}P \leqslant 0.05$ by Student's *t*-test; $n = 3$/group).

persal of biomolecules at distal sites has become more clearly defined in a variety of pathogenic states [17]. Our findings indicate that microvesicles shed during infection enable the dispersal of TF to TF-negative endothelial cells and that simvastatin limits this mechanism of transfer. Whether inhibition of the uptake of TF is protective, or alternatively, whether uptake is a mechanism of clearance that inhibition would impair, remains to be fully clarified. *In vivo*, simvastatin improved survival and diminished procoagulant TF activity in a murine model of bacterial sepsis [37], suggesting that the overall effect of statin treatment would be to blunt rather than prolong the procoagulant state. In the current work, inhibition of uptake *in vitro* is modest (20%). However, inhibition of dispersal *in vivo* would be in the context of the additional pleotropic effects of statins, such as the inhibition of TF transcription [38], and in this context, may contribute to limiting pathogenesis.

Our findings indicate that constitutive sources release TF^+ microvesicles in response to invasion by one of the most commonly identified etiologic agents of sepsis. Patient populations at an increased risk of death due to severe infection are characterized by a procoagulant status [39] that has been associated with an elevation in circulating TF^+ microvesicles [40]. The significance of our finding is that the distribution of procoagulant TF appears to be an early event initiated by constitutive sources. Although therapies that singly target procoagulant molecules have met with limited success, our work builds upon previous findings that statin pharmacology could address severe infection on multiple fronts, including improvement in cardiovascular and immune function and suppression of TF transcription from inducible sources [41,42]. Taken together, these findings will contribute to the on-going assessment of the safety and efficacy of statin use in the treatment of severe infection and provide strategies for advancing new therapeutic approaches.

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