HHS Public Access
Author manuscript
Crit Care Med. Author manuscript; available in PMC 2016 July 01.

Published in final edited form as:
Crit Care Med. 2015 July ; 43(7): e230–e240. doi:10.1097/CCM.0000000000000993.

Drug Repurposing Screen Identifies Foxo1-Dependent Angiopoietin-2 Regulation in Sepsis

Chandra C. Ghosh, PhD[1], **Kristina Thamm, MSc**[2], **Anthony V. Berghelli**[1], **Claudia Schrimpf, MD**[3], **Manish R. Maski, MD**[1], **Tanaz Abid**[1], **Katelyn E. Milam**[1], **Augustine Rajakumar, PhD**[1], **Ansgar Santel, PhD**[4], **Jan T. Kielstein, MD**[2], **Asif Ahmed, MD**[5], **David Thickett, MD**[6], **Keqin Wang, PhD**[6], **Maureen Chase, MD**[7], **Michael W. Donnino, MD**[7], **William C. Aird, MD**[1], **Hermann Haller, MD**[2], **Sascha David, MD**[1,2], and **Samir M. Parikh, MD**[1]

[1]Center for Vascular Biology Research, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA.

[2]Department of Nephrology and Hypertension, Medical School Hannover, Hannover, Germany.

[3]Department of Heart, Thoracic, Transplantation and Vascular Surgery, Medical School Hannover, Hannover, Germany.

[4]Silence Therapeutics AG, Berlin, Germany.

[5]Department of Reproductive and Vascular Biology, Aston University, Birmingham, United Kingdom.

[6]Academic Department of Anaesthesia, Pain, and Critical Care, Heart of England NHS Foundation Trust, Birmingham, United Kingdom.

[7]Division of Critical Care and Department of Emergency Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA.

Abstract

Objective—The recent withdrawal of a targeted sepsis therapy has diminished pharmaceutical enthusiasm for developing novel drugs for the treatment of sepsis. Angiopoietin-2 is an endothelial-derived protein that potentiates vascular inflammation and leak-age and may be involved in sepsis pathogenesis. We screened approved compounds for putative inhibitors of angiopoietin-2 production and investigated underlying molecular mechanisms.

Design—Laboratory and animal research plus prospective placebo- controlled randomized controlled trial (NCT00529139) and retrospective analysis (NCT00676897).

Setting—Research laboratories of Hannover Medical School and Harvard Medical School.

Patients—Septic patients/C57Bl/6 mice and human endothelial cells.

For information regarding this article, david.sascha@mh-hannover.de.
Drs. Ghosh, Thamm and David, Parikh contributed equally to the work.

The remaining authors have disclosed that they do not have any potential conflicts of interest.

Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's website (http://journals.lww.com/ccmjournal).

Interventions—Food and Drug Administration–approved library screening.

Measurements and Main Results—In a cell-based screen of more than 650 Food and Drug Administration–approved compounds, we identified multiple members of the 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitor drug class (referred to as statins) that suppressed angiopoietin-2. Simvastatin inhibited 3-hydroxy-3-methyl-glutaryl-CoA reductase, which in turn activated PI3K-kinase. Downstream of this signaling, PI3K-dependent phosphorylation of the transcription factor Foxo1 at key amino acids inhibited its ability to shuttle to the nucleus and bind *cis*-elements in the angiopoietin-2 promoter. In septic mice, transient inhibition of angiopoietin-2 expression by liposomal siRNA in vivo improved absolute survival by 50%. Simvastatin had a similar effect, but the combination of angiopoietin-2 siRNA and simvastatin showed no additive benefit. To verify the link between statins and angiopoietin-2 in humans, we performed a pilot matched case-control study and a small randomized placebo-controlled trial demonstrating beneficial effects on angiopoietin-2.

Conclusions—3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitors may operate through a novel Foxo1-angiopoietin-2 mechanism to suppress de novo production of angiopoietin-2 and thereby ameliorate manifestations of sepsis. Given angiopoietin-2's dual role as a biomarker and candidate disease mediator, early serum angiopoietin-2 measurement may serve as a stratification tool for future trials of drugs targeting vascular leakage.

Keywords

angiopoietin-2; endothelium; Foxo1; sepsis; statin; Tie2

Despite modern antimicrobials, mortality from severe sepsis remains high at 30–50% (1). Dozens promising preclinical agents have failed in clinical trials. The recent market withdrawal of drotrecogin α following a large negative clinical trial (2) has eliminated the only targeted therapy for sepsis.

Angiopoietin-2 (Angpt-2) is markedly induced in sepsis, and its circulating levels are associated with disease severity and mortality (3–8). Largely synthesized by endothelial cells (ECs), premade Angpt-2 is stored in Weibel Palade bodies and rapidly released following inflammatory stimuli (9). During inflammation, Angpt-2 inhibits signaling through Tie2, a receptor expressed by ECs that is otherwise tonically active in the adult vasculature (10, 11). Induction of Angpt-2 sensitizes the microvasculature to inflammation and destabilizes quiescent ECs (12, 13). Furthermore, clinical results suggest that Angpt-2 in the most critically ill subjects rises not only from the release of preformed protein by stimulated ECs but also ongoing biosynthesis of new protein through unknown mechanisms (8).

Inhibition of Tie2 signaling appears to be critical for this leak-inflammation response because its stimulation with excess endogenous ligand, Angpt-1—or with a structurally unrelated peptide agonist—abrogates vascular leakage, attenuates inflammation, and improves survival in models of sepsis (14–16). Genetic deletion of ANGPT2 or a targeted antibody improves survival in experimental sepsis, ameliorates organ dysfunction, and

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

prevents vascular leakage (8, 17); protective effects are also observed in models of chronic infection (18).

On the basis of hypothesis that Angpt-2 may be an important mediator of septic phenotypes and recognizing the high barrier for bringing new drugs to market in critical illness, we sought inexpensive compounds with known safety profile that inhibit Angpt-2 production. Screening a Food and Drug Administration drug library, we identified statins, a class of drugs that blocks HMG-CoA reductase to lower low-density lipoprotein-cholesterol levels. Statins are also thought to exert important but incompletely understood effects on the vascular endothelium (19). Finally, given the unexplained discrepancy between beneficial effects in numerous preclinical models (20–22) and recent evidence suggesting lack of efficacy in clinical trials (23–27), we chose to investigate this drug class.

MATERIALS AND METHODS

A detailed description of the Methods is provided in the online supplement (Supplemental Digital Content 1, http://links.lww.com/CCM/B269).

Mouse Studies

The respective Institutional Animal Care and Use Committees approved all experiments. We used 8-week male C57BL/6J mice and ANGPT-2 heterozygous mice on a 129Sv/C57BL/6 mixed background and their wild-type (WT) littermates (gift of S. Wiegand, Regeneron Pharmaceuticals, and Tarrytown, NY). We induced experimental sepsis either with lipopolysaccharide from *Escherichia coli* serotype O111:B4 IP or by cecal ligation and puncture as described previously (28). Survival was followed more than 96 hours. A subset of animals was euthanized 16 hours after sepsis induction for further organ analysis. When simvastatin was compared with a vehicle control, the drug was given at a concentration of 0.2 μg/g body weight IP 24 hours before sepsis induction. Estimated circulating concentrations are reported in Supplemental Table 1 (Supplemental Digital Content 2, http://links.lww.com/CCM/B270).

Patient Cohorts (Retrospective and Randomized Placebo Controlled Trial)

Please see online supplement (Supplemental Digital Content 1, http://links.lww.com/CCM/B269) for details on the retrospective (NCT00529139) and prospective randomized placebo controlled trial (NCT00676897).

Inclusion criteria are summarized in Table 1.

Cell Culture Studies

Human umbilical vein ECs (HUVECs), human microvascular ECs, and the monocyte cell line U937 were cultured according to the manufacturer's instructions. Further details regarding reagents and readouts are provided in the online supplement (Supplemental Digital Content 1, http://links.lww.com/CCM/B269).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Molecular Analysis

Tissue (Angpt-2) and cellular (Angpt-2, von Willebrand factor [vWF], Foxo1) immunofluorescence were performed as previously described (8). Details on immunofluorescence, Western analysis, quantitative polymerase chain reaction, gel shift assays, and chromatin immunoprecipiation are available in the online supplement (Supplemental Digital Content 1, http://links.lww.com/CCM/B269) for.

Statistical Analysis

Statistical significance was evaluated by independent samples *t* test unless otherwise noted. Survival data were analyzed by log-rank test. Clinical data are presented as median (quartile 1–quartile 3) or percentage. In the case-control study, subjects were matched for sepsis severity and age. Longitudinal changes of Angpt-2 in the randomized controlled trial were analyzed by two-sided unpaired Mann-Whitney *U* test. Comparison between baseline characteristics was calculated by Fisher exact test and Mann-Whitney *U* test. Spearman correlation was used for Angpt-2 and soluble vascular cell adhesion molecule/sESelectin. All experimental results are presented as mean±SEM and two-tailed *p* value of less than 0.05 were considered to indicate statistical significance. Analysis and graph generation were performed in GraphPad Prism 6.0 (La Jolla, CA).

Study Approval

The respective Institutional Animal Care and Use Committees approved all animal experiments. Both human trials have been approved by the Institutional Review Boards for Hanover Medical School and Beth Israel Deaconess Medical Center and are registered at http://www.clinicaltrial.gov (NCT00529139 and NCT00676897 “Statin Therapy in the Treatment of Sepsis”).

RESULTS

FDA-Library Screening Identifies Simvastatin as a Potent Inhibitor of Angpt-2

ECs are the primary source of Angpt-2 in the body (29). Therefore, we applied an FDA-drug library for 24 hours to HUVECs and measured secreted Angpt-2 by enzyme-linked immunosorbent assay. Results were normalized for plate, and assay controls and are presented as fold versus median (Fig. 1*A*; Supplemental Table 2, Supplemental Digital Content 3, http://links.lww.com/CCM/B271). Two HMG-CoA reductase inhibitors, lovastatin and simvastatin, reduced Angpt-2 two- to three-fold. In vitro studies with simvastatin confirmed dose-dependent inhibition of Angpt-2 in different EC types with maximal inhibition observed at 10 μM (Fig. 1*B*) after 24 hours (Fig. 1*C*; Supplemental Fig. 1, Supplemental Digital Content 4, http://links.lww.com/CCM/B272). Simvastatin also suppressed Angpt-2 produced by ECs stimulated with lipopolysaccharides, phorbol ester, or conditioned medium from cultured monocytes (Supplemental Fig. 2, Supplemental Digital Content 4, http://links.lww.com/CCM/B272). The metabolite downstream of HMG-CoA reductase, mevalonate, reversed simvastatin’s effect on Angpt-2 (Fig. 1*D*), providing evidence to support HMG-CoA reductase dependence rather than a pleiotropic mechanism. Costaining of ECs for the Weibel Palade body proteins vWF and Angpt-2 showed an initial

accumulation of Angpt-2 following 1 hour of simvastatin (Supplemental Fig. 3, Supplemental Digital Content 4, http://links.lww.com/CCM/B272), suggesting impaired release as previously reported (30). However, by 24 hours of simvastatin exposure, intraendothelial Angpt-2, but not vWF, was also depleted (Fig. 1*E*).

Simvastatin Reduces Angpt-2 Transcription and Binding of the Transcription Factor Foxo1 in a PI3K/AKT-Dependent Manner

Consistent with the protein results, simvastatin dose-dependently reduced Angpt-2 mRNA abundance (Fig. 2*A*). Simvastatin was able to not only phosphorylate AKT but also required PI3 kinase activity to significantly suppress Angpt-2 (Supplemental Fig. 4, Supplemental Digital Content 4, http://links.lww.com/CCM/B272). Both, Krüppel-like factor-2 (KLF2) and Foxo1 have been described as potential transcriptional regulators of ANGPT2 in the literature (31, 32). Although simvastatin strongly induced KLF2 mRNA (Supplemental Fig. 5*A*, Supplemental Digital Content 4, http://links.lww.com/CCM/B272) as previously reported (33, 34), knockdown of KLF2 during simvastatin exposure did not restore Angpt-2 expression (Fig. 2*B*).

The 5′ region of ANGPT2 contains poorly resolved but powerful *cis*-elements (35). An in silico search revealed two putative Foxo1 binding sites (Fig. 2*C*; Supplemental Fig. 6, Supplemental Digital Content 4, http://links.lww.com/CCM/B272). We tested biotin-labeled and biotin-unlabeled DNA probes against these two sites in HUVEC nuclear extracts. Competition with six-fold excess of unlabeled probe established the specific bands of interest in gel shift assays (Fig. 2*D*). Application of simvastatin (10 μM) for 24 hours attenuated both bands, indicative of a decrease in bound protein (Fig. 2*E*). Chromatin immunoprecipitation with anti-Foxo1 antibody revealed significantly less binding of Foxo1 in the ANGPT2 promoter in simvastatin-treated cells (Fig. 2*F*). These results show that Foxo1 binds to distinct regions in the 5′ region of the ANGPT2 locus and that simvastatin attenuates this binding.

Simvastatin Induces Foxo1 Phosphorylation

Simvastatin exposure led to Foxo1 exclusion from the nucleus (Fig. 3, *A* and B), an event that is mediated by serine/threonine phosphorylation. Indeed, simvastatin induced endogenous Foxo1 phosphorylation at Ser-256 (Fig. 3, *C* and *D*), as well as phosphorylation of ectopically expressed WT Foxo1 (Ad-Foxo1; Fig. 3, *E* and *F*). As expected, when a triple-phosphorylation mutant Foxo1 (Ad-TM-Foxo1, Thr-24-Ala, Ser-256-Ala, and Ser-319-Ala)—constitutively active with regard to its function as a transcription factor—was expressed in ECs, phosphorylation of Foxo1 was undetectable, even in the presence of simvastatin (Fig. 3, *G*).

Foxo1 Phosphorylation Is Critical for the Suppression of Angpt-2 by Simvastatin

Transfection of ECs with control (βGal), WT Foxo1 (Ad-Foxo1), or triple-mutant Foxo1 (Ad-TM-Foxo1) adenovirus revealed progressive induction of Angpt-2 transcript and released protein (Fig. 4, *A* and *B*). Cells transfected with control adenovirus and then treated with simvastatin (10 μM) exhibited a marked reduction in Angpt-2 (left bars). The suppressive effect of simvastatin was even dominant to WT Foxo1 overexpression (Ad-

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Foxo1; middle bars). In contrast, cells transfected with triple-mutant Foxo1 (Ad-TM-Foxo1) were completely resistant to the Angpt-2-suppressive effect of simvastatin (right bars).

Simvastatin Suppresses Angpt-2 During Sepsis and Improves Sepsis Survival

We and others have shown that Angpt-2 contributes to adverse outcomes in experimental sepsis (8, 17) and that simvastatin improves survival in these models (20–22). As an initial test for potential interactions, we found that Angpt-2 was induced in endotoxemic mice, and that simvastatin pretreatment (–24 hr) was able to suppress Angpt-2 induction (Fig. 5*A*). Angpt-2 heterozygous mice are protected from death and end-organ injury following different models of sepsis (8). In Angpt-2 heterozygotes, simvastatin did not suppress Angpt-2 further (Supplemental Fig. 7, Supplemental Digital Content 4, http://links.lww.com/CCM/B272).

We then tested the interaction of simvastatin and Angpt-2 using a specific liposomal Angpt-2 siRNA delivery system that targets the pulmonary endothelium (36) in a polymicrobial model induced by cecal ligation and puncture (Fig. 5*B*). Simvastatin improved survival as anticipated (Fig. 5*C*, blue line). Depletion of Angpt-2 (blue dotted line) protected mice from sepsis-related mortality to a comparable extent as simvastatin (red line). But the combination of simvastatin and Angpt-2 siRNA (red dotted line) had no additive effect, consistent with a shared mechanism of action.

Statin Use Is Associated With Lower Circulating Angpt-2 Among Critically Ill Patients

In septic shock, eventual nonsurvivors not only have marked Angpt-2 elevation early in disease but also levels tend to *rise even higher* over time (8). To exemplary test the concept that statins suppress Angpt-2 in clinically meaningful contexts, we first studied a cohort of chronic simvastatin users (20–40 mg/d) on maintenance treatment from a previously published clinical trial (clinicaltrials.gov NCT00529139) who presented to medical care with pulmonary sepsis. Both groups had similar Acute Physiology and Chronic Health Evaluation II score, indicative of comparable disease severity. Subsequent inpatient mortality was lower in the statin group although this did not reach statistical significance. A higher proportion of males (39% vs 76%; $p = 0.049$) and a higher prevalence of pre-existing congestive heart failure (0% vs. 77%; $p < 0.001$) were present in the statin group (Table 2). Nonetheless, chronic statin users exhibited a lower Angpt-2 level than their counterparts (Fig. 6*A*).

To address confounding from gender distribution, heart failure, and other potential residual differences in the retrospective study, we then performed two independent exploratory randomized placebo-controlled trials in which statin-naïve patients presenting with sepsis to the emergency department received either simvastatin 40 mg orally once daily or a placebo pill. Because of the small numbers in each trial and the comparable study designs, the results were analyzed in aggregate. Baseline characteristics did not differ between treatment groups (Table 3), and there was only one death. To assess the safety of this intervention, we tested liver function (alanine transaminase or aspartate transaminase) and creatine phosphokinase levels at baseline and at 48 hours post initiation of statin therapy. We found that these levels were similar in both groups (all $p > 0.05$) and that none rose into the abnormal range. We

observed a significant reduction in circulating Angpt-2 over time and a trend toward improved tumor necrosis factor-α levels in the statin group (Fig. 6, *B* and *C*). Consistent with the concept that Angpt-2 is an upstream mediator of endothelial inflammation (12), the change in Angpt-2 over time was strongly associated with the change in soluble vascular cell adhesion molecule-1 ($r = 0.50$; $p = 0.01$) and sEselectin ($r = 0.54$; $p = 0.006$).

DISCUSSION

Using an unbiased screen, we found that statins attenuated the production of Angpt-2 by ECs, an emerging mechanistic target in sepsis-related vascular leakage (3, 8). Subsequent cellular studies revealed a novel mechanism of statin action in the endothelium, ie, phosphorylation of the transcription factor Foxo1 that prevents its nuclear translocation and subsequent binding to the ANGPT2 promoter. Studies in septic WT and Angpt-2 heterozygous mice revealed the physiological significance of statin-mediated Angpt-2 suppression. Finally, the potential medical impact of these findings was exemplified by two independent studies of individuals hospitalized with critical illness.

Since the initial reports of statin benefits in preclinical sepsis models (20–22), a number of putative molecular mechanisms have been identified, involving the endothelium and other cell types (summarized in [37]). Among the several endothelial pathways implicated in sepsis, results from Angpt-2 overexpression studies, chemically inflamed and septic Angpt-2 knockout mice, and infected mice treated with an Angpt-2–neutralizing antibody all strongly suggest that this molecule is a major effector of the vascular pathophysiology in sepsis (3, 8, 12, 17, 18, 38). Furthermore, these loss- and gain-of-function experimental findings have been corroborated by more than 20 independent clinical studies in sepsis and related diseases (summarized in [39]). Interestingly, Angpt-2 appears to be both a potent biomarker of outcome and a promising candidate disease mediator. Therefore, although statins clearly have multiple vascular and nonvascular salutary effects, the importance of Angpt-2 in sepsis is supported by a large body of independently reported experimental and human evidence.

Seeking pathways beyond lipid-lowering to account for statin benefits in cardiovascular disease, Kureishi et al (40) observed activation of endothelial nitric oxide synthase (eNOS) and statin-induced angiogenesis in a chronic ischemia mouse model, and Pleiner et al (41) showed simvastatin-dependent preservation of endothelial-NO-dependent flow responses. However, eNOS-null mice respond to different noxious stimuli with *less* vascular leakage and *less* cellular inflammation, arguing against a putative statin-eNOS mechanism of benefit in experimental sepsis. Focusing on vascular permeability, groups led by Jacobsen and van Niew Amerongen independently found that endothelial monolayers pretreated with simvastatin fortify barrier function against inflammatory mediators of permeability (19, 42). Statins appear to remodel the endothelial cytoskeleton and intercellular junctions to tighten the barrier by using the same Rho family GTPases that are modulated downstream of the Angpt-2 receptor, Tie2 (3, 8, 14, 43, 44).

In experimental sepsis, Angpt-1 is reduced, Angpt-2 is induced, and the phosphorylation of Tie2 and Akt are reduced (14). Activation of Tie2 by Angpt-1 leads to Akt-mediated Foxo1 phosphorylation at both Ser-256 and Thr-24 and a consequent reduction in Angpt-2 (31).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Presently, simvastatin induced phosphorylation of Foxo1 at Ser-256 and Thr-24 and reduced Foxo1 binding to the ANGPT2 promoter. Together, the results support a mechanism in which statin-mediated Foxo1 phosphorylation retards the nuclear trafficking of Foxo1 and inhibits its DNA binding to the ANGPT2 promoter. Given the recent demonstration that the global knockout of Foxo1 is rescued by EC-specific reconstitution, statin-dependent modulation of Foxo1 may exert its most potent impact on the endothelium (45). Perhaps not surprisingly, Angpt-2 was one of the most reliable indicators of Foxo1 activity in both the gain- and the loss-of-function mouse models reported by Dharaneeswaran et al (45).

Synthesizing the present results, the transition from healthy to septic vasculature may be instigated by an initial reduction in Tie2 signaling driven by the cytokine-mediated rapid release of preformed Angpt-2 protein (9). Impairment of Tie2 signaling, in turn, relieves the otherwise tonic suppression of Foxo1, leading to sustained Angpt-2 biosynthesis that further destabilizes blood vessels toward a leaky, inflammatory phenotype (Fig. 7). Although the cytokine-dependent release of preformed Angpt-2 protein is well-known (9), serial Angpt-2 measurements from critically ill patients clearly suggest that the sickest patients exhibit ongoing Angpt-2 production (3, 8). Because Angpt-2 antagonizes Tie2 in the context of inflammation, a profound initial release of preformed Angpt-2 could initiate a feedback loop that sustains (and perhaps amplifies) the deleterious state of Tie2 signaling impairment. Thus, primary prevention of Angpt-2 induction may be more effective clinically than efforts to intervene after an Angpt-2/Tie2 feedback loop has been established. Intriguingly, approximately 40 human studies suggest an association between chronic prior statin use and improved outcomes among patients with infection (summarized in [46]), whereas the results of acutely administered statins have been mixed or even negative (24–27, 46). In our mouse studies, 24-hour pretreatment with statins was intended to model chronic dosing because this duration of treatment potently reduced Angpt-2 expression in vitro.

Several questions merit further investigation. First, the pharmacology of statins in sepsis is problematic because first-pass metabolism, particularly after oral administration, limits systemic endothelial availability. Therefore, the present results should spur new thinking about ways to deliver existing statin drugs to the endothelium (eg, nanoparticles) or consider novel pharmacological approaches to targeting pulmonary endothelial HMG-CoA reductase. Second, there may be additional mechanistic links, such as statin-driven regulation of Foxo1-modulating serine-threonine phosphatases and the prevalence of Foxo1 mutancy. Third, although our in vivo results argue that Angpt-2 is a major effector down-stream of simvastatin in sepsis, additional studies are needed to address the contribution of Angpt-2 to the chronic cardiovascular effects of statins. Finally, the trial results presented here help address some of the shortcomings of our own retrospective analysis, but both investigations merit larger and better-powered follow-up studies that focus on Angpt-2 as a potential stratifier for statin benefit.

CONCLUSIONS

The present results elucidate the molecular apparatus that sustains ongoing Angpt-2 biosynthesis in the septic endothelium. Through an unbiased approach, they identify statins as potential inhibitors of Angpt-2 and propose a novel mechanism for this widely used drug

class. In the setting of recent negative clinical trial results with statins in ICU situations, our results offer two concrete implications—first, pharmacologically attenuating the Angpt-2-Tie-2 feedback loop may require a preventative approach; and second, early (point of care) measurement of circulating Angpt-2 levels may help stratify subjects responsive to adjunctive statin treatment for future clinical trials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank S.A. Karumanchi for use of the Food and Drug Administration drug library and J. Wenger for statistical review of our data.

S.D. is supported by the German Research Foundation (DA 1209/4-1), Else Kröner-Fresenius Stiftung (2013_A154), and Silence Therapeutics; S.M.P. by the National Institutes of Health (R01-HL093234, R01-HL125275, and R01-DK095072), and the American Diabetes Association (1-13-BS-141).

Dr. Maski is employed by Beth Isreal Deaconess Medical Center. Dr. Milam is employed by Beth Isreal Deaconess Medical Center. Dr. Rajakumar received support for article research from Howard Hughes Medical Institute (HHMI). Dr. Santel is employed by Silence Therapeutics and GmbH, has patent, received royalties, and has stock with Silence Therapeutics plc. Dr. Chase received support for article research from the National Institutes of Health (NIH) and NIGMS-K23 Mentored Patient oriented Research Career Development Award, 2013–2018). Dr. Donnino consulted for the American Heart Association, received grant support from the NIH and American Heart Association, and received support for article research from the NIH. Dr. Parikh consulted for Boston Clinical Research Institute and received support for article research from the NIH. His institution received grant support from the NIH.

REFERENCES

1. Martin GS, Mannino DM, Eaton S, Moss M. The epidemiology of sepsis in the United States from 1979 through 2000. N Engl J Med. 2003; 348(16):1546–1554. [PubMed: 12700374]
2. Ranieri VM, Thompson BT, Barie PS, et al. PROWESS-SHOCK Study Group. Drotrecogin alfa (activated) in adults with septic shock. N Engl J Med. 2012; 366:2055–2064. [PubMed: 22616830]
3. Parikh SM, Mammoto T, Schultz A, et al. Excess circulating angiopoietin-2 may contribute to pulmonary vascular leak in sepsis in humans. PLoS Med. 2006; 3:e46. [PubMed: 16417407]
4. Calfee CS, Gallagher D, Abbott J, et al. NHLBI ARDS Network: Plasma angiopoietin-2 in clinical acute lung injury: Prognostic and pathogenetic significance. Crit Care Med. 2012; 40:1731–1737. [PubMed: 22610178]
5. Giuliano JS Jr, Lahni PM, Harmon K, et al. Admission angiopoietin levels in children with septic shock. Shock. 2007; 28:650–654. [PubMed: 18092380]
6. Kümpers P, Lukasz A, David S, et al. Excess circulating angiopoietin-2 is a strong predictor of mortality in critically ill medical patients. Crit Care. 2008; 12:R147. [PubMed: 19025590]
7. Ricciuto DR, dos Santos CC, Hawkes M, et al. Angiopoietin-1 and angiopoietin-2 as clinically informative prognostic biomarkers of morbidity and mortality in severe sepsis. Crit Care Med. 2011; 39:702–710. [PubMed: 21242795]
8. David S, Mukherjee A, Ghosh CC, et al. Angiopoietin-2 may contribute to multiple organ dysfunction and death in sepsis*. Crit Care Med. 2012; 40:3034–3041. [PubMed: 22890252]
9. Fiedler U, Scharpfenecker M, Koidl S, et al. The Tie-2 ligand angiopoietin-2 is stored in and rapidly released upon stimulation from endothelial cell Weibel-Palade bodies. Blood. 2004; 103:4150–4156. [PubMed: 14976056]
10. Wong AL, Haroon ZA, Werner S, et al. Tie2 expression and phosphorylation in angiogenic and quiescent adult tissues. Circ Res. 1997; 81:567–574. [PubMed: 9314838]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

11. Yuan HT, Khankin EV, Karumanchi SA, et al. Angiopoietin 2 is a partial agonist/antagonist of Tie2 signaling in the endothelium. Mol Cell Biol. 2009; 29:2011–2022. [PubMed: 19223473]
12. Fiedler U, Reiss Y, Scharpfenecker M, et al. Angiopoietin-2 sensitizes endothelial cells to TNF-alpha and has a crucial role in the induction of inflammation. Nat Med. 2006; 12:235–239. [PubMed: 16462802]
13. Scharpfenecker M, Fiedler U, Reiss Y, et al. The Tie-2 ligand angiopoietin-2 destabilizes quiescent endothelium through an internal autocrine loop mechanism. J Cell Sci. 2005; 118:771–780. [PubMed: 15687104]
14. David S, Ghosh CC, Kümpers P, et al. Effects of a synthetic PEGylated Tie-2 agonist peptide on endotoxemic lung injury and mortality. Am J Physiol Lung Cell Mol Physiol. 2011; 300:L851–L862. [PubMed: 21421750]
15. David S, Park JK, Meurs Mv, et al. Acute administration of recombinant Angiopoietin-1 ameliorates multiple-organ dysfunction syndrome and improves survival in murine sepsis. Cytokine. 2011; 55:251–259. [PubMed: 21531574]
16. Witzenbichler B, Westermann D, Knueppel S, et al. Protective role of angiopoietin-1 in endotoxic shock. Circulation. 2005; 111:97–105. [PubMed: 15611372]
17. Ziegler T, Horstkotte J, Schwab C, Pfetsch V, Weinmann K, Dietzel S, Rohwedder I, Hinkel R, Gross L, Lee S, et al. Angiopoietin 2 mediates microvascular and hemodynamic alterations in sepsis. J Clin Invest. 2013; 123:3436–3445.
18. Tabruyn SP, Colton K, Morisada T, et al. Angiopoietin-2-driven vascular remodeling in airway inflammation. Am J Pathol. 2010; 177:3233–3243. [PubMed: 20952594]
19. van Nieuw Amerongen GP, Vermeer MA, Nègre-Aminou P, et al. Simvastatin improves disturbed endothelial barrier function. Circulation. 2000; 102:2803–2809. [PubMed: 11104736]
20. Ando H, Takamura T, Ota T, et al. Cerivastatin improves survival of mice with lipopolysaccharide-induced sepsis. J Pharmacol Exp Ther. 2000; 294:1043–1046. [PubMed: 10945857]
21. Merx MW, Liehn EA, Janssens U, et al. HMG-CoA reductase inhibitor simvastatin profoundly improves survival in a murine model of sepsis. Circulation. 2004; 109:2560–2565. [PubMed: 15123521]
22. Merx MW, Liehn EA, Graf J, et al. Statin treatment after onset of sepsis in a murine model improves survival. Circulation. 2005; 112:117–124. [PubMed: 15998696]
23. Kruger PS, Harward ML, Jones MA, et al. Continuation of statin therapy in patients with presumed infection: A randomized controlled trial. Am J Respir Crit Care Med. 2011; 183:774–781. [PubMed: 20959555]
24. Papazian L, Roch A, Charles PE, et al. STATIN-VAP Study Group: Effect of statin therapy on mortality in patients with ventilator-associated pneumonia: A randomized clinical trial. JAMA. 2013; 310:1692–1700. [PubMed: 24108510]
25. Patel JM, Snaith C, Thickett DR, et al. Randomized double-blind placebo-controlled trial of 40 mg/day of atorvastatin in reducing the severity of sepsis in ward patients (ASEPSIS Trial). Crit Care. 2012; 16:R231. [PubMed: 23232151]
26. Truwit JD, Bernard GR, Steingrub J, Matthay MA, Liu KD, Albertson TE, Brower RG, Shanholtz C, Rock P, Douglas IS, et al. Rosuvastatin for sepsis-associated acute respiratory distress syndrome. N Engl J Med. 2014; 370:2191–2200. [PubMed: 24835849]
27. McAuley DF, Laffey JG, O'Kane CM, et al. HARP-2 Investigators; Irish Critical Care Trials Group: Simvastatin in the acute respiratory distress syndrome. N Engl J Med. 2014; 371:1695–1703. [PubMed: 25268516]
28. Rittirsch D, Huber-Lang MS, Flierl MA, et al. Immunodesign of experimental sepsis by cecal ligation and puncture. Nat Protoc. 2009; 4:31–36. [PubMed: 19131954]
29. Maisonpierre PC, Suri C, Jones PF, et al. Angiopoietin-2, a natural antagonist for Tie2 that disrupts *in vivo* angiogenesis. Science. 1997; 277:55–60. [PubMed: 9204896]
30. Hilbert T, Poth J, Frede S, et al. Anti-atherogenic effects of statins: Impact on angiopoietin-2 release from endothelial cells. Biochem Pharmacol. 2013; 86:1452–1460. [PubMed: 24041741]
31. Daly C, Wong V, Burova E, et al. Angiopoietin-1 modulates endothelial cell function and gene expression via the transcription factor FKHR (FOXO1). Genes Dev. 2004; 18:1060–1071. [PubMed: 15132996]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

32. Kawanami D, Mahabeleshwar GH, Lin Z, et al. Kruppel-like factor 2 inhibits hypoxia-inducible factor 1alpha expression and function in the endothelium. J Biol Chem. 2009; 284:20522–20530. [PubMed: 19491109]
33. Parmar KM, Nambudiri V, Dai G, et al. Statins exert endothelial atheroprotective effects via the KLF2 transcription factor. J Biol Chem. 2005; 280:26714–26719. [PubMed: 15878865]
34. Sen-Banerjee S, Mir S, Lin Z, et al. Kruppel-like factor 2 as a novel mediator of statin effects in endothelial cells. Circulation. 2005; 112:720–726. [PubMed: 16043642]
35. Hegen A, Koidl S, Weindel K, et al. Expression of angiopoietin-2 in endothelial cells is controlled by positive and negative regulatory promoter elements. Arterioscler Thromb Vasc Biol. 2004; 24:1803–1809. [PubMed: 15284088]
36. Santel A, Aleku M, Keil O, et al. A novel siRNA-lipoplex technology for RNA interference in the mouse vascular endothelium. Gene Ther. 2006; 13:1222–1234. [PubMed: 16625243]
37. Terblanche M, Almog Y, Rosenson RS, et al. Statins and sepsis: Multiple modifications at multiple levels. Lancet Infect Dis. 2007; 7:358–368. [PubMed: 17448939]
38. Bhandari V, Choo-Wing R, Lee CG, et al. Hyperoxia causes angiopoietin 2-mediated acute lung injury and necrotic cell death. Nat Med. 2006; 12:1286–1293. [PubMed: 17086189]
39. Parikh SM. Dysregulation of the angiopoietin-Tie-2 axis in sepsis and ARDS. Virulence. 2013; 4:517–524. [PubMed: 23652985]
40. Kureishi Y, Luo Z, Shiojima I, et al. The HMG-CoA reductase inhibitor simvastatin activates the protein kinase Akt and promotes angiogenesis in normocholesterolemic animals. Nat Med. 2000; 6:1004–1010. [PubMed: 10973320]
41. Pleiner J, Schaller G, Mittermayer F, et al. Simvastatin prevents vascular hyporeactivity during inflammation. Circulation. 2004; 110:3349–3354. [PubMed: 15520323]
42. Jacobson JR, Dudek SM, Birukov KG, et al. Cytoskeletal activation and altered gene expression in endothelial barrier regulation by simvastatin. Am J Respir Cell Mol Biol. 2004; 30:662–670. [PubMed: 14630613]
43. David S, Ghosh CC, Mukherjee A, Parikh SM. Angiopoietin-1 requires IQ domain GTPase-activating protein 1 to activate Rac1 and promote endothelial barrier defense. Arterioscler Thromb Vasc Biol. 2011; 31:2643–2652. [PubMed: 21885850]
44. Mammoto T, Parikh SM, Mammoto A, et al. Angiopoietin-1 requires p190 RhoGAP to protect against vascular leakage in vivo. J Biol Chem. 2007; 282:23910–23918. [PubMed: 17562701]
45. Dharaneeswaran H, Abid MR, Yuan L, et al. FOXO1-mediated activation of Akt plays a critical role in vascular homeostasis. Circ Res. 2014; 115:238–251. [PubMed: 24874427]
46. Kruger PS, Venkatesh B. Are there any benefits from statin treatment for the septic patient? Curr Atheroscler Rep. 2014; 16:378. [PubMed: 24277655]

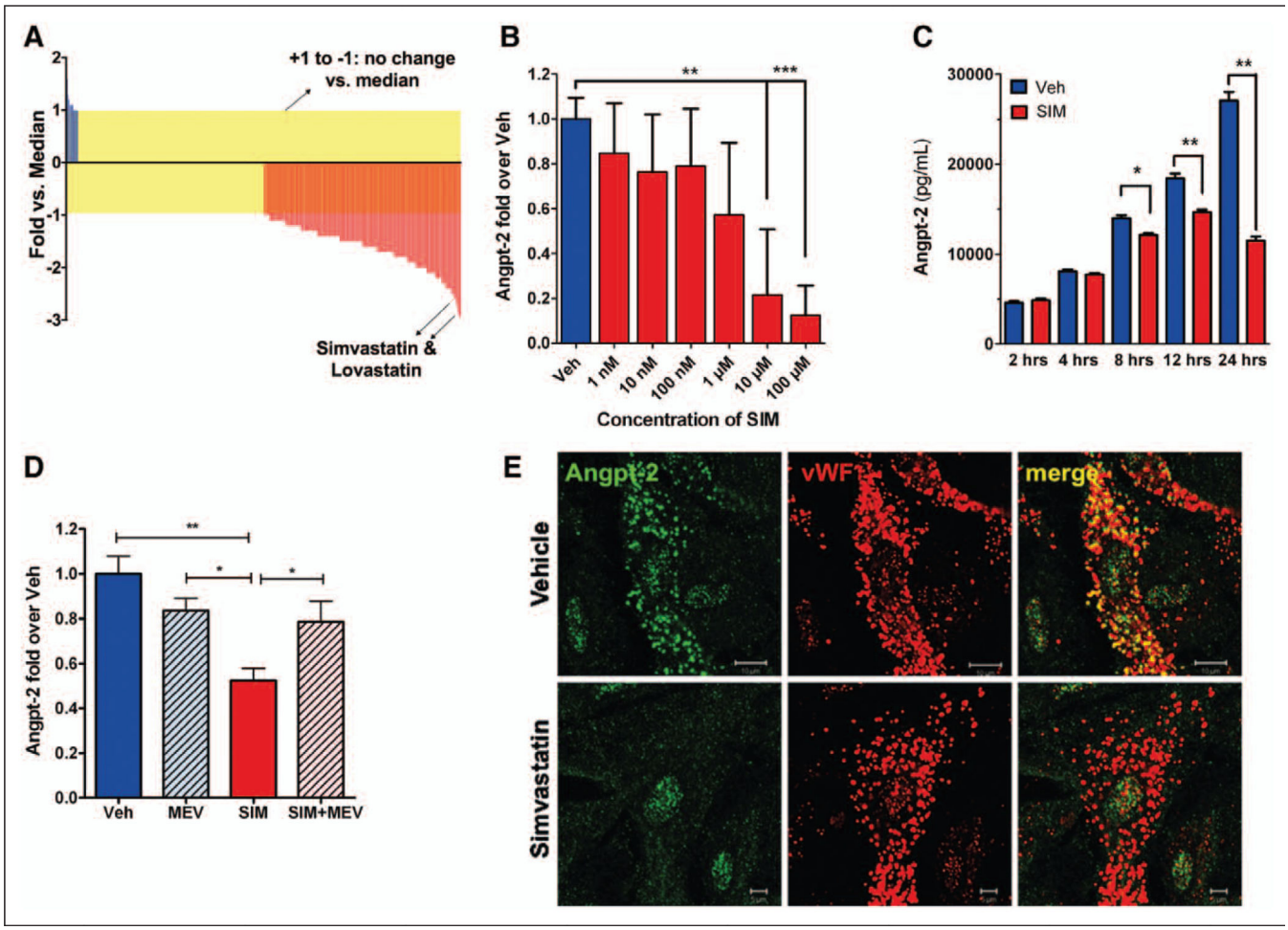

Figure 1.

Food and Drug Administration (FDA) library screening identifies simvastatin (SIM) as a potent inhibitor of Angpt-2 production. **A**, Human umbilical vein endothelial cells (HUVECs) grown in a 96-well format were treated with an FDA-drug library for 24 hr, and secreted Angpt-2 protein was measured by enzyme-linked immunosorbent assay (ELISA). Results were analyzed as the fold-change relative to the median value and ordered from strongest inducers (left, *blue bars*) to strongest inhibitors (right, *red bars*). The yellow window spans ±1-fold change. **B**, Simvastatin (SIM, 10 μM) was applied to HUVECs for 24 hr at indicated doses and secreted Angpt-2 protein was measured by ELISA. **C**, Simvastatin (10 μM) was applied to HUVECs for indicated durations, after which Angpt-2 protein levels in the conditioned media were measured by ELISA $^{*}p < 0.05$, $^{**}p < 0.01$. **D**, HUVECs were treated with SIM (10 μM) or an excess of mevalonate (MEV, 200 μM), and secreted Angpt-2 protein was measured by ELISA. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. **E**, HUVECs were stained for Angpt-2 (*green*) and a marker of Weibel Palade bodies (ie, von Willebrand Factor, red) 24 hr after vehicle or SIM (10 μM) treatment.

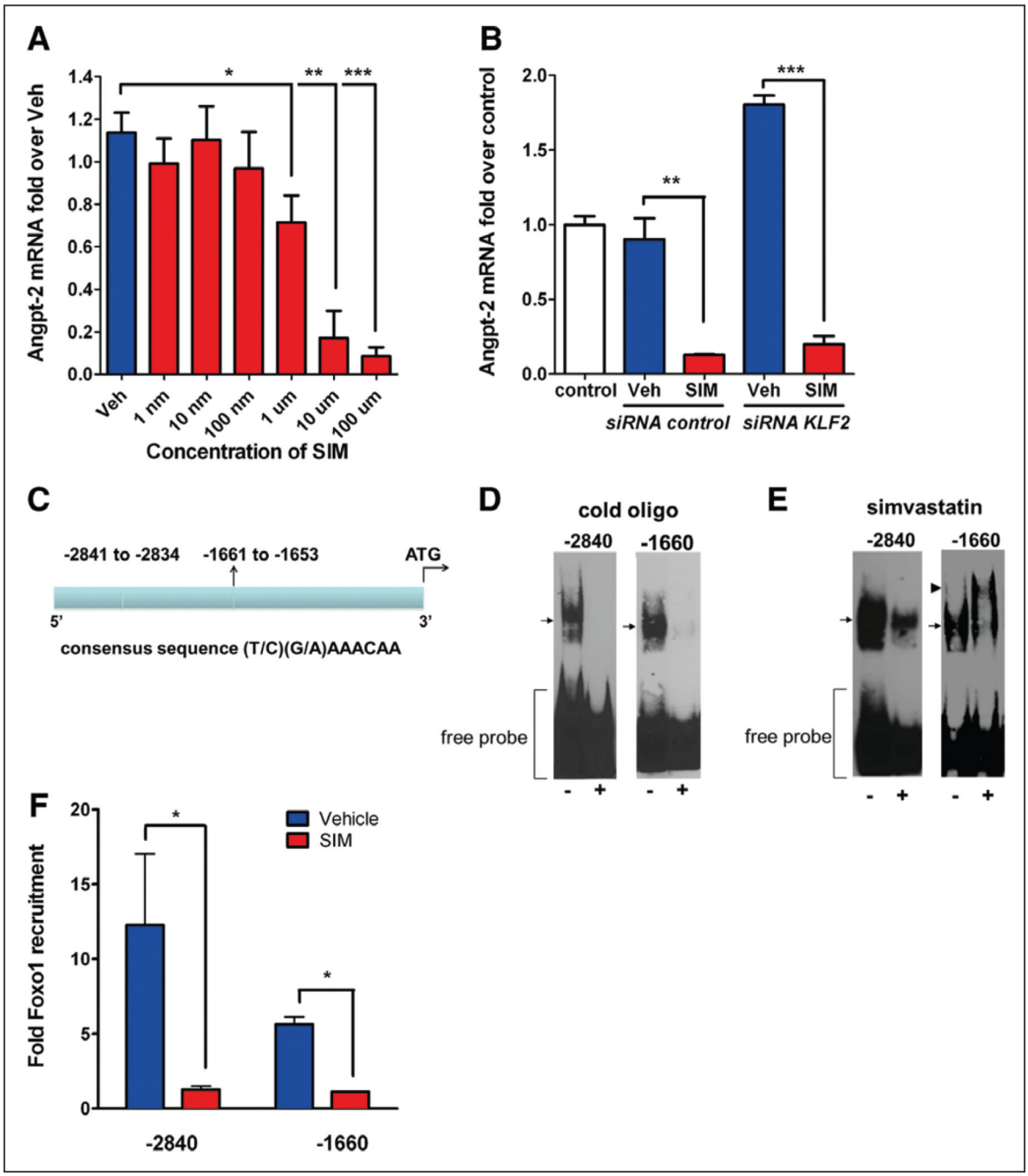

Figure 2.

Simvastatin reduces angiopoietin-2 (Angpt-2) transcription and binding of the transcription factor Foxo1. **A**, Angpt-2 mRNA concentrations were measured via real-time polymerase chain reaction (RT-PCR) 24 hr after applying simvastatin (SIM) at indicated concentrations to human umbilical vein endothelial cells (HUVECs). **B**, SIM (10 μM) was applied for 24 hr to HUVECs pretreated with control or Krüppel-like factor-2 (KLF2) siRNA and Angpt-2 mRNA was measured by RT-PCR. **C**, Putative Foxo1 binding sites were identified at –2,840 and –1,660 in the three kilobases 5′ to the translational start site of human ANGPT2 by

TFsearch and cross-checked by aligning the consensus Foxo1 binding sequence (in bold). **D**, Nuclear extracts of HUVECs were incubated with biotin labeled DNA probes specific for the –2,840 and –1,660 sites of ANGPT2 in the presence (+) or absence (–) of six-fold excess unlabeled probes. Arrows indicate the specific band eliminated by competition. **E**, Nuclear extracts of HUVECs either treated with vehicle (–) or SIM (10 μM, +) for 24 hr were prepared and incubated with biotin-labeled DNA probes specific for the –2,840 and –1,660 sites of ANGPT2. Arrows indicate the band of interest and arrowhead indicates a non-specific band. **F**, HUVECs treated with SIM (10 μM) for 24 hr were gently lysed and chromatin immunoprecipitation was performed with anti-Foxo1. Results of RT-PCR to quantify ANGPT2 promoter concentration are shown for the –2,840 and –1,660 sites. *$p < 0.05$, ***$p < 0.001$.

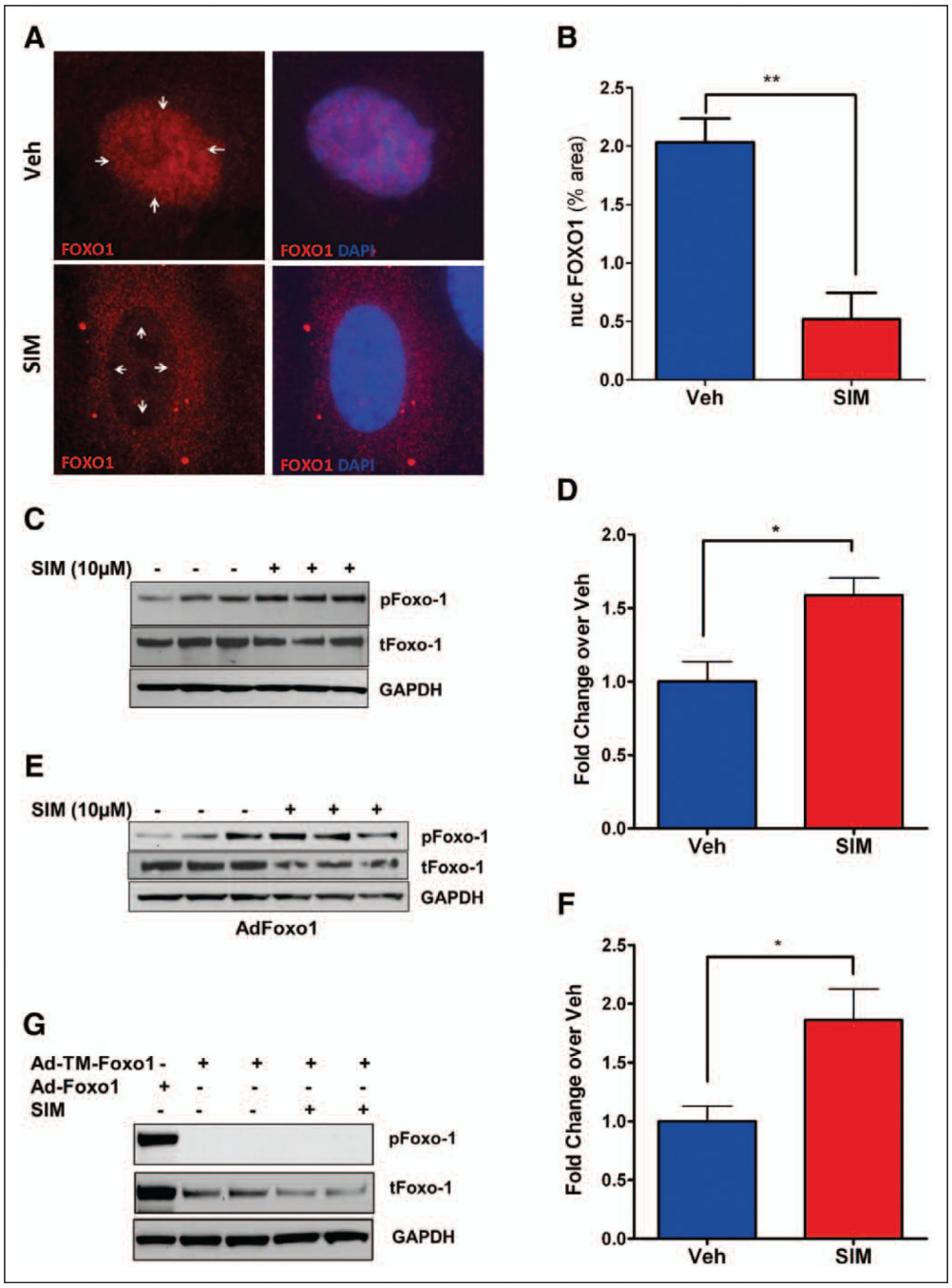

Figure 3.

Simvastatin prevents nuclear Foxo1 translocation by phosphorylation. **A**, Endothelial cells (ECs) treated with simvastatin (SIM, 10 μM) were stained for Foxo1 (*red*) and nuclei (4',6-diamidino-2-phenylindole, *blue*). White arrows indicate the edge of nuclear staining. **B**, Planimetric quantification of staining results by surveying 10 high-powered fields (×40) per slide. *$p < 0.05$ and **$p < 0.01$. **C**, HUVEC lysates 24 hr after SIM treatment (10 μM) were immunoblotted with anti-pSer^{256}-Foxo1 (pFoxo-1), anti-Foxo1 (tFoxo-1), and anti-GAPDH as a loading control. **D**, Densitometric quantification of the above results. **E**,

Immunoblotting as described above for lysates of ECs infected with a virus encoding wild-type Foxo1 (AdFoxo1) for 24 hr and treated with SIM (10 μM) for 24 hr or vehicle. **F**, Quantification of the above results. **G**, Immunoblotting as described above for lysates of ECs infected with a virus encoding triple-phosphorylation mutant Foxo1 (TM Foxo1) before 24-hr treatment with SIM (10 μM) or vehicle. *$p < 0.05$.

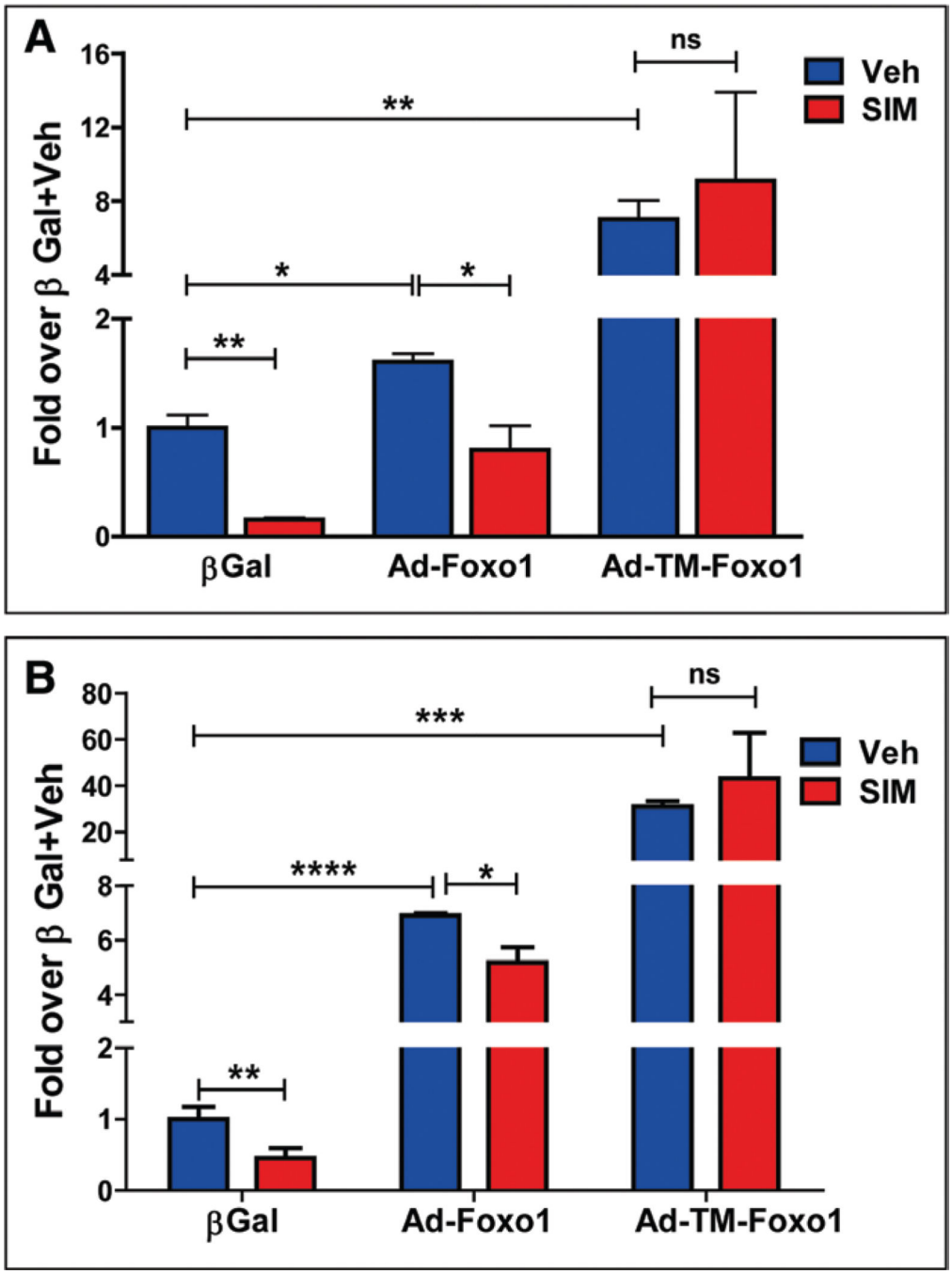

Figure 4.

Foxo1 phosphorylation is critical for the suppression of Angpt-2 by simvastatin. **A**, Real-time polymerase chain reaction for Angpt-2 24 hr after treating virally transduced human umbilical vein endothelial cells with simvastatin (SIM, 10 μM) or vehicle. "βGal" is a control virus expressing β-galactosidase; "AdFoxo1" is a virus encoding wild-type Foxo1; and "Ad-TM-Foxo1" is a virus encoding the triple phosphorylation constitutively active mutant Foxo1. **B**, ELISA for secreted Angpt-2 protein for the above conditions. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

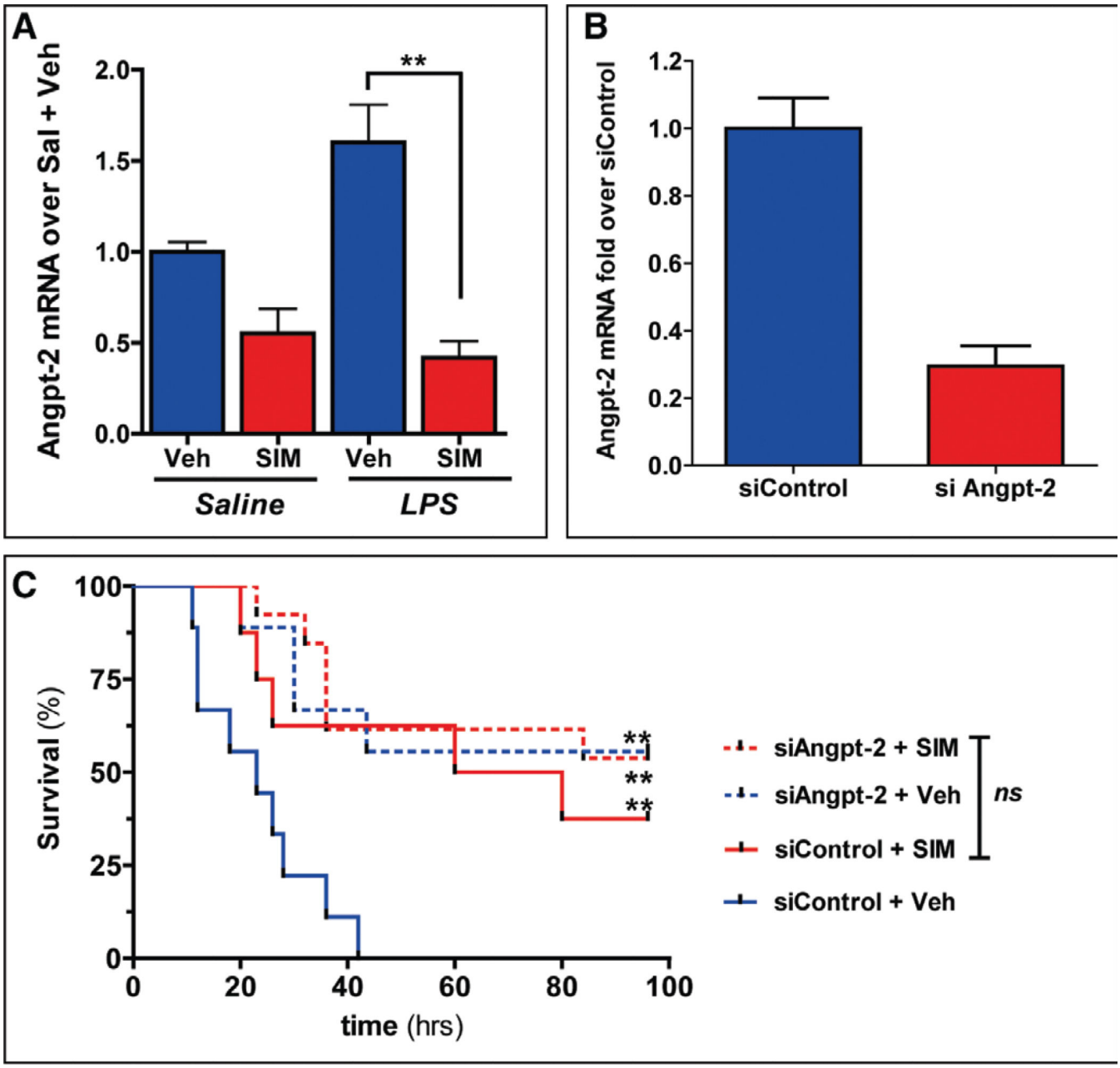

Figure 5.

Simvastatin (SIM) suppresses Angpt-2 during sepsis and improves sepsis survival. **A**, Adult male C57BL/6 mice treated with SIM (0.2 μg/g body weight [BW] IP) or vehicle (Veh) 24 hr before the injection with gram-negative endotoxin (lipopolysaccharides [LPS] from *Escherichia coli* IP) or saline control. Angpt-2 mRNA was measured 16 hr after LPS by real-time polymerase chain reaction (RT-PCR) in lung homogenates. (n = 4–9 mice per group, $^{**}p < 0.01$). **B**, Angpt-2 mRNA was measured by quantitative RT-PCR from murine lungs 48 hr after treatment with an Angpt-2 or control siRNA specifically targeting the pulmonary endothelium (n = 9 mice per group, $^{**}p < 0.01$). **C**, Adult male C57BL/6 mice (n = 10 mice per each of the four study arms) were treated with SIM (200 μg/kg BW IP) versus

vehicle solution and control siRNA versus Angpt-2 siRNA as described in the *Materials and Methods* section. Survival after cecal ligation and puncture (CLP) was followed. $^{**}p < 0.01$ by log-rank test for all conditions versus animals treated with control siRNA and vehicle. Survival following CLP was statistically indistinguishable among the remaining groups.

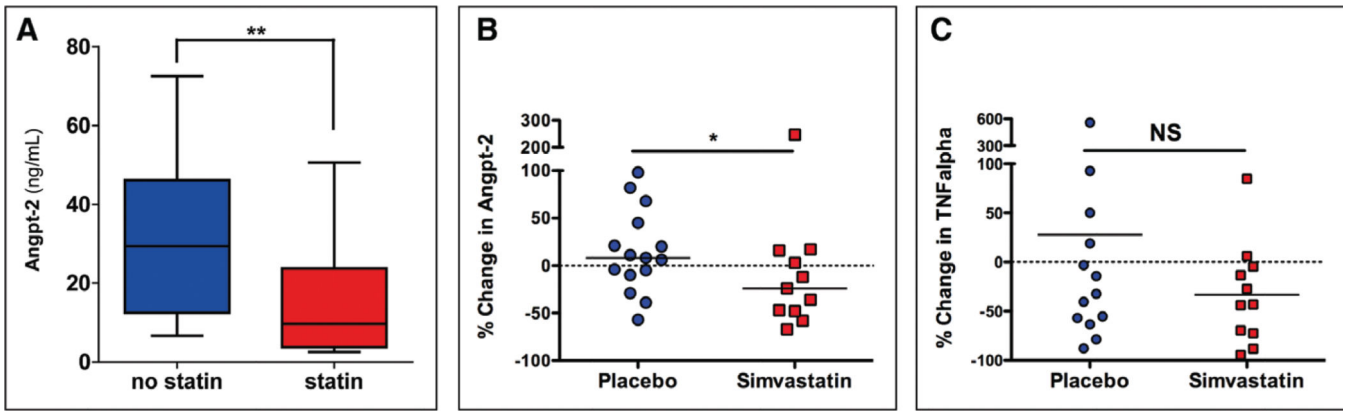

Figure 6.

Chronic or acute statin use is associated with lower circulating Angpt-2 among critically ill patients. **A**, Circulating Angpt-2 concentration at the time of enrollment in a case-control study of critically ill subjects comparing chronic statin users versus statin-naïve individuals. **B**, Change in circulating Angpt-2 over the first 48–72 hr after randomization in a placebo controlled clinical trial of statin-naïve septic individuals receiving 40 mg simvastatin orally once daily or placebo pill (*$p < 0.05$ and **$p < 0.01$).

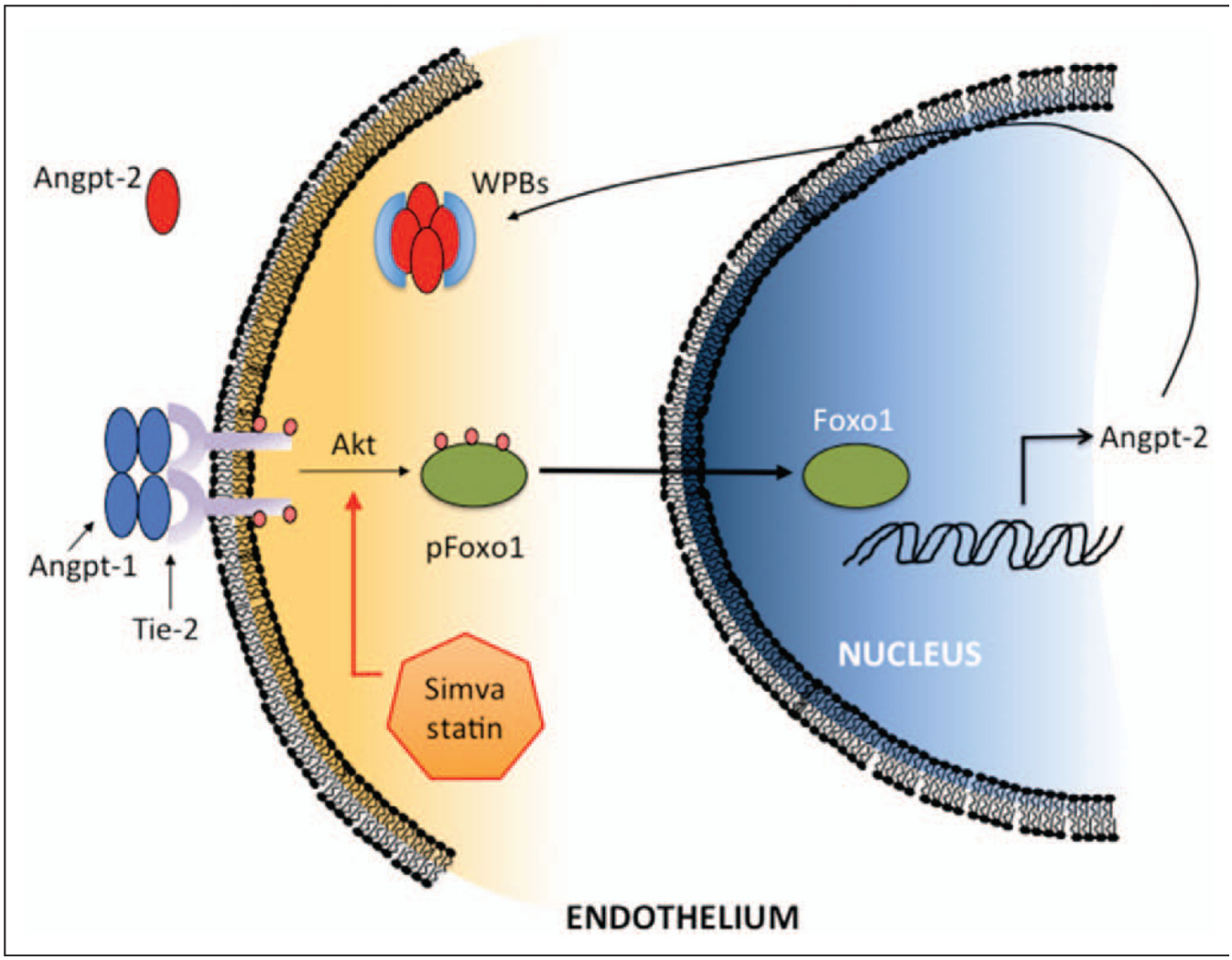

Figure 7.

Proposed mechanism for statin-mediated suppression of ongoing Angpt-2 production during sepsis. In endothelium of quiescent blood vessels, Tie2 is tonically expressed and activated. Through downstream activation of Akt, Foxo1 is phosphorylated and Angpt-2 transcription is suppressed. During the initial phase of sepsis, pre-formed Angpt-2 stored in Weibel Palade bodies (WPBs) is rapidly exocytosed in response to early acute-phase cytokines. Excess Angpt-2 impairs Tie2 signaling, which in turn, reduces Foxo1 phosphorylation and enables it to drive the transcription of the ANGPT2 gene. Simvastatin favors the phosphorylation of Foxo1, thus attenuating the production of Angpt-2.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Table 1

Enrollment Criteria for Sepsis-Simvastatin Randomized Trials

Adult (> 18 yr) patients	Concomitant statin, cyclosporine or digoxin use
Confirmed or suspected infection	Pregnant
Two or more systemic inflammatory response syndrome criteria:	Liver transaminase elevation > 3× normal
Temperature > 100.4°F or < 96.8°F	Creatinine phosphokinase > 6× normal
Heart rate > 90 bpm	Unable to take enteral medication
Respiratory rate > 20 breaths/min or	
Oxygen saturation < 90% on supplemental oxygen	
White blood cell count > 12,000 or < 4,000 cells/μL or > 10% bandemia	
NB: for the septic shock cohort, shock was defined as vasopressor use > 1 hr	

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Table 2

Baseline Characteristics of Retrospective Case-Control Study

Characteristics	No Statin (n = 13)	Statin (n = 13)	p
Age (yr)	51 (36–62)	56 (47–63)	0.64
Men (%)	39	76	0.049
Race (% Caucasian)	100	100	1.0
Comorbidity (%)			
Congestive heart failure	0	77	< 0.001
Prior stroke/transient ischemic attack	8	0	0.31
Chronic lung disease	15	31	0.35
Chronic kidney disease	46	38	0.69
Cancer	8	0	0.31
Diabetes	46	23	0.21
Disease severity at enrollment			
Acute Physiology and Chronic Health Evaluation II	31 (27–34)	30 (27–34)	0.97
Lactate (mg/dL)	2.8 (1.7–4.8)	4.4 (1.5–10.5)	0.48
In-hospital mortality (%)	46.2	15.4	0.096

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Table 3

Baseline Characteristics of the Simvastatin Randomized Placebo-Controlled Clinical Trial

Characteristics	Placebo (n = 15)	Simvastatin (n = 11)	p
Age (yr)	54 (47–62)	56 (53–72)	0.11
Males (%)	27	45	0.42
Race (% Caucasian)	80	91	1.0
Comorbidity (%)			
Congestive heart failure	13	18	1.0
Prior stroke/transient ischemic attack	27	0	0.11
Chronic lung disease	13	27	0.62
Chronic kidney disease	33	18	0.66
Cancer	7	36	0.13
Diabetes	20	9	0.61
Sepsis severity at enrollment			
Acute Physiology and Chronic Health Evaluation II	18 (3–20)	16 (9–21)	0.46
Lactate (mg/dL)	1.9 (1.3–2.4)	2.5 (1.5–3.1)	0.11
In-hospital mortality (%)	6	0	1.0