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Human Serum Albumin Is an Essential Component of the Host Defense Mechanism Against *Clostridium difficile* Intoxication

Alessandra di Masi,[1,a] Loris Leboffe,[1] Fabio Polticelli,[1,2] Federica Tonon,[3] Cristina Zennaro,[3] Marianna Caterino,[4,5] Pasquale Stano,[1] Stephan Fischer,[6] Marlen Hägele,[7] Martin Müller,[7] Alexander Kleger,[7] Panagiotis Papatheodorou,[6,8,b] Giuseppina Nocca,[9,10] Alessandro Arcovito,[9] Andrea Gori,[11] Margherita Ruoppolo,[4,5,12] Holger Barth[6,a] Nicola Petrosillo,[13] Paolo Ascenzi,[1] and Stefano Di Bella[13]

[1]Department of Sciences, Roma Tre University, Roma, Italy; [2]National Institute of Nuclear Physics, Roma Tre Section, Roma, Italy; [3]Department of Medical, Surgery and Health Sciences, University of Trieste, Trieste, Italy; [4]Department of Molecular Medicine and Medical Biotechnology, University of Napoli "Federico II", Napoli, Italy; [5]Associazione Culturale DiSciMuS RCF, Casoria, Napoli, Italy; [6]Institute of Pharmacology and Toxicology, University of Ulm Medical Center, Ulm, Germany; [7]Department of Internal Medicine I, University of Ulm Medical Center, Germany; [8]Institute of Experimental and Clinical Pharmacology and Toxicology, University of Freiburg, Freiburg, Germany; [9]Institute of Biochemistry and Clinical Biochemistry, Catholic University of Sacred Heart, Roma, Italy; [10]Institute of Chemistry of Molecular Recognition, CNR, Roma, Italy; [11]Clinic of Infectious Diseases, San Gerardo Hospital, University of Milano-Bicocca, Monza, Italy; [12]CEINGE Biotecnologie Avanzate, Napoli, Italy; [13]2nd Infectious Diseases Division, National Institute for Infectious Diseases "L. Spallanzani", Roma, Italy

Background. The pathogenic effects of *Clostridium difficile* are primarily attributable to the production of the large protein toxins (*C difficile* toxins [Tcd]) A (TcdA) and B (TcdB). These toxins monoglucosylate Rho GTPases in the cytosol of host cells, causing destruction of the actin cytoskeleton with cytotoxic effects. Low human serum albumin (HSA) levels indicate a higher risk of acquiring and developing a severe *C difficile* infection (CDI) and are associated with recurrent and fatal disease.

Methods. We used a combined approach based on docking simulation and biochemical analyses that were performed *in vitro* on purified proteins and in human epithelial colorectal adenocarcinoma cells (Caco-2), and *in vivo* on stem cell-derived human intestinal organoids and zebrafish embryos.

Results. Our results show that HSA specifically binds via its domain II to TcdA and TcdB and thereby induces their autoproteolytic cleavage at physiological concentrations. This process impairs toxin internalization into the host cells and reduces the toxin-dependent glucosylation of Rho proteins.

Conclusions. Our data provide evidence for a specific HSA-dependent self-defense mechanism against *C difficile* toxins and provide an explanation for the clinical correlation between CDI severity and hypoalbuminemia.

Keywords. *Clostridium difficile* toxins; human serum albumin; TcdA; TcdB; zebrafish.

Clostridium difficile is a Gram-positive, anaerobic, spore-forming, toxin-producing bacillus that has emerged as a major contributor to nosocomial infections in Western countries [1]. Approximately 15%–25% of antibiotic-associated diarrhea cases result from *C difficile* infection (CDI) [2]. The clinical manifestations of CDI range from asymptomatic colonization and mild diarrhea to toxic megacolon and life-threatening fulminant colitis [3]. In addition to antibiotic treatments, the primary risk factors for CDI include advanced age, impaired humoral immunity, renal disease, and hypoalbuminemia. In particular, an association between low human serum albumin (HSA) levels and CDI development, severity, and mortality rates has been highlighted [4–7] (Supplementary Table S1), refuting the hypothesis that hypoalbuminemia is merely a consequence of CDI-induced protein-losing enteropathy.

The pathogenic effects of *C difficile* are primarily caused by the production in the intestine of toxins A (TcdA) and B (TcdB) [8], which monoglucosylate and inactivate the Rho GTPases of host cells, causing several cytopathic effects and ultimately colonocyte death and loss of intestinal barrier function [9, 10].

In this study, we show that HSA acts as a self-defense mechanism towards CDI by binding both TcdA and TcdB and inducing a conformational change, thus promoting their autoproteolysis outside the intestinal epithelial cells. This prevents toxin internalization and the consequent cytotoxic and cytopathic effects, both in vitro and in vivo, providing an explanation for the clinical correlation between CDI severity and hypoalbuminemia.

MATERIALS AND METHODS

Cell Culture and Reagents

Human epithelial colorectal adenocarcinoma (Caco-2) cells were cultured as previously described [11]. HSA (66 kDa) was obtained from Sigma-Aldrich (St. Louis, MO). TcdA (308 kDa)

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[a]Alessandra di Masi and Holger Barth are senior authors of this work and contributed equally to the corresponding author Paolo Ascenzi.

[b]Present Affiliation: Institute of Pharmaceutical Biotechnology, University of Ulm, Germany; Institute of Pharmacology and Toxicology, University of Ulm Medical Center, Germany.

Correspondence: P. Ascenzi, MD, Department of Sciences, Roma Tre University, 00146 Roma, Italy (ascenzi@uniroma3.it).

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and TcdB (270 kDa) were from Enzo Life Science (Farmingdale, NY). Recombinant TcdA and TcdB (strain VPI 10463) were expressed in *Bacillus megaterium* (strain WH320; MoBiTec, Göttingen, Germany). For details, see Supplementary Data.

Intoxication of Human Epithelial Colorectal Adenocarcinoma Cells

For the (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay [12], Caco-2 monolayers were placed in serum-free medium and treated for 24 hours with 8 and 16 µg/mL commercial toxins and/or 3.3 and 6.6 mg/mL HSA. For details, see Supplementary Data. To evaluate cells rounding [13], cells were preincubated for 1 hour with 6.6 mg/mL HSA, washed, and then treated with 16 ng/mL recombinant toxins.

Transepithelial Electrical Resistance Measurements

HSA (6.6 mg/mL) and 2 ng/mL recombinant toxins were added apically to confluent cells. Transepithelial electrical resistance (TEER) measurements were performed as described elsewhere [14].

In Vivo Intoxication Experiments Using the Zebrafish *Clostridium difficile* Model

Experiments performed with zebrafish conformed to the Italian guidelines (Dgl 26/2014), in accordance with European Union legislation (2010/63/UE). The Ethics Committee for animal experimentation of the University of Trieste approved the experimental protocols (no. PO3118ZEN16). Embyos were microinjected with 0.5 mg/mL HSA, and then 8 µg/mL commercial TcdB was added to the medium and incubated for 24 hours at 28.5°C [15]. For experimental details, see [15] and Supplementary Data.

Intoxication of Stem Cell-Derived Human Intestinal Organoids

Induced human intestinal organoids were derived from plugged human hair from a healthy donor [16]. Organoids were incubated for 4 hours at 37°C with 16 ng/mL recombinant TcdA-TcdB mixture and/or 6.6 mg/mL HSA. For experimental details, see Supplementary Data.

Western Blot

Caco-2 cell monolayers were exposed to 2 µg/mL commercial toxins for 24 hours in the absence or presence of 3.3 and 6.6 mg/mL HSA. Immunoblots were performed as described elsewhere [12]. For details, see Supplementary Data.

Native Gel Electrophoresis and Immunoblot

Commercial TcdB (1 µg) was incubated for 20 minutes at 37°C in 50 mM HEPES pH 7.4 in the absence or presence of 1 µg of HSA. Alternatively, commercial 2.8×10^{-7} M TcdB was incubated for 1 hour at 37°C in HEPES buffer and 1 µM inositol hexakisphosphate ($InsP_6$) (Sigma-Aldrich) in the absence or presence of 5.6×10^{-7} M HSA. Gels were either stained with silver nitrate or used for immunoblotting.

Mass Spectrometry Analysis

Reduction, carbamidomethylation, and enzymatic reactions were performed as previously described [17]. The peptide mixtures were characterized using the EASY-nLC II chromatographic separation system coupled with an LTQ-Orbitrap-XL mass spectrometer (Thermo Fisher Scientific, San Jose, CA). For details, see Supplementary Data.

Binding Assay

The formation of the HSA-toxin complexes was monitored spectrofluorimetrically between 300 and 400 nm using an excitation wavelength of 280 mm. For details, see Supplementary Data.

Structural Model of *Clostridium difficile* Toxin B and Protein-Protein Docking

The TcdB amino acid sequence (AGG91641.1) and the TcdA (Protein Data Bank Identification: 4R04) and HSA 3-dimensional structures were used for bioinformatic analyses. For detail, see Supplementary Data.

Data Analysis

Results are shown as the mean ± standard deviations (SDs) derived minimally from 3 independent experiments. For details, see Supplementary Data.

RESULTS

Human Serum Albumin Rescues Human Epithelial Colorectal Adenocarcinoma Caco-2 Cells From Intoxication With TcdB and With a TcdA-TcdB Mixture

TcdB has been reported to be more cytotoxic than TcdA in human intestinal Caco-2 cells [18, 19], whereas TcdA appears to facilitate the translocation of TcdB from the gut into submucosal areas, where it may play a role in inducing inflammatory damage [18]. These findings explain the more severe phenotype observed when Caco-2 cells are treated with a combination of TcdA and TcdB (present data; [18]). To analyze the effect of HSA on *C difficile*-induced intoxication, we treated confluent Caco-2 cells (placed in serum-free medium) with either TcdB or a TcdA-TcdB mixture in the presence of tolerated doses of HSA (Supplementary Figure S1A). As expected, the TcdB treatment caused a cell viability of 41% ($P < .001$) and 36% ($P < .001$) after exposure to 8 and 16 µg/mL, respectively, compared with that in TcdB-untreated cells (Figure 1A). Upon pretreatment with 3.3 or 6.6 mg/mL HSA and the subsequent exposure to 8 µg/mL TcdB, cell viability significantly increased up to 83% and 85%, respectively, compared with that in HSA-untreated cells exposed to TcdB ($P < .001$). Upon pretreatment with 3.3 or 6.6 mg/mL HSA and a subsequent exposure to 16 µg/mL TcdB, cell viability increased to 80% and 86%, respectively, compared with that in HSA-untreated cells exposed to TcdB ($P < .001$) (Figure 1A). These results indicate that HSA doubled the percentage of cell survival upon Caco-2 intoxication

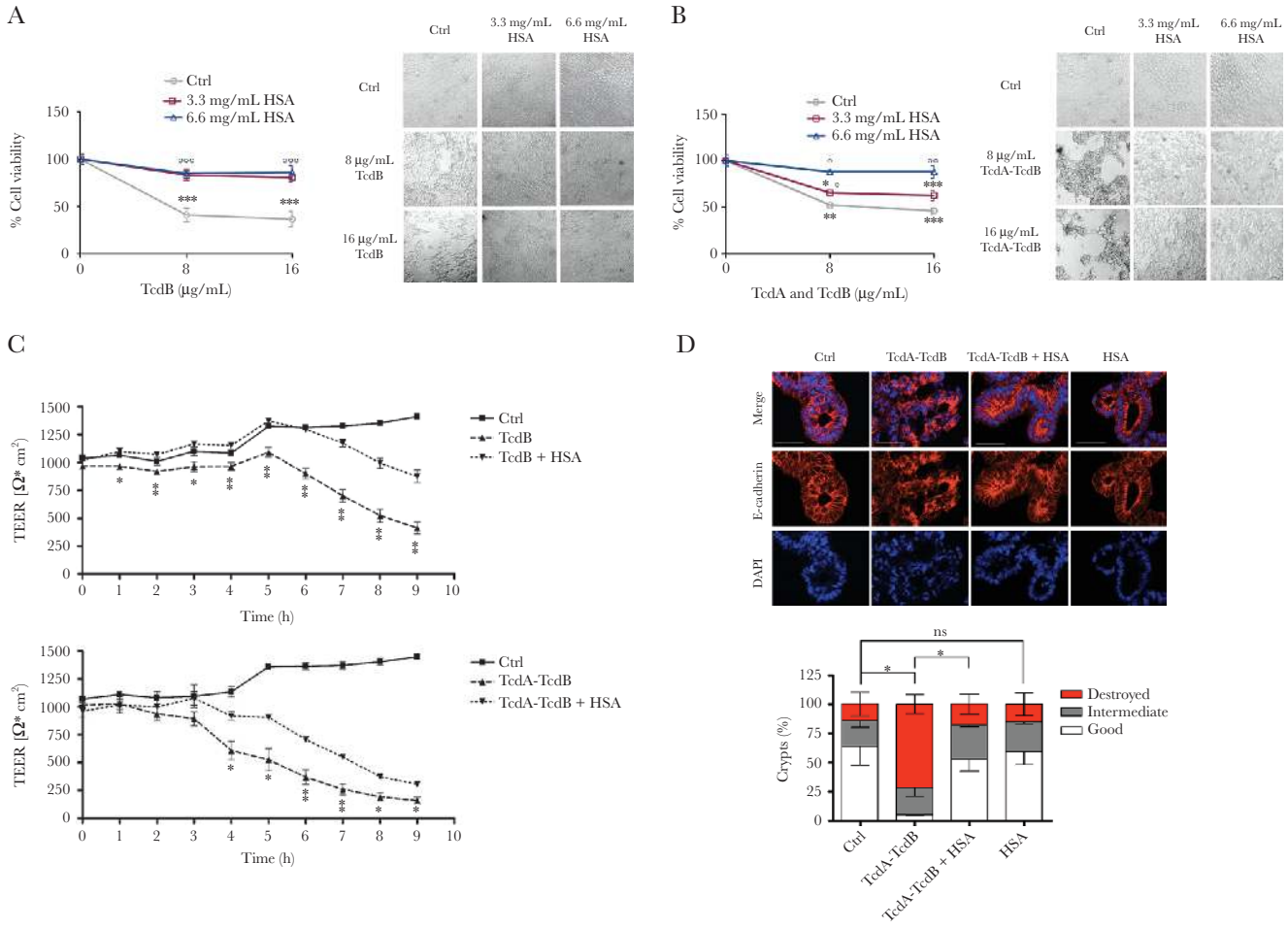

Figure 1. Human serum albumin (HSA) protects human Caco-2 cells and human intestinal organoids from intoxication with *Clostridium difficile* toxins (Tcd). (A) Caco-2 cells were either untreated or pretreated with 3.3 mg/mL or 6.6 mg/mL HSA for 1 hour and subsequently exposed to 8 µg/mL (corresponding to 3.0×10^{-8} M) or 16 µg/mL (corresponding to 6.0 × 10–8 M) TcdB for 24 hours. (B) Caco-2 cells were either untreated or pretreated with 3.3 mg/mL (corresponding to 5.0×10^{-5} M) or 6.6 mg/mL (corresponding to 5.0×10^{-5} M) HSA for 1 hour and subsequently exposed to 8 µg/mL (corresponding to 2.6×10^{-8} M for TcdA and 3.0×10^{-8} M for TcdB) or 16 µg/mL (corresponding to 5.2×10^{-8} M for TcdA and 6.0×10^{-8} M for TcdB) TcdA-TcdB for 24 hours. Data are presented as the mean values of the percentage of cell survival derived from 3 independent experiments ± standard deviation ([SD] Student's *t* test: *, $P < .05$, **, $P < .01$, and ***, $P < .001$ compared with the control in the absence of HSA; °, $P < .05$ compared with cells treated with 8 µg/mL toxin(s) in the absence of HSA; °°, $P < .01$ compared with cells treated with 16 µg/mL toxin(s) in the absence of HSA; °°°, $P < .001$ compared with cells treated with either 8 µg/mL or 16 µg/mL toxin(s) in the absence of HSA). Images show the morphologies of Caco-2 cells that were untreated or pretreated with HSA and then exposed to *C difficile* toxin(s). Images were acquired using a Leica microscope (Leica Microsystems, Heidelberg, Germany); magnification, ×20. (C) Human Caco-2 cells were grown to confluence. Then, cells were either left untreated (control, which is the same in the upper and lower panel), treated with toxin (TcdB or TcdA-TcdB), or treated with toxin and HSA (TcdB + HSA or TcdA-TcdB + HSA). When HSA was -present, cells were preincubated for 1 hour with 6.6 mg/mL HSA. Due to the high sensitivity of transepithelial electrical resistance (TEER) measurements, low toxin concentrations (2 ng/mL TcdB or 2 ng/mL TcdB plus 2 ng/mL TcdA) were chosen. The TEER was measured at the indicated time points. Raw resistance data, transformed into unit area resistance by subtracting background resistance, were multiplied by the surface area (0.3 cm^2). Values are given as the means ± SD (n = 3). Student's *t* test; *, $P < .05$ and **, $P < .01$ compared with cells treated with toxin(s) alone and treated with toxin(s) in the presence of HSA. (D) Stem cell-derived intestinal organoids were incubated with TcdA-TcdB in the absence or presence of HSA for 4 hours at 37°C, and untreated organoids served as controls. Miniguts were fixed in the wells and frozen sections were prepared. Samples were then incubated with an anti-E-cadherin antibody, followed by incubation with a fluorescently labeled secondary antibody. (Top Panel) Representative images of the morphologies of human intestinal organoids after incubation with TcdA-TcdB in the absence or presence of HSA; E-cadherin was stained with a fluorescence-labeled secondary antibody (red), and nuclei were stained with 4′,6-diamidino-2-phenylindole ([DAPI] blue). Bar = 50 µm. (Bottom Panel) The quantitative analysis of E-cadherin expression in crypt structures, and the classification of crypts were performed using the following classification: those with clear cell borders and well definable lumina were defined as "good"; those having a partial organized E-cadherin structure or an easily definable lumen were defined as "intermediate"; and those displaying a strongly disorganized E-cadherin structure with a ragged and difficult to identify lumen were defined as "destroyed". Every definable crypt was counted and assigned to one of these categories blinded. Images, acquired by the ApoTome Axio Z1 imager microscope (Carl Zeiss, Oberkochen, Germany), were derived from 2 independent experiments with at least 8 pictures per sample. Values are given as the means ± SD (n = 2). Student's *t* test; *, $P < .05$ compared with cells treated with toxin(s) only.

with TcdB. The preincubation of TcdB with HSA for 1 hour at 37°C before treating Caco-2 cells (Supplementary Figure S2A) did not result in significant differences from the data obtained treating cells with HSA 1 hour before intoxication (Figure 1A). This finding indicated that the HSA-TcdB direct interaction influences toxicity.

Similarly, Caco-2 cells treated with the TcdA-TcdB mixture showed a significant reduction in cell survival (49%) compared with that of controls ($P < .01$ at 8 μg/mL; $P < .001$ at 16 μg/mL) and exhibited more damage than cells treated with TcdB alone (Figure 1B). However, when cells were pretreated with 3.3 mg/mL HSA and subsequently exposed to 8 and 16 μg/mL TcdA-TcdB mixture, cell viability was significantly increased by ~64% ($P < .05$) compared with that of TcdA-TcdB-treated cells (1.3-fold increase). The percentage of viable cells significantly increased to 88% in Caco-2 cells pretreated with 6.6 mg/mL of HSA and then exposed to 8 μg/mL ($P < .05$) or 16 μg/mL ($P < .01$) TcdA-TcdB (2-fold increase) (Figure 1B).

The results obtained after treating Caco-2 cells for 48 hours with either TcdB or TcdA-TcdB (Supplementary Figure S2B) in the presence of HSA confirmed its protective role towards *C. difficile*-induced intoxication. Remarkably, incubation of Caco-2 cells in serum-free medium for 24 and 48 hours did not affect cell viability (Supplementary Figure S1).

Human Serum Albumin Is Able to Protect Confluent Caco-2 Epithelium From Toxin-Induced Disruption

The TEER measurements were performed using a confluent monolayer of Caco-2 cells mimicking the intestinal epithelium. The decrease in TEER values after exposure to either TcdB (Figure 1C, top panel) or the TcdA-TcdB mixture (Figure 1C, lower panel), which indicates a loss of cell epithelial integrity, was significantly reduced in cells preincubated with HSA (Figure 1C). This result confirmed the ability of HSA to protect the epithelium of intestinal cells from toxin-induced disruption. Of note, HSA alone did not affect epithelial integrity (Supplementary Figure S1B).

Human Serum Albumin Reduces the Toxic Effects of TcdA-TcdB in Human Induced Pluripotent Stem Cell-Derived Intestinal Organoids and in Zebrafish

The findings in Caco-2 cells were verified in stem cell-derived induced human intestinal organoids [16]. According to previous data [20], visible and severe alterations have been observed in organoids upon exposure to a TcdA-TcdB mixture. In particular, disruption of the epithelial barrier (as measured by an abnormal distribution of E-cadherin, a regulator of epithelial morphology and intestinal barrier function) was observed in toxin-treated miniguts, whereas untreated organoids displayed a normal epithelial barrier (Figure 1D). The toxic effect on intestinal crypts was diminished by the addition of HSA, as indicated by the increased number of healthy crypts, similar to the controls (Figure 1D, bottom panel). It is notable that HSA alone had no effect on the morphology of the intestinal organoids (Figure 1D).

The ability of HSA to counteract *C difficile* intoxication was confirmed in vivo using zebrafish embryos, a widely accepted model for the study of infectious diseases [15, 21]. Embryonic infection with the TcdA-TcdB mixture resulted in the death of all treated animals, even at low toxin concentrations (data not shown). Zebrafish embryos exposed to 8 μg/mL TcdB for 24 and 48 hours displayed significantly lower survival rates than vehicle-treated embryos, and toxic effects on embryo development, heart function, and the circulatory system were observed (Figure 2). Kaplan-Meier curves for the zebrafish embryos exposed to TcdB for 24 or 48 hours showed a 45% survival rate, in contrast to the 65% survival rate observed in animals pretreated with HSA before TcdB exposure ($P < .0001$). Both the vehicle and the HSA injection alone did not affect embryo survival (95%) (Figure 2A). The TcdB-treated embryos showed a 17% decrease in heart rate (expressed as the number of beats per minute [bpm]) compared with that of vehicle-treated animals, whereas embryos exposed to both TcdB and HSA displayed a significant increase in heart rate compared with that of TcdB-treated embryos ($P < .01$ and $P < .05$ at 24 and 48 hours, respectively; Figure 2B, left panel). Moreover, embryos exposed to TcdB showed an ~30% increase in pericardial area compared with that of controls and of embryos treated with both TcdB and HSA ($P < .01$ and $P < .05$ at 24 and 48 hours, respectively; Figure 2B, right panel). Intracranial hemorrhaging and severe vascular impairments in the tail region were observed in 38% and 50% of TcdB-treated animals after 24 and 48 hours, respectively. HSA pretreatment reduced this percentage to a normal value of 10% ($P < .05$ at 24 and 48 hours compared with TcdB-treated animals) (Figure 2C and D). Because albumin is not expressed in zebrafish [22], the HSA-dependent protective effects observed here are directly attributable to the exogenous HSA.

Human Serum Albumin Reduces the Toxin-Induced Glucosylation of Rho GTPase Substrates in Caco-2 Cells

The analysis of the toxin-catalyzed modification of human Rac1 is a well established method for monitoring the glucosyltransferase activity of TcdA and TcdB in the cytosol of host cells [23–25]. Indeed, once in the host cytosol, the toxins undergo $Insp_6$-dependent autocatalytic cleavage with the consequent release and activation of their glucosyltransferase domain (GTD) [10]. We analyzed the glucosylation status of Rac1 in cells treated with TcdB or the TcdA-TcdB mixture in the absence and presence of HSA (Figure 3A). The levels of nonglucosylated Rac1 were significantly reduced in cells treated with either TcdB (~3.3-fold, $P < .01$) or TcdA-TcdB mixture (~2.7-fold, $P < .05$) compared with control levels. In contrast, cells pretreated with HSA before toxin exposure showed similar levels of nonglucosylated Rac1 as controls. These data indicated that the cytosolic TcdA and TcdB enzymatic activities in infected host cells were reduced in the presence of HSA, resulting in low levels of intracellular nonglucosylated proteins [22].

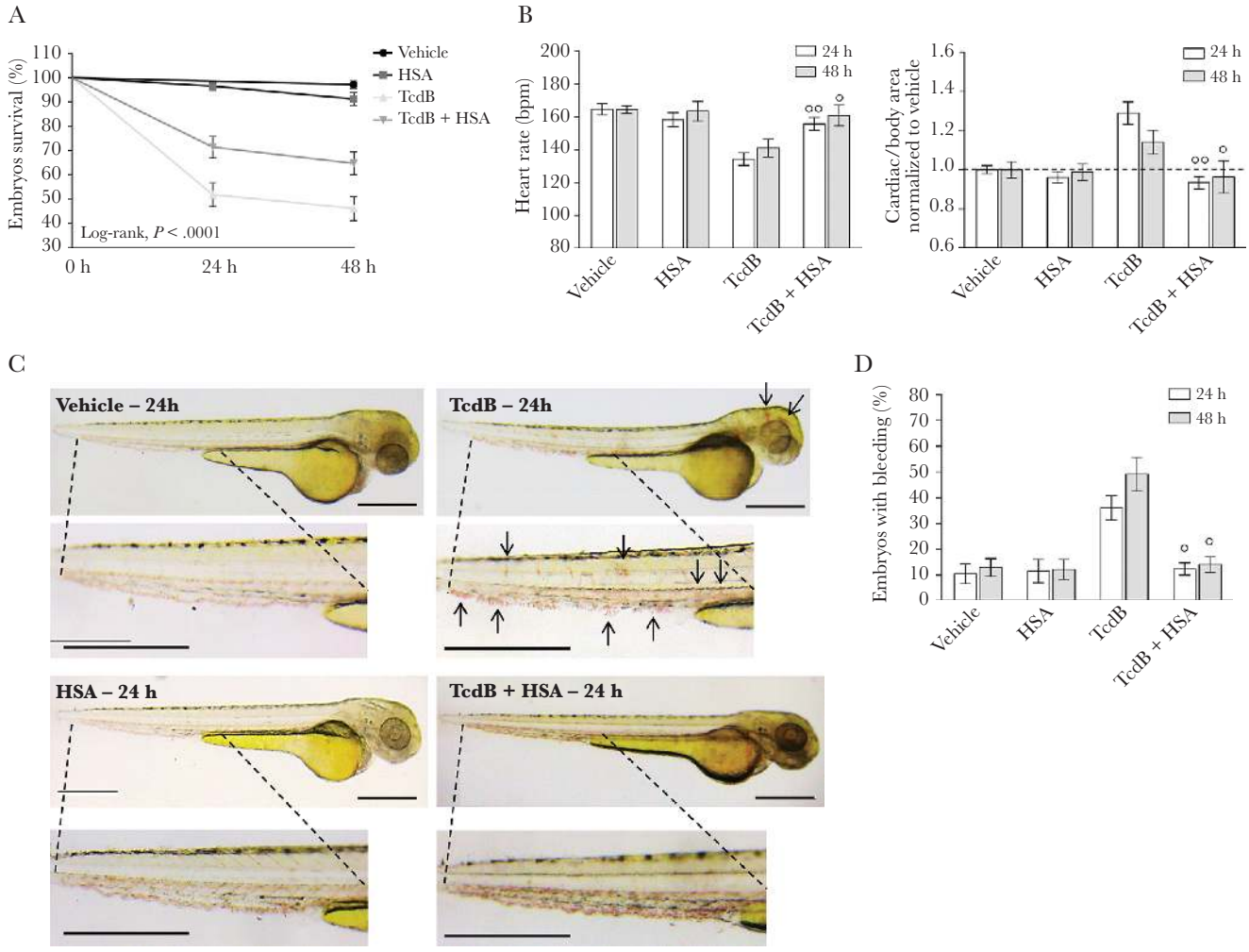

Figure 2. Protective effect of human serum albumin (HSA) on the CDI zebrafish model. (A) Kaplan-Meier curve showing zebrafish embryo survival 24 and 48 hours after treatment with *Clostridium difficile* toxin B(TcdB), HSA, TcdB + HSA, or vehicle. Data are presented as the means of 3 independent experiments ± standard error of the mean ([SEM] n = 110 for each group) (analysis of variance [ANOVA], $P < .0001$ TcdB vs TcdB + HSA). (B, left panel) Evaluation of the zebrafish embryo heart rate. The graph represents the heart rate of zebrafish embryos 24 and 48 hours after treatment with 8 µg/mL (corresponding to 3.0×10^{-8} M) TcdB in the absence and presence of 0.5 mL/mL (corresponding to 8.3×10^{-6} M) HSA. The HSA pretreatment protects cardiac function from the cardiotoxic effects of TcdB. Data are presented as the means ± SEM (n = 30 for each group) (ANOVA test, °°, $P < .001$ and °, $P < .05$ compared with TcdB-treated embryos in the absence of HSA. (Right Panel) Evaluation of pericardial edema in zebrafish embryos 24 and 48 hours after exposure to the vehicle, HSA, TcdB, or TcdB + HSA. The histogram displays the ratio of cardiac to total body area values. The values, which were normalized to the vehicle group, are presented as the means ± SEM (n = 30 for each group) (ANOVA, °°°, $P < .001$ and °, $P \leq 0.05$ compared with TcdB-treated embryos in the absence of HSA). Only embryos that were treated with TcdB alone exhibited pericardial edema compared with vehicle-treated embryos. (C) Representative images of zebrafish embryos that had been exposed to the vehicle, TcdB, HSA, or both TcdB and HSA for 24 hours. The TcdB-treated embryos (top right image) showed extensive cerebral hemorrhaging and severe vascular impairments in the tail region (black arrows); the TcdB + HSA-treated animals (bottom right image) did not show significant signs of vascular changes, similar to vehicle- and HSA-treated animals. Scale bar = 0.5 mm. (D) The histogram shows the number of zebrafish embryos that exhibited bleeding. A significantly increased percentage of TcdB-treated embryos exhibited bleeding compared with embryos treated with TcdB + HSA. Data are presented as the means ± SEM (n = 30). °, $P \leq 0.05$ compared with TcdB-treated embryos in the absence of HSA. Leica S8APO and DM2000 microscopes (Wetzlar, Germany) were used to perform morphological analyses and to take pictures.

Human Serum Albumin Binds TcdB Outside the Cells and Prevents Its Internalization

Cellular levels of TcdB and HSA were measured to determine whether the protective effect of HSA was due to the extracellular sequestration of TcdA and TcdB or to inhibition of their intracellular activity (Figure 3B). High levels of TcdB were observed in Caco-2 cells treated with either TcdB or the TcdA-TcdB mixture, suggesting that TcdB was internalized by the host cells in the absence of HSA. However, when cells were pretreated with HSA, 6- ($P < .001$) and 2-fold ($P < .01$) reduction in TcdB levels were observed in cells exposed to TcdB or the TcdA-TcdB mixture, respectively. Moreover, HSA internalization was also reduced in cells pretreated with HSA before TcdB or TcdA-TcdB exposure. Indeed, 2- and 1.6-fold reductions ($P < .001$) were measured in cell-associated HSA levels compared with those of cells that were treated with TcdB or the TcdA-TcdB mixture alone, respectively.

To further evaluate whether the interaction between toxin(s) and HSA occurs at the extracellular or intracellular level,

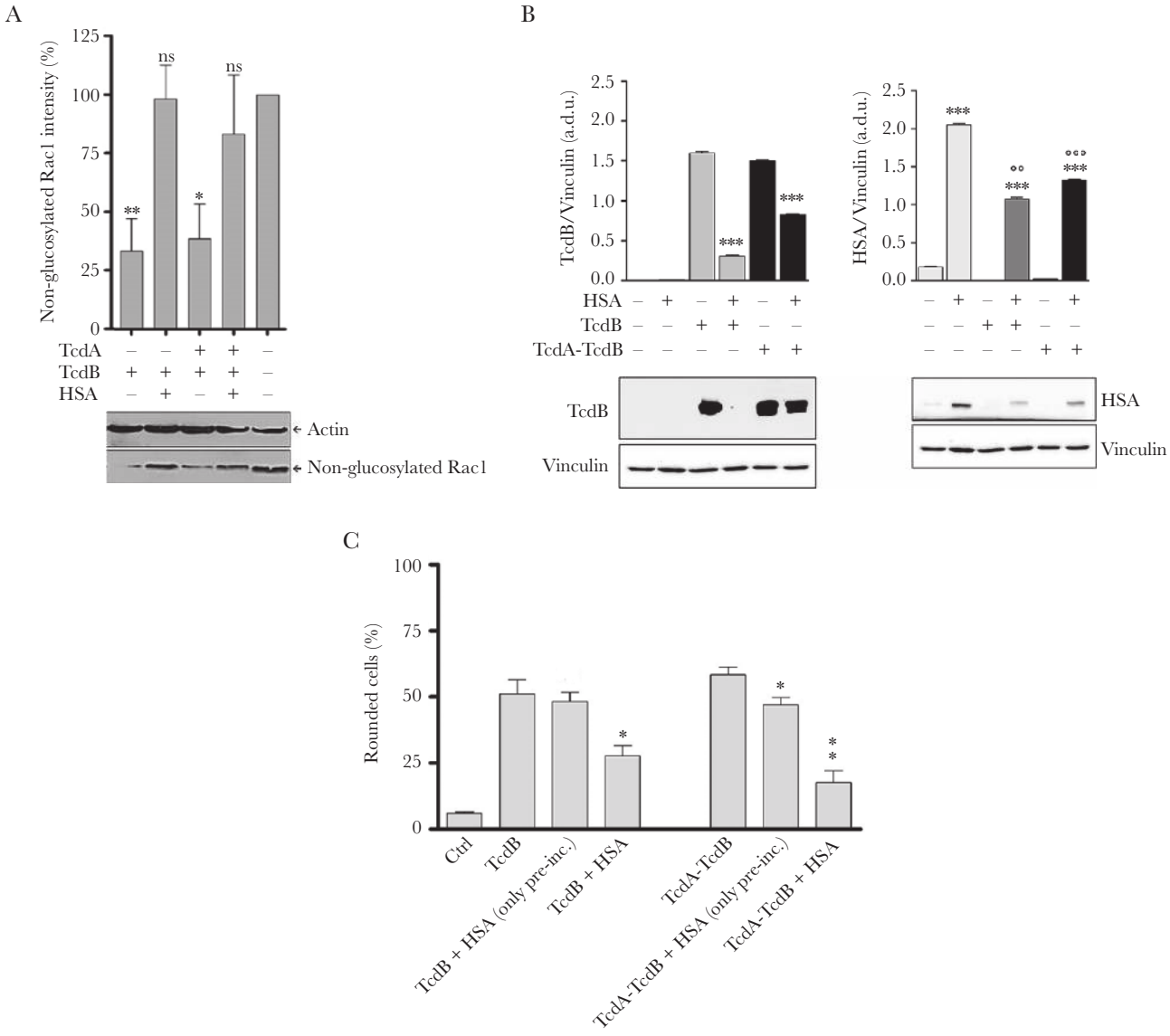

Figure 3. Human serum albumin (HSA) binds *Clostridium difficile* toxin B(TcdB) outside the cell and prevents its internalization. (A) Caco-2 cells were preincubated with HSA for 1 hour and subsequently treated with either TcdB or the TcdA-TcdB mixture for 2 hours. Nonglucosylated Rac1 levels were detected by immunoblotting with an anti-Rac1 antibody, and actin was used as a loading control. The graph shows the mean nonglucosylated Rac1 levels derived from three independent experiments normalized to actin, ±standard deviation (SD) (Student's *t* test; *, $P < .05$ and **, $P < .01$ compared with the control, untreated cells). (B) Immunoblot analysis of intracellular TcdB and HSA levels in Caco-2 cells. Cells were either untreated or pretreated with HSA for 1 hour and subsequently exposed to TcdB or the TcdA-TcdB mixture for 2 hours. Immunoblots were performed using anti-TcdB, anti-HSA, and antivinculin antibodies. Blots are representative of the results obtained, and quantitative data represent the mean values from 3 independent experiments normalized to vinculin ± SD (Student's *t* test; ***, $P < .001$ compared with the relative control; °°, $P < .01$ and °°°, $P < .001$ compared with cells treated with HSA but not *C difficile* toxins). (C) Caco-2 cells were preincubated with 6.6 mg/mL HSA for 1 hour, washed 3 times to remove the HSA, and treated with either 16 ng/mL TcdB or TcdA-TcdB mixture. As a negative control, cells were left untreated. For the HSA control, cells were preincubated with 6.6 mg/mL has, and the toxins (either TcdB alone or TcdA-TcdB) were added in the presence of HSA. After 2.5 hours, the number of rounded cells was determined. The values are given as the means ± SD (n = 3). Student's *t* test; *, $P < .05$ and **, $P < .01$ compared with cells treated with toxin(s) only.

Caco-2 cells were incubated for 1 hour in a medium containing 6.6 mg/mL HSA, which was then removed and replaced with fresh medium containing 16 ng/mL of either TcdB or TcdA-TcdB mixture. In parallel, the toxin(s) were applied to cells that still had HSA in the medium. We observed that the simultaneous presence in the medium of both HSA and *C difficile* toxin(s) was crucial for their HSA-dependent neutralization (Figure 3C), because the percentage of rounded cells was significantly reduced ($P < .05$ for TcdB; $P < .01$ for TcdA-TcdB) compared with that of cells in which HSA was removed before toxin addition to the medium. This result indicated the following: (1) the presence of either TcdB or TcdA-TcdB mixture in the medium caused reduced internalization of HSA compared with that in cells treated only with HSA; (2) the presence of

HSA in the medium is required for reducing internalization; and (3) the HSA-toxin interaction occurs outside the cell.

Human Serum Albumin Binds TcdA and TcdB

The observed protective effects of HSA on *C difficile*-induced host cell damage in vitro and in vivo were achieved due to the ability of HSA to bind TcdA and TcdB in a noncooperative, saturating, and dose-dependent manner (Figure 4A). Indeed, the K_D values for TcdA and TcdB binding to HSA were 0.24 ± 0.04 and 0.44 ± 0.12 nM, respectively. It is notable that the toxin fluorescence decreased as the HSA concentration increased, suggesting that HSA binding induced a conformational change in the toxin structure.

The electrophoretic migration of TcdB, HSA, and the HSA-TcdB mixture was evaluated using native polyacrylamide gel electrophoresis (PAGE) to assess the formation of the HSA-TcdB complex (Figure 4B). As expected, in addition to its monomeric form, TcdB formed a dimer in solution [26], whereas HSA showed its characteristic pattern of multiple bands [27]. The hybridization of the filter with anti-TcdB and anti-HSA antibodies revealed overlapping signals in the HSA-TcdB mixture (red line). A shift in the electrophoretic mobility of HSA was observed in this mixture, as indicated by the hybridization of the filter with the anti-HSA antibody. A fourth band was present in the HSA-TcdB mixture at a level corresponding to the TcdB monomer, as shown in both the silver-stained native gel and the Western blot.

Prediction of TcdA-HSA and TcdB-HSA Binding Region

Molecular docking studies were performed to predict the HSA-TcdA and HSA-TcdB recognition regions. HSA is an all-α-helical protein containing 9 fatty acid (FA) binding sites (named FA1 to FA9), which display considerable topological differences and ligand affinity [28]. TcdA and TcdB share common enzymatic activities and a multimodular domain structure [29, 30] (Figure 4C): region A is located at the *N*-terminus and contains a 63-kDa GTD responsible for the glucosylation of host GTPases; region B is located at the *C*-terminus and consists of combined repeated oligopeptides (CROPs) that form the receptor-binding domain (RBD); region C corresponds to a cysteine protease domain (CPD) that promotes the autocatalytic cleavage of the toxins; and region D, also termed the delivery domain (DD), is involved in the translocation of the toxins into the cytosol and their binding to target cells. It is notable that the CROP domain extends from the base of the CPD and interacts with the DD domain, protecting *C difficile* toxins from autoproteolysis [30]. Our docking analyses predicted an interaction between the hydrophobic portions of the DD domains of both TcdA and TcdB and domain II of HSA (Figure 4C). Both complexes may be stabilized by hydrogen bonds and electrostatic interactions. It is interesting to note that the regions of both toxins that were predicted to interact with HSA are responsible for the interaction between the RBD (containing the CROP region) and the DD domains, which regulates toxin autoproteolysis [30]. In fact, once bound to the host receptor, the toxin-receptor complex enters the cell through endocytosis, and endosomal acidification is required for toxin translocation into the host cytosol [31]. Both endosomal acidification and binding of host $InsP_6$ to the toxins allow the allosteric activation of the CPD due to the exposure of hydrophobic regions located within the DD [25, 32]. Consequently, the GTD is released into the host cytosol and promotes the glucosylation of host Rho GTPases [33].

The autocatalytic cleavage of *C difficile* toxins can be simulated in vitro [25]. In the absence of dithiothreitol (DTT), $InsP_6$ alone is able to cause toxin cleavage only at concentrations at least 16-fold higher (ie, 2 μM) than those used in the presence of DTT [25]. To mimic physiological cellular conditions, we evaluated TcdB cleavage using a buffer containing suboptimal concentration of $InsP_6$ (ie, 1 μM) and lacking DTT. This buffer composition was not sufficient to induce TcdB cleavage at either pH 7.4 (Figure 4D) or pH 5.0 (Supplementary Figure S3), indicating that the acidification of the milieu is necessary but not sufficient to induce toxin cleavage in the absence of DTT. Thus, we evaluated whether HSA was able to modulate the $InsP_6$-dependent autocatalytic cleavage of TcdB (Figure 4D, Supplementary Figure S3). The results obtained showed that HSA enhanced $InsP_6$-dependent TcdB autoproteolysis, resulting in the release of the *N*-terminal GTD to a similar extent as 2 mM DTT (Supplementary Figure S3). A ~60-kDa TcdB fragment appeared in the sample containing TcdB and a suboptimal concentration of $InsP_6$ (ie, 1 μM) at pH 7.4, and higher levels were observed in the sample containing TcdB, $InsP_6$, and HSA at pH 7.4 (Figure 4D). A suboptimal concentration of $InsP_6$ was chosen to highlight the role of HSA in $InsP_6$-dependent autoproteolytic cleavage of TcdB, which is promoted by $InsP_6$ concentrations ranging from 2 to 100 μM [25, 32]. Mass spectrometry analysis of this fragment indicated that it corresponds to the *N*-terminal region of TcdB (band C4; Supplementary Figures S4 and S5; Supplementary Table S2). Therefore, upon HSA binding, TcdB undergoes a conformational change at physiological pH, resulting in the $InsP_6$-dependent autoproteolytic cleavage in extracellular environments where $InsP_6$ is present [34]. It is notable that the mass spectrometry analysis confirmed the presence of both TcdB and HSA in the highest-molecular-weight band (eg, bands C1 and C2; Supplementary Figures S4 and S5; Supplementary Table S2) that was not dissociated by SDS-PAGE denaturing conditions and heating [35, 36].

DISCUSSION

In recent years, many studies have demonstrated a significant association between low serum HSA levels and CDI development, thus strengthening the hypothesis that hypoalbuminemia predisposes patients to CDI and, in turn, CDI aggravates hypoalbuminemia, generating an autosustained vicious cycle (see

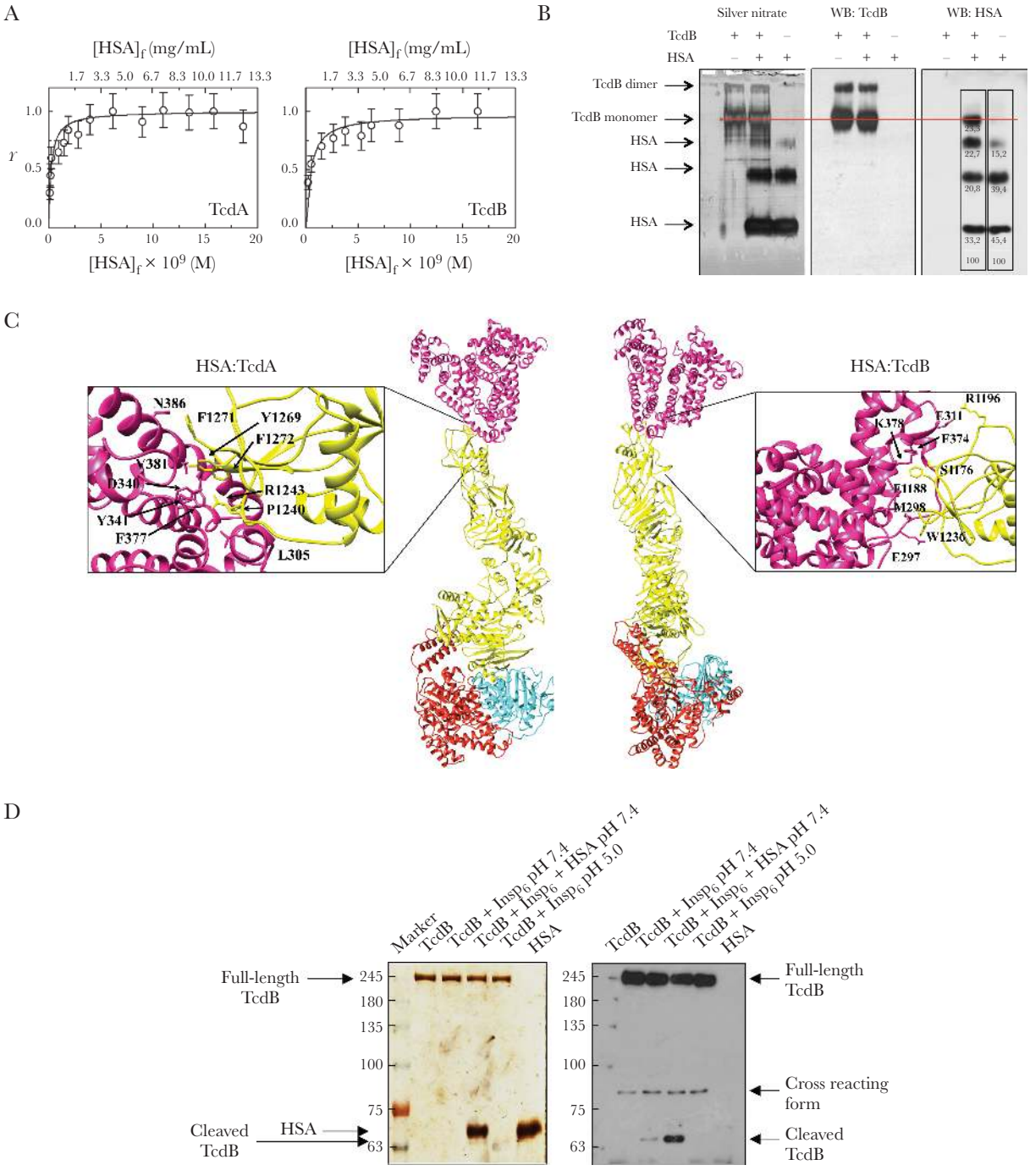

Figure 4. Human serum albumin (HSA) binds *Clostridium difficile* toxins (Tcd) and induces their cleavage. (A) TcdA and TcdB binding to HSA. Data were plotted against HSA-free conditions and were analyzed using nonlinear regression, according to Eq. 1 (see Materials and Methods of Supplementary Data). (B) Native gel electrophoresis was used to evaluate the formation of the HSA-TcdB complex. One microgram of TcdB was incubated with 1 microgram of HSA (1:1 ratio) in 50 mM HEPES at pH 7.4 for 20 minutes. As controls, the same amounts of TcdB and HSA were run alone. After blotting, filters were incubated with an anti-HSA antibody, stripped, and incubated with the anti-TcdB antibody. The hybridization of the filter with anti-TcdB and anti-HSA antibodies revealed that the signals from the mixture overlapped (red line). (C) Molecular aspects of the TcdA and TcdB interactions with HSA. (Left) Overall view of the putative complex between HSA (Protein Data Bank Identification [PDB ID]: 1AO6) (deep pink) and TcdA (PDB ID, 4R04; red, glucosyltransferase domain; cyan, cysteine protease domain; yellow, delivery domain). (Right) Overall view of the putative complex between HSA and the model of TcdB (red, GTD; cyan, CPD; yellow, DD) obtained by docking simulations. The residues involved in HSA-TcdA and HSA-TcdB interactions are highlighted. Images were drawn with the UCSF Chimera-package. (D) Sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblot analyses of HSA-induced TcdB proteolysis. Commercial TcdB (2.8×10^{-7} M) was incubated for 1 hour at 37°C in 50 mM HEPES pH 7.4 supplemented with 1 μM $InsP_6$, in the absence or presence of 5.6×10^{-7} M of HSA (the ratio of HSA/TcdB was 2). The experiment was performed in duplicate so that 1 gel was stained with silver nitrate and the other was used for immunoblotting, probing the filter with the anti-*C difficile* TcdB antibody specific for the *N*-terminus of the toxin. (Left Panel) Silver nitrate staining; (Right Panel) immunoblot using an anti-TcdB antibody.

references in Supplementary Table S1). However, the mechanism by which hypoalbuminemia promotes a more severe form of CDI is not yet understood. In this study, we report for the first time that HSA acts as a self-defense mechanism against *C difficile* toxin-dependent infection.

Our results demonstrate that HSA reduces the cytotoxic and cytopathic effects of TcdB and the TcdA-TcdB mixture on Caco-2 cells in a dose-dependent manner. This protective effect of HSA has also been demonstrated using the stem cell-derived human intestinal organoid model. The *C difficile* toxins cause a loss of the epithelial barrier in human miniguts and the disorganization of E-cadherin, which is a regulator of epithelial morphology and intestinal barrier function [20]. Accordingly, HSA reduced TcdA-TcdB-induced changes in the integrity and structure of the intestinal organoid, protecting intestinal crypts from toxin-dependent damage. Our results showed that HSA inhibits toxin internalization, reducing the free intracellular toxin levels. Indeed, when Caco-2 cells were treated with TcdB or with TcdA-TcdB mixture in the presence of HSA, the toxin-dependent glucosylation of Rac1 [25] was significantly reduced, thus supporting the capability of HSA to rescue cells from intoxication. The ability of HSA to counteract *C difficile* intoxication has finally been confirmed in vivo using zebrafish embryos. HSA pretreatment increased the survival rate of TcdB-treated zebrafish embryos (65% versus 45%) and significantly reduced extraintestinal TcdB-induced effects, such as cardiotoxicity, intracranial hemorrhaging, and severe vascular impairments in the tail region.

The protective effects of HSA on *C difficile*-induced host cell damage, both in vitro and in vivo, are due to the capability of HSA to bind TcdA and TcdB. The similar affinity of toxins for HSA reflects the fact that they share the common ABCD modular structure, with 47% sequence identity and 66% sequence similarity [10, 29, 30]. Our docking analyses predicted that domain II of HSA interacts with hydrophobic portions of the DD domain of *C difficile* toxins, specifically the region between the RBD (containing the CROP region) and the DD domains. HSA binding to the DD domain of TcdB may cause the displacement of the CROP region, enhancing $InsP_6$ binding to CPD [30, 37]. In turn, this leads to the toxin autoproteolysis, the release of GTD from the toxin, and the inhibition of toxin internalization in Caco-2 cells in miniguts and zebrafish embryos.

CONCLUSIONS

In addition to their intestinal effects, *C difficile* toxins contribute to extraintestinal complications of CDI, including ascites, pleural effusion, cardiopulmonary arrest, hepatic abscess, acute respiratory distress syndrome, and renal failure [38–41]. Thus, given the potential role of *C difficile* toxins in the development of systemic CDI complications [10], HSA may act as a buffer that can partially neutralize the toxins that reach the bloodstream after being produced in the colon. If this buffer is inadequate (ie, in patients with hypoalbuminemia), the fraction of toxins that can be neutralized is decreased. Therefore, greater pathogenic effects are expected, thus in part explaining the

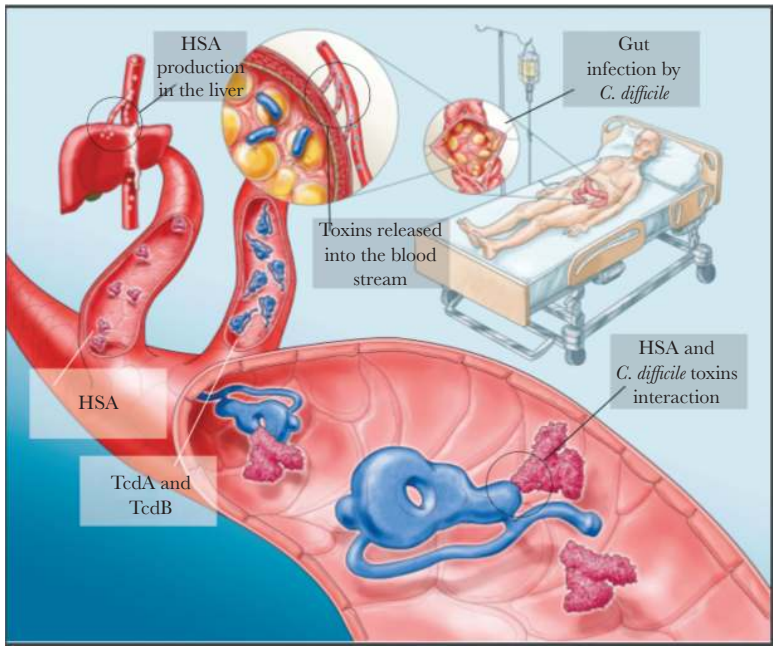

Figure 5. Schematic depiction of the proposed mechanism by which human serum albumin (HSA) acts as a "buffer system" in the bloodstream of patients with *Clostridium difficile* (*C. difficile*) infection. Abbreviation: Tcd, *C. difficile* toxin.

increased clinical severity and poor outcomes associated with CDI in patients with hypoalbuminemia (Figure 5).

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Author contributions. A. d. M., L. L., and S. F. performed and analyzed the in vitro experiments and the biochemical assays; F. P. and L. L. performed the bioinformatic analyses; A. A., G. N., L. L., P. A., and P. S. performed and analyzed the binding assays; F. T. and C. Z. performed and analyzed the zebrafish experiments; M. C. and M. R. performed and analyzed the mass spectrometry experiments; S. F., M. H., M. M., A. K., P. P., and H. B. performed experiments with the minigut model and purified *C difficile* toxins; S. D. B. analyzed the clinical data; A. G. and N. P. contributed reagents/materials/analysis tools; A. d. M., C. Z., F. P., H. B., M. R., N. P., P. A., and S. D. B. conceived and designed the experiments; and A. d. M., H. B., P. A., and S. D. B. wrote the paper.

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