CENTRO DE INVESTIGACIÓN Y DE ESTUDIOS AVANZADOS DEL INSTITUTO POLITÉCNICO NACIONAL

UNIDAD ZACATENCO

DEPARTAMENTO DE BIOMEDICINA MOLECULAR

"El papel de cortactina y HS1 en la hiper-permeabilidad vascular durante la sepsis"

T E S I S

Que presenta

M. en C. ALEXANDER GARCÍA PONCE

para obtener el grado de

DOCTORADO EN CIENCIAS

EN LA ESPECIALIDAD DE

BIOMEDICINA MOLECULAR

Director de Tesis

Dr. Michael Schnoor

Ciudad de México Agosto de 2017

CENTRO DE INVESTIGACIÓN Y DE ESTUDIOS AVANZADOS DEL INSTITUTO POLITÉCNICO NACIONAL

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"The role of cortactin and HS1 in vascular hyperpermeability during sepsis"

T H E S I S

presented by

M. Sc. ALEXANDER GARCÍA PONCE

To obtain the degree

PH. D. IN SCIENCES

IN THE SPECIALTY

MOLECULAR BIOMEDICINE

Thesis Director

Michael Schnoor

México, D.F. August, 2017

DEDICATORIA

Quiero dedicar este trabajo a todas aquellas personas que con quienes he cruzado caminos, en especial a mi familia, pareja y amigos.

Gracias a todos por su apoyo incondicional y sus palabras de aliento cuando fueron necesarias.

AGRADECIMIENTOS

Quiero agradecer al Dr. Michael Schnoor por haber sido mi mentor durante 6 años, en los cuales siempre me guio con paciencia y sabiduría, aconsejándome a lo largo de este camino que era totalmente desconocido para mí.

Agradezco también de forma especial a nuestra auxiliar de laboratorio, la Dra. Hilda Vargas, ya que sin su labor día a día, el laboratorio no funcionaría y además también, gracias por siempre tener la disposición de ayudar los estudiantes en todo lo posible.

También quiero agradecer a mis compañeros de laboratorio, ya que siempre han sido una fuente de retroalimentación y apoyo importante para mí. Agradecimientos especialmente a la M. en C. Sandra Denisse Chánez, a la M. en C. Karla Fabiola Castro, al Dr. Alí Francisco Citalán Madrid y al Dr. Eduardo Vadillo Rosado.

Agradezco también a CONACYT, al CINVESTAV y las siguientes instituciones por el apoyo económico durante mi preparación doctoral para asistir a conferencias y estancias importantes para mi formación académica.

Cinvestav

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ASIP
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Resumen

La sepsis continúa siendo un importante problema de salud a nivel mundial y en México. La tasa de mortalidad de esta enfermedad alcanza incluso el 30% de los casos en nuestro país. Esto se debe principalmente a la falta de tratamientos efectivos que prevengan el desarrollo de síndrome de disfunción multi-orgánica. En este estudio evaluamos el papel de las proteínas de unión a actina cortactina y HS1, que están involucradas en el proceso de transmigración trans-endotelial de leucocitos en el modelo de sepsis murino de punción y ligación del ciego (CLP). Nuestros resultados demuestran que la deficiencia tanto de cortactina como de HS1 tienen un efecto protector después de la inducción de sepsis letal mediante CLP. Adicionalmente, encontramos que este efecto, se asocia con una disminución en el número de leucocitos que son capaces de infiltrar el pulmón. Análisis histológico de estos tejidos mostraron que los ratones deficientes de HS1 presentan pocos hallazgos de inflamación y daño 24 horas después de la inducción de sepsis. A su vez, observamos que el corte de la proteína PARP, empleado como marcador de apoptosis, es menor en los ratones deficientes de HS1 y cortactina en comparación con los ratones WT. Finalmente, confirmamos mediante microscopía intravital del músculo cremaster que incluso después de una infección y estímulo inflamatorio sistémicos, los leucocitos son incapaces de abandonar el sistema vascular para infiltrarse en el tejido para llevar a cabo su función. Nuestros resultados son los primeros en demostrar que las proteínas de unión a actina cortactina y HS1 disminuyen la mortalidad y el daño orgánico inducido por sepsis, debido a la incapacidad de los leucocitos de alcanzar los tejidos inflamados.

Abstract

Sepsis remains an important health problem worldwide and in Mexico. Mortality rates for this disease reach up to 30% of total cases in our country. This is a consequence of the inefficacy of current treatments to prevent multi organ dysfunction syndrome. In this study, we evaluated the role of the actin-binding proteins cortactin and HS1, involved in leukocyte transendothelial migration, in a model of murine cecal-ligation and puncture (CLP) sepsis. Our results demonstrate that cortactin and HS1 deficiency have a protective effect after sepsis induced by lethal CLP. Additionally, we found that this effect is associated with reduced numbers of infiltrating leukocytes in the lung. Histological analysis of these tissues showed that HS1-deficient mice had less inflammation and tissue damage 24 h after sepsis induction. We also observed that the cleavage of PARP, used as a marker of apoptosis, is reduced in both cortactin and HS1 deficient animals when compared to WT animals. Finally, we confirmed by cremaster muscle intravital microscopy that even after systemic infection and inflammatory stimuli, leukocytes are unable to leave the blood vessels in order to infiltrate the underlying tissue to exert their functions. Our results are the first to demonstrate that the actin binding proteins cortactin and HS1 diminished mortality and organ damage induced by CLP sepsis due to the inability of leukocytes to infiltrate inflamed tissues.

Introduction

Sepsis is the leading cause of death in most intensive care units (ICUs) over the world. However, in Mexico, few epidomiological studies exist addressing sepsis and thus, this condition has been understimated. The most recent study was published by Carrillo-Esper and colleagues in 2009 [7], where it was shown that sepsis mortality in ICUs in 25 states climbed up to 30%. This high mortality is considered to be a consequence of the poor understanding of sepsis and lack of effective treatments. Moreover, treatment in ICUs implicates increased costs making sepsis an important health problem worldwide and a burden on public health systems. Thus, it is of utmost importance to better understand sepsis pathophysiology and find new therapeutic approaches to treat this complex condition.

There are several definitions for sepsis. In 2014, sepsis was defined as a systemic, non-specific and exacerbated inflammatory response to microbial infection, and “severe sepsis” was defined as sepsis in conjunction with multi/organ dysfunction (MOD) including hypo-perfusion [8, 9] However, in 2017 the definition changed according to the international guidelines generated by the Surviving Sepsis Campaign consensus “Sepsis-3”, and it is now defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection, and the term “severe sepsis” is no longer used [10]. The worst stage of sepsis is known as “septic shock” defined as sepsis with persisting hypotension requiring vasopressor therapy to maintain arterial pressure.

Pathophysiology of sepsis

Sepsis begins with an infection that enters the bloodstream and disseminates systemically, thus activating the immune system all over the body. The immune response to sepsis is both pro-inflammatory and anti-inflammatory at the same time (Figure 1) [4, 8, 9]. The activation of the immune system during the inflammatory stage leads to the production of pro-inflammatory cytokines and chemokines, such as IL-1, -6, -8, -10, TNF-α, monocyte chemoattractant protein 1 (MCP-1), the complement system and acute phase molecules such as C-reactive protein. During this phase, endothelial cells get activated causing the opening of cell contacts and subsequently increased vascular permeability which facilitates leakage of fluids and proteins into the tissues [11, 12]. Additionally, endothelial cells express adhesion molecules on their apical membrane supporting the adhesion of neutrophils to the endothelial surface that is followed by extravasation of these cells into the tissues. Recruitment and activation of leukocytes is not only required for pathogen clearance but may also contribute to tissue damage by excessive secretion of proteases and reactive oxygen species (ROS). On the other hand, release of

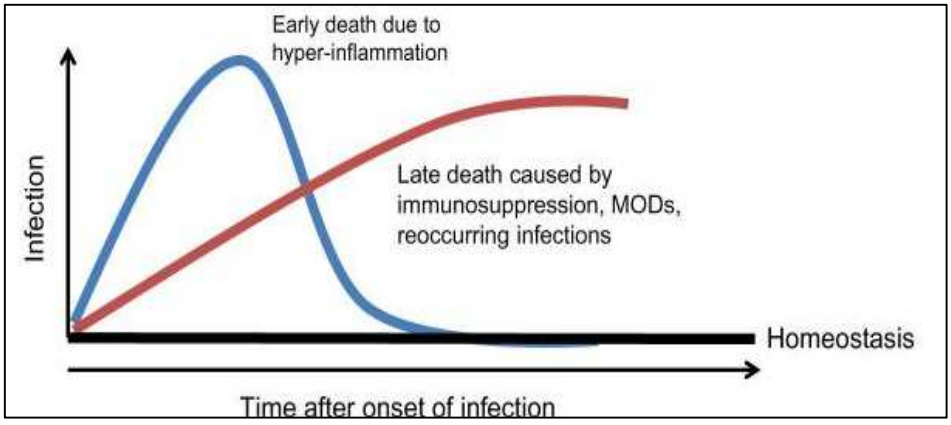

Figure 1 Stages of sepsis. The pro-inflammatory and anti-inflammatory responses to sepsis usually occur together. Innate immune cells release high amounts of pro-inflammatory cytokines. Premature death in sepsis is a consequence of the hyperinflammatory stage. As sepsis progresses, there is a systemic deactivation of the immune system (CARS) normally responsible for restoring homeostasis after the inflammatory state. However, this leads to immunosuppression and high risk of secondary infections and death is caused by the inability of the host to clear infection [4].

complement factors and damage-associated molecular patterns (DAMPs) [10] serve as important chemoattractants for leukocytes, which will be activated during prolonged periods resulting in excessive influx of these cells into the tissues to exert their function without the anti-inflammatory response being able to properly regulate this process. Other factors such as coagulation proteins further enhance organ damage during sepsis by reducing tissue perfusion and oxygen uptake, as consequence of enhanced clot formation. which eventually blocks the capillaries and reduce blood pressure.

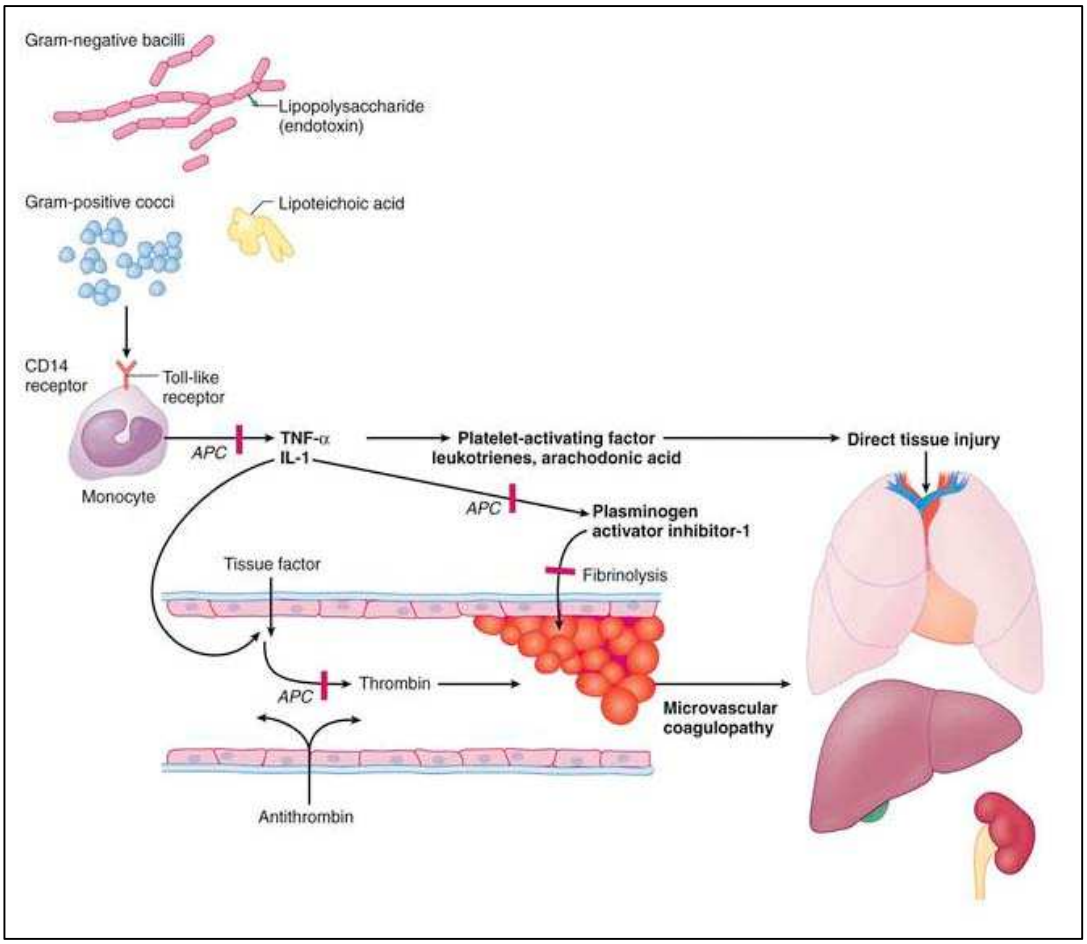

Fig. 2 Sepsis-induced Multiple Organ Dysfunction (MODs) and death. Common inflammation and coagulation pathways involved in sepsis-induced tissue damage that often leads to death. Organs usually affected by this pathology are the heart, lungs, liver and kidneys, most likely because of the high content of blood vessels. *http://www.clevelandclinicmeded.com/medicalpubs/diseasemanagement/infectious-disease/sepsis/*

In some cases, increased coagulation during sepsis can result in disseminated intravascular coagulation (DIC), further contributing to multple organ dysfunction syndrome (MODS) via activation of tissue factor on endothelial cells and production of thrombin,x (Figure 2). As the inflammatory phase progresses, patients that have not been treated appropriately develop the compensatory anti-inflammatory response syndrome (CARS) or immunosuppressed phase, in which, cells from the immune system suffer from increased apoptosis, reduced antigen presentation, cytotoxic function and cytokine secretion. In general, immune cells get exhausted and are not able to respond to additional microbial infections increasing deaths. Recovery after this phase is very hard to achieve, but if it happens subjects will have an increased rate of mortality in the future [13, 14]. Novel therapheutic strategies are trying to target molecules involved in this immunossupresive stage [13]. However, it is important to consider that immunologic therapies need to be individualized, as one patient may behave different from another.

Endothelial cells and vascular permeability during homeostasis and inflammation

As mentioned before, endothelial cells get activated by pro-inflammatory cytokines during sepsis. Activation induces the expression of adhesion molecules on the apical cell surface and the opening of interendothelial cell contacts which evetually leads to edema formation due to increased vascular permeabilty (Figure 3) [11, 15]. Cell contact stability is largely regulated by transmembranal protein complexes known as tight junctions (TJ) and adherence junctions (AJ) which control the passage of ions, water, proteins and cells between endothelial cells (paracellular). TJ are formed by homophilic interactions between members of the claudin family such as claudin-3 and -5, as well as occludins and junctional adhesion molecules (JAM). On other hand, AJ are mostly comprised of vascular endothelial cadherin (VE-cadherin) [16, 17]. TJ and AJ are finely regulated by actin cytoskleton dynamics because these molecular complexes are linked to

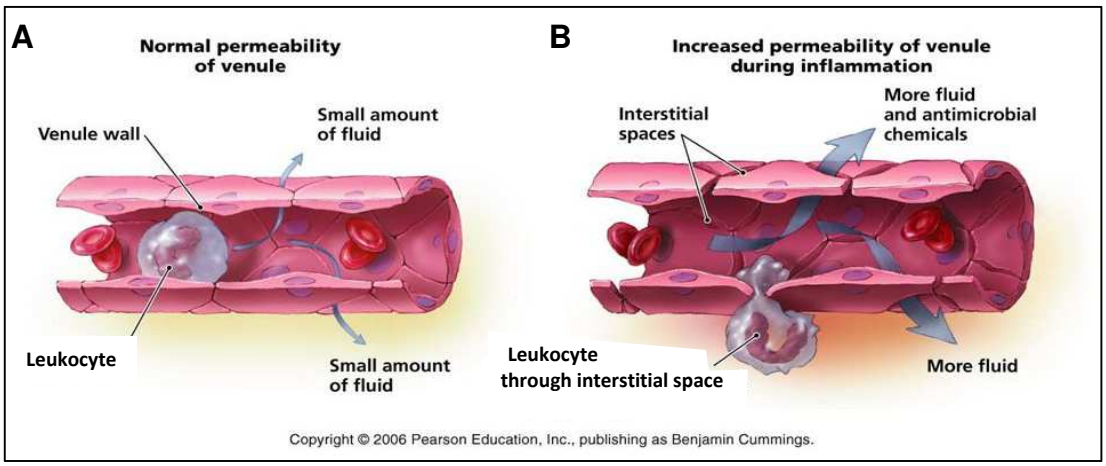

Fig. 3 Vascular permeability of venules. A) A venule under homeostatic conditions where cell contacts prevent leakage of large amounts of fluid into the tissues and cells circulate with no adhesion on the endothelia surface. **B)** Under inflammatory conditions, cell contacts are opened and the interstitial space between them increases leading to increased fluid leakage and transmigration of leukocyte to the site of inflammation.

filamentous actin by different adaptor proteins such as members of the zonula occludens family (ZO-1, -2 and -3) in the case of TJ, and members of the catenin family (α-,β- and γ- catenin) in the case of AJ. Regulation of endothelial contacts requires the activity of small GTPases, primarly members of the Rho familiy, such as Rac1, Cdc42 and RhoA which are known to control lamellipodia, filopodia and stress fiber formation thus having a direct impact on actin dynamics. For example, Rac1 and Cdc42 are involved in barrier stabilization by controling cortical actin formation [18-20].This process involves other molecules such as IQ-motif-containing, GTPase activating protein 1 (IQGAP1) which controls the active states of Rac1 and Cdc42, and p21-activated kinase (PAK), a Rac effector, that activates LIM kinase and inhibits the actin-severing protein cofilin-1, thus enhancing barrier properties. On the other hand, it is well known that RhoA destabilizes endothelial barriers by generating pulling forces on endothelial cell contacts via actin stress fibers, a mechanism in which ROCK, the downstream effector of RhoA, phosphorylates myosin II to activate it's ATPase activity and generate intracellular contraction that facilitates the loosening of intercellular contacts

(Figure 4) [6, 19]. Several studies have proven that blocking of this signaling cascade results in improved endothelial barrier properties and reduced vascular permeability [21-24].

Clearly, the actin cytoskeleton plays a pivotal role in endothelial barrier regulation, and another class of molecules, known as actin-binding proteins

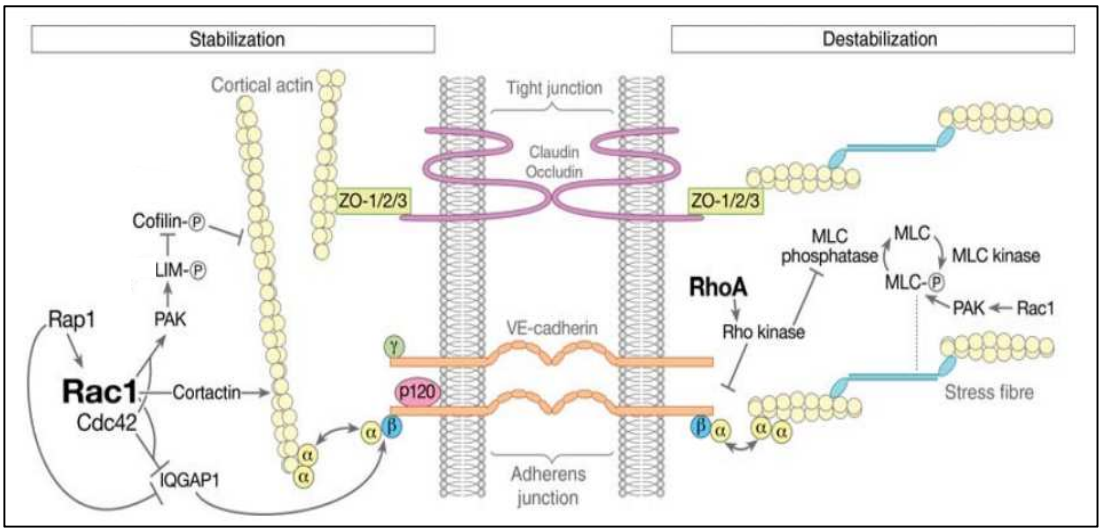

Fig. 4. Mechanisms of endothelial cell contact regulation. Endothelial contacts are formed by TJ and AJ which are connected to the actin cytoskeleton via adaptor proteins. Given this characteristic, changes in actin dynamics regulate stabilization/destabilization of the endothelial barrier where several molecules including small GTPases are involved [6]. While active Rap1 is a major stabilizer of endothelial contacts (left), active RhoA contributes to cell contact destabilization (right).

(ABP) regulate cytoskeleton remodelling and have direct impact on barrier homeostasis. One of these proteins is cortactin, which is an ABP with the ability to directly bind F-actin filaments and support the branching process as a weak activator of the Arp2/3 complex. Additionally, via its SH3 (Src homology-3) domain, cortactin can bind other molecuels involved in endothelial barrier regulation such as ZO-1 [25] and myosin light chain kinase (MLCK) [26]. Cortactin has also been associated with the regulation of permeability and leukocyte recruitment in vivo by controlling GTPase activation and ICAM-1 clustering in endothelial cells [27]. We have recently

also published mechanistic insights demonstrating that cortactin controls the RhoA/ROCK pathway, actomyosin contractility and in consequence in the integrity of both endothelial and epithelial barriers [28, 29], as discussed below in more detail.

Leukocyte recruitment to sites of inflamamtion

Following adherence, leukocytes require a chemokine gradient to complete the process of transmigration. The chemokine family members are classified according to the position of two cysteine residues present in their primary structure. The ELR+ CXC chemokines including IL-8 (CXCL-8) and growth-related oncogene (Gro) attract mainly neutrophils, whereas the C-C chemokines such as the monocyte chemotactic protein-1 are chemotactic for a variety of leukocytes. The specificity of chemokine-induced leukocyte chemotaxis is related to differential expression of chemokine receptors, a superfamily of G-protein-coupled receptors (GPCRs), on specific leukocyte subsets [30]. Thus, interactions between specific chemokines and leukocytes are the mechanisms that allow the host response to deliver specific subsets of leukocytes to localized areas of infection. Leukocyte transendothelial migration (TEM), required to reach infected/inflammed tissues, is critical for infection resolution and wound healing. To this end, these cells have to undergo a series of interactions with endothelial cells, a process termed “leukocyte extravasation cascade”. This process requires that both leukocytes and endothelial cells express and/or activate adhesion molecules on their surface to start interacting with one another (Figure 5). The initial contact and subsequent rolling of tethered leukocytes are mediated by endothelial selectins such as E- and P-selectin with their leukocyte ligands PSGL1 (P-selectin glycoprotein ligand 1), L-selectin and ESL1 (E-selectin ligand 1). These interactions trigger activation of integrins such as LFA-1 (Lymphocyte function-associated antingen 1), MAC-1 (Macrophage antigen 1) and VLA4 (Very late antigen 4) which in turn, induce slow rolling and arrest of leuckoytes. Integrin activation in leukocytes is further promoted by sensing of

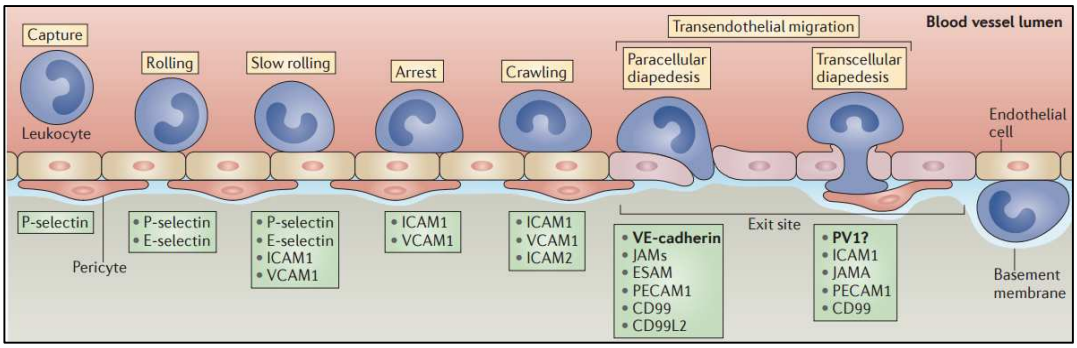

Fig. 5. Leukocyte extravasation cascade. Schematic representation of the multi-step process by which leukocytes reach inflamed/injured tissues, showing the adhesion molecules involved in each step [3].

chemokines presented on the endothelial apical surface. Subsequent crawling on the endothelial surface guides the leukocytes to the spots of damaged or inflammed tissue where they will eventually transmigrate, a process termed diapadesis that can occur via two different routes: paracellularly across endothelial cell contacts or transcellularly across the body of a single endothelial cell. During paracellular migration leukocytes passing through opened cell junctions are enriched on their surface with CD99, PECAM-1 and JAM-A or ICAM-1 [31, 32], molecules known to regulate this process. Paracellular migration is largely regulated by VE-cadherin, this comes from an elegant study showing that Tyr731 needs to be dephosphorylated as leukocytes transmigrate leading to VE-cadherin internalization. This is mediated by Src-homology domain phosphatase 2 (SHP-2) and subsequent endocytosis of VE-cadherin-catenin complexes via the AP-2 (adaptins) complex [33]. Vascular-Endothelial Protein Tyrosine Phospatase (VE-PTP) is associated with the VE-cadherin-catenin complex while junctions are stable; however, when leukocytes adhere to the endothelial apical cell surface, dissociation of VE-PTP from VE-cadherin occurs to enhance leukocyte TEM in a plakoglobin-dependent manner [34]. The actin cytoskeleton is also involved in the internalization of the VE-cadherin-catenin complex as RhoA-induced pulling forces trigger internalization of VE-cadherin through clatrin-coated vesicles (CCV) in a

p120-catenin-dependent manner [35, 36]. On the other hand, transcellular transmigration requires membrane fusion events at sites where there are no cellular contacts, and PV-1 (plasmalalemma vesicle-associated protein 1) has been proposed as canditate marker for this particular transmigration event [3, 37]. Transcellular migration also requires the formation of certain structures called Invadosome-like protrusions (ILPs) which are similar to podosomes and invadopodia, however, no matrix degradation properties have been described for ILPs yet. They have been proposed as “probing” structures that leukocytes use to find sites on the endothelial surface suitable for transcellular migration [38]. Transcellular TEM is also controlled by caveolae, as it has been shown in primary human dermal microvascular endothelial cells (HDMVECs) that at sites of transcelullar migration, caveolin-1 appears to favor this process; and down-regulation of this protein rather promotes paracellular migration [39]. Both trans- and paracellular TEM are supported by the vesicular compartment known as LBRC (Lateral border recycling compartment) from endothelial cells that localizes near the cell surface surrounding leukocytes and contains CD99, PECAM-1 and JAM-A [36]. However, LBRC relies on microtubule dynamics, as dissolution of these structures with demecolcine (DCN) prevented either of the two types of TEM in HUVECs. There are special cellular mechanisms that usually prevent vascular leakage when leukocytes transmigrate [3, 40], however, when not controlled properly, as in septis, excessive transmigration can result in edema formation and tissue hypoperfusion.

Cortactin and HS1 in TEM

As in endothelial cells, leukocytes require extensive rearangements of the actin cytoskeleton during transmigration that is also regulated by ABP. Leukocytes express the cortactin homologue HS1 (Hematopoietic Lineage Cell-Specific protein-1) that is known to exert several functions in hematopoietic cells. HS1 has a similar domain structure as cortactin, but contains only 3 F-actin-binding tandem repeats in contrast to cortactin, which

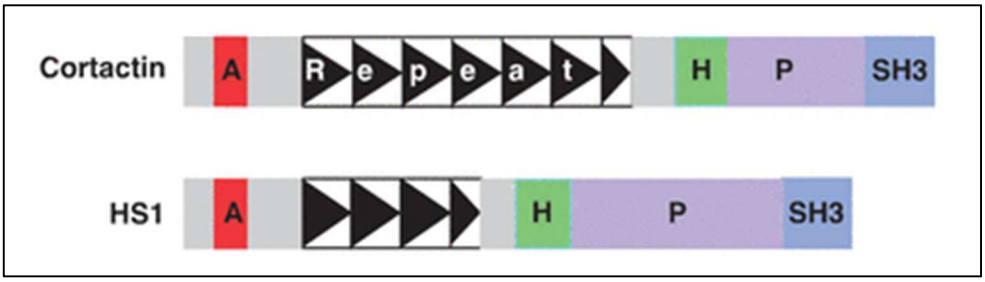

Fig. 6 Cortactin and HS1 domain structures. Schematic representation of cortactin and HS1 domain structure. A, acidic domain; H, helical domain; P, proline-rich region; SH3, Src homology domain; triangles, 37-aminoacid tandem F-actin binding repeats [2]

contains 6.5 repeats [2] (Figure 6). Additionally, HS1 has a nuclear localization signal (NLS) for nuclear translocation after tyrosine phosphorylationwhere where it may be involved in regulating BCR-induced apoptosis [41, 42], Cortactin can also localize to the nucleus when acetylated, but its nuclear functions remain elusive [43]. Although these two proteins contain an SH3 domain in their C-terminal domain with a similarity of 86%, their interaction patterns differ, probably because of the differential expression patterns in tissues [44]. In spite of these differences, Cortactin and HS1 share many similarities, for example, they both can bind the Arp2/3 complex with their N-terminal DDW motif (tryptophan-containing motif) in order to activate actin polymerization *in vitro*. However, HS1 is less efficient than cortactin in this regard [45]. In addition to this, both cortactin and HS1 are known to localize and concentrate at podosomes in osteoclasts [46] and dendritic cells, respectively [47, 48]. Furthermore, both proteins have been shown to play important roles for leukocyte transmigration [5, 27]. We published that cortactin deficiency in endothelial cells not only enhanced microvascular permeability by defective activation of Rap1, but also increased leukocyte rolling velocities, and decreased the number of adherent and transmigrated cells inside venules of TNFα-inflammed cremaster. This was a consecuence of reduced ICAM-1 enriched docking structures that require the activity of the small GTPases RhoG [27, 49]. HS1 is also involved in leukocyte TEM but it is only expressed on hematopoietic cells. HS1-deficient

mice showed impaired leukocyte adhesion and recruitment in the venules of CXCL1-stimulated cremaster [5]. In this study, the authors analyzed at which step of the leukocyte transmigration cascade HS1-deficient cells were being affected. They did not observe effects on P-selectin-mediated adhesion but found that *in vitro* adhesion to ICAM-1 coated surfaces was reduced after CXCL1 treatment. Additionally, CXCL1 chemokine-induced arrest assays *in vivo* which induce activation of integrins mediated by GPCR and subsequent leukocyte attachment to cremaster venules resulted in less adherent cells in HS1 KO animals suggesting that HS1 is required for proper activation of LFA-1. These results were confirmed by soluble ICAM-1 binding assays after CXCL1 treatment of isolated WT and HS1-KO neutrophils, where HS1 neutrophils showed reduced ICAM-1 surface binding (Figure 7). These changes were not caused by differential expression of adhesion molecules as flow cytometry analysis of different leukocyte adhesion molecules did not show important differences between WT and KO animals. Hemograms also showed that HS1-deficient animals had increased numbers of circulating neutrophils (0.85 ± 0.25 K/ul) compared to WT littermates (0.54 ± 0.22). These results highlight cortactin and HS1 as key regulators of leukocyte TEM during inflammation. However, these studies have focused on localized inflammatory stimuli (cremaster injection with pro-inflammatory and chemotactic mediators). Analyzing the role of these molecules during a systemic inflammatory response such as sepsis where excessive luekocyte infiltration into tissues is critical will be important to determine if these proteins can be used as potential therapeutic targets to treat patients suffering from sepsis.

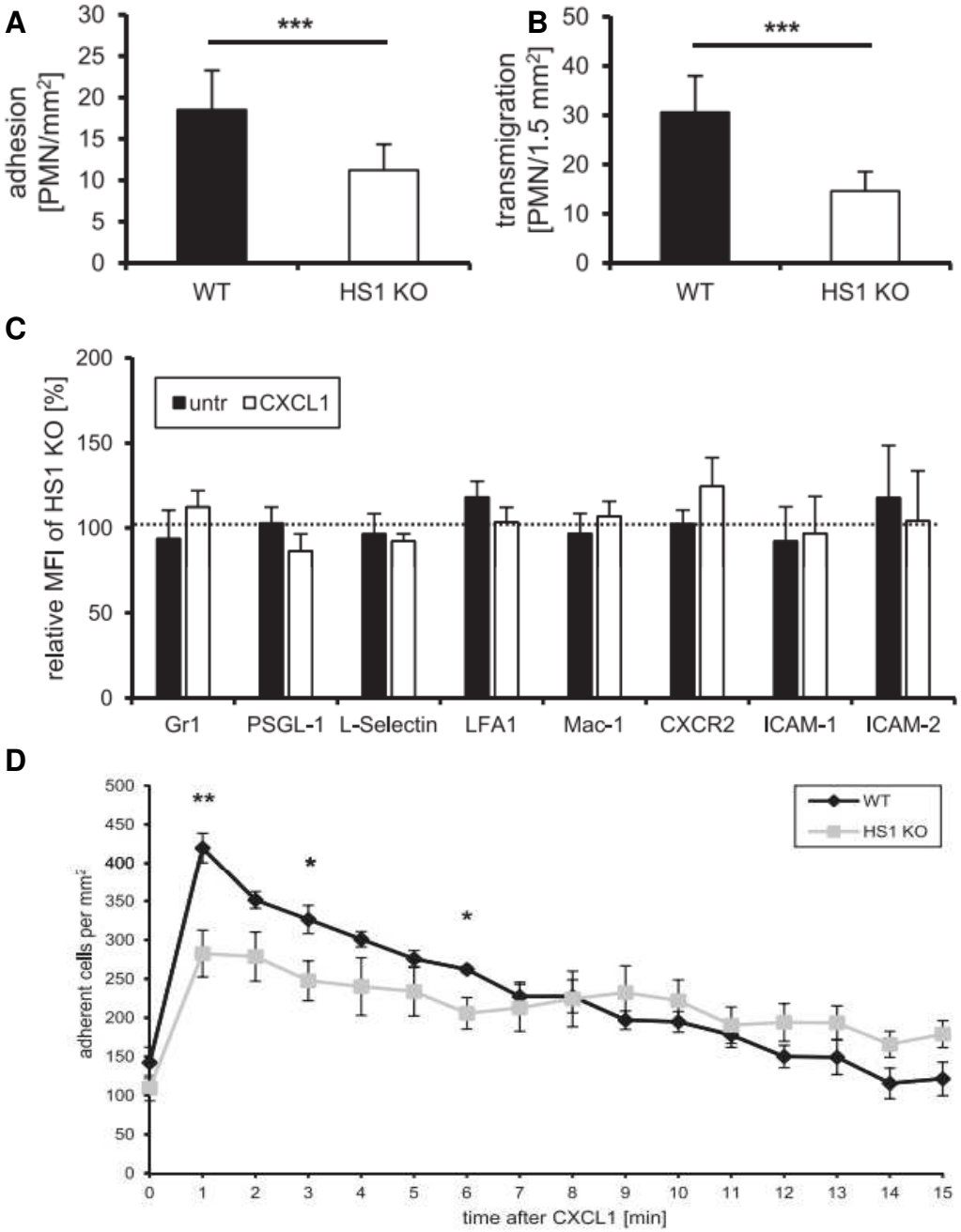

Fig 7. HS1 deficiency affects leukocyte adhesion and transmigration but not the expression of adhesion molecules [5]. (A) The number of adherent cells counted in a designated area, and **(B)** the number of transmigrated cells in inflamed cremaster muscles of WT and HS1-KO mice. **(C)** Relative mean fluorescence intensities of different surface adhesion molecules involved at different steps of the extravasation cascade in untreated or CXCL-1-treated HS1-KO neutrophils normalized to WT (set to 100%). **(D)** Chemokine arrest assay showing the numbers of adherent leukocytes in the cremaster every 60s after CXCL1 injection.

Animal models of sepsis

Studying sepsis pathophysiology in humans has proven to be a challenge, therefore, animal models are needed to further understand and characterize how sepsis develops. Several animal models have been established that have been reviewed recently with each one showing certain advantages and disadvantages [50-53]. It is important to consider that the host response to different models may change depending on the animal type, strain, age, weight and environment. Endotoxemia-induced sepsis has been one of the most used models over the years. This model usually consists of an intraperitoneal or intravenous injection, or intratracheal instillation of LPS to induce activation of TLR-4. This model has different advantages, with reproducibility being the most important, and it induces the activation of the innate immune system. However, mice have increased resistance to LPS while humans are very sensitive, making it difficult to compare the results between the two species. Also, this model does not allow to analyze the hyperdynamic cardiovascular state observed in human sepsis. Finally, human sepsis is usually of polymicrobial nature with several different bacterial components involved, whereas the endotoxin model is restricted to LPS-mediated signaling leading to very early and prominent increases of inflammatory mediators that does not resemble the slow inflammatory response observed in human sepsis [1, 50-52].

An alternative to LPS-induced endotoxemia is the administration of live bacteria such as *E. coli, S. aureus or P. aeruginosa* [54]. An advantage of this model is the choice of pathogen that allows relating disease features to a specific organism. It is also very easy to do and highly reproducible. Additionally, combinations of different pathogens can be used to better simulate polymicrobial human sepsis. In spite of these advantages, intravenous administration of live bacteria also comes with disadvantages as it usually does not lead to colonization and replication of bacteria which is

critical to mimic human sepsis, and immune responses vary in between strains [54].

Since both endotoxemia and bacterial injection induce exaggerated early inflammation that is different to what occurs in humans, other models of sepsis were developed, such as cecal ligation and puncture (CLP), which is considered nowadays as the gold-standard for the study of sepsis. In this model, inflammation arises from polymicrobial infection and involves tissue necrosis. This method consists of midline laparotomy and exteriorization of the cecum which is then ligated and punctured to expose feces [55]. An advantage of this model is the modulation of severity of inflammation and mortality by varying the portion of cecum ligated and the number of punctures and needle size to make the punctures, all of which defines the number of bacteria released into the peritoneum. Moreover, CLP leads to a slow increase in pro-inflammatory mediators similar to what is observed in human sepsis. However, CLP also has some disadvantages, such as increased variation from one experimenter to the other due to variations in their surgical technique. Additionally, stool consistency and differences in microbiota can vary the response, and in some mice the infected area is limited by fibrin deposition leading to intra-abdominal abscess formation as a way to contain the leakage from the cecum, and failure in this process leads to increased mortality [56].

Colon ascendens stent peritonitis (CASP) is another example of peritoneal sepsis induced by host polymicrobial challenge [57]. CASP also consists of a midline laparotomy, but unlike CLP, a stent of determined diameter is implanted into the colon ascendens of the mouse leading to constant feces leakage into the peritoneum, thus avoiding abscess formation. However, it lacks the presence of necrotic tissue and can be even harder to reproduce than CLP (Table 1). Clearly no model is perfect, but CLP is considered the best so far because it is easy, cheap and better represents what happens during human sepsis. Additionally, CLP consistently induces lung tissue

damage, a key feature we pretrend to study here because lungs contain a large number of blood vessels, capillaries and venules, sites for leukocyte extravasation. Another reason to study lungs is that acute lung injury and dysfunction are very common in human sepsis and are responsible for many sepsis-derived deaths.

Animal models of sepsis

Animal model	Advantage	Disadvantage
LPS injection	Simple, sterile; some similarities with human sepsis pathophysiology	Early and transient increases in inflammatory mediators more intense than in human sepsis
CLP or CASP	Early silent period; moderate and delayed peak of mediators; multiple bacterial flora	Age and strain variability; early hemodynamic period in some models
Clinically relevant CLP	Replication of clinical risk factors	Difficulty in analyzing pathophysiological pathways
Infusion or instillation of exogenous bacteria	Early hyperdynamic state	No change in intrarenal microcirculation; need large animals; labor-intensive

Table 1. Advantages and disadvantages of animal sepsis models [1]. The table describes the main important differences between most commonly used and well-characterized sepsis models in animals.

Implications of leukocytes and endothelial cells in sepsis

Evidence for the importance of endothelial cells and leukocytes in sepsis comes from different studies. There are *in vivo* data using different models of sepsis which have proven the importance of the RhoA/ROCK axis in sepsis-induced organ damage. For example, inhibition of ROCK with the inhibitor Y-27632 attenuated lung edema formation and neutrophil transmigration induced by intravenous injection of LPS [58]. Another example involving ROCK in sepsis-induced lung damage in rats showed that inhibition of this molecule resulted in less caspase-3 cleavage and apoptosis measured *in situ* by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays. Additionally, the authors found reduced numbers of neutrophils and protein concentrations in broncheoalveolar lavage fluids from animals treated with Y-27632 [59]. Another study showed the importance of vascular permeability and leukocyte activation in lungs of rats instilled intratracheally with LPS to induce acute lung injury (ALI) and treated with intravenous injection of heparin. The authors found that this treatment reduced lung

edema, myeloperoxidase (MPO) activity and secretion of tumor necrosis factor-α (TNF-α) and IL-6 [60]. Additionally, the same group found later that treatment of mice, subjected to intraperitoneal injection of LPS as sepsis model, with unfractioned heparin attenuated inflammation in the lungs presumably by regulating the RhoA/ROCK1 pathway [61].

Leukocytes are ideally suited for the elimination of pathogenic microorganisms because of their large storage of proteolytic enzymes and rapid production of reactive oxygen species to degrade internalized pathogens [62]. When these lytic factors and pro-inflammatory cytokines are released from tissue-infiltrating leukocytes local damage will ensue [63-66].

The mechanism by which inappropriately activated leukocytes induce injury involves production of elastase [67] and superoxide ions [68]. *In vitro* and *in vivo* data indicate that activated leukocytes are capable of inflicting considerable endothelial injury via combined action of these agents, probably acting synergistically [69]. Leukocytes incubated with endotoxin are capable of causing ALI when reinjected into animals [70]. Furthermore, endotoxin primes leukocytes to produce an enhanced respiratory burst in response to a second activating stimulus [71]. This priming effect was shown by the ability of trace amounts of endotoxin to act synergistically in producing lung injury in animals whose leukocytes have been exposed to small amounts of a chemotactic peptide [72]. Injection of either agent alone did not cause lung injury in this model demonstrating that sub-lethal doses of endotoxin can still provoke significant injury in the presence of other predisposing factors.

The release of pro-inflammatory mediators including TNF-α and IL-1 during the early phases of sepsis makes the endothelium and the underlying tissue more susceptible to neutrophil-mediated killing, presumably because TNF-α and IL-1 increase leukocyte adhesiveness and recruitment. Additionally, continuous presence of proinflammatory cytokines cause endothelial dysfunction including actin rearrangement into contractile stress fibers and

internalization of junctional molecules that increase vascular permeability and induce activation of endothelial adhesion molecules to arrest circulating leukocytes [73]. Clearly, leukocyte TEM plays a pivotal role in sepsis pathophysiology and this is why it will be important to determine in an animal model of CLP sepsis whether modulating TEM can result in better outcomes.

Justification

Given the regulatory functions of cortactin and HS1 during leukocyte-endothelial interactions, it is tempting to speculate that these proteins also play a crucial role during sepsis that is characterized by an exacerbated inflammatory response resulting in increased vascular permeability and leukocyte infiltration into inflamed tissues. Based on this knowledge, we will use cortactin- and HS1-deficient mice to investigate if and how these proteins are involved in the development of lung injury during sepsis.

Hypothesis

Cortactin and HS1 are involved in sepsis-induced lung damage through regulation of endothelial permeability and leukocyte recruitment.

General Objective

To examine potential molecular mechanisms by which cortactin and HS1 affect endothelial barrier function and leukocyte recruitment during sepsis.

Particular Objectives

1. To establish the CLP-induced sepsis model in C57BL/6 mice and evaluate if cortactin- or HS1-deficiency affect survival of septic mice and the inflammatory response.
2. To study how cortactin and HS1 affect leukocyte counts and leukocyte-endothelial cell interactions in sepsis
3. To analyze vascular permeability and leukocyte recruitment in lungs of control and septic cortactin- and HS1-KO mice.
4. To determine the expression and activation of cortactin, HS1 and apoptosis markers in lungs under septic conditions as potential mechanisms controlling permeability and leukocyte recruitment in the presence or absence of cortactin or HS1.

Materials and Methods

Reagents and antibodies

All chemicals were purchased from the following companies: ROCHE, J.T. Baker, Affymetrix, Macron, Sakura, Sigma, Polysciences, Invitrogen, Fermentas, Qiagen, Bio-Rad and Life Technologies.

All buffers were prepared in deionized water purified using a Mili-Q-system (Millipore).

Buffers and Solutions

Transfer buffer	20% methanol 25 mM Tris 192 mM glycine pH 8.3
SDS-PAGE Running buffer	25 mM Tris 192 mM glycine 0.1% SDS pH 8.3
5X SDS Loading sample buffer	250 mM Tris-HCl pH6.8 10%SDS 30% glycerol 5% β-mercaptoethanol 0.02% bromophenol blue
TBS	*150 mM NaCl*

	10 mM Tris *pH 8.0*
TBS-T	*100 ml 10x TBS* *0.1% Tween20*
PBS	*138 mM NaCl* *3 mM KCl* *8.1 mM Na_2HPO_4* *1.5 mM KH_2PO_4*
PBS-T	*100ml 10x PBS* *0.05% Tween20*
Blocking Buffer	*5 % skim-milk or BSA* *TBS-T*
RIPA Lysis Buffer	20mM Tris-HCl pH 7.5 150mM NaCl 1mM Na_2EDTA 1mM EGTA 1% NP-40 1% sodium deoxycholate cOmplete™ protease inhibitor cocktail and PhosSTOP® (Roche

Antibodies		
Millipore	*Anti-Cortactin clone 4F11*	*Cat # 05-180*
BD Bioscience	*Anti-CD31 clone MEC 13.3*	*Cat # 553370*
	Anti-CD62L clone MEL-14	*Cat # 553152*
Santa Cruz	*Anti-VE-Cadherin (C19)*	*sc-6458*
	Goat anti-mouse IgG-HRP	*sc-2005*
	Goat anti-rabbit IgG-HRP	*sc-2004*
Cell Signaling	*Anti-HS1 (Rodent Specific)*	*# 4557*
	Anti-ROCK1 (C8F7)	*# 4035*
	Anti-ROCK2 (D1B1)	*# 9029*
	Anti-PARP (46D11)	*# 9532*
	Anti-Caspase 3	*# 9662*
BioLegend	*TruStain fcX™ anti-mouseCD16/32 (93)*	*Cat # 101319*
	Anti-CD45-APC/Cy7 (30F11)	*Cat # 103116*
	Anti-Ly6G/Ly6C	*Cat # 108408*
	Anti-CD11b	*Cat # 101208*
Invitrogen	*Alexa Fluor 488 goat anti-rabbit IgG (H+L)*	*A11008*
	Alexa Fluor 488 goat anti-mouse IgG (H+L)	*A11001*
	Alexa Fluor 488 rabbit anti-goat IgG (H+L)	*A11078*
	Alexa Fluor 568 goat anti-rabbit IgG (H+L)	*A11011*
	Alexa Fluor 568 rabbit anti-mouse IgG (H+L)	*A11061*

Animals

Male C57BL/6J WT (The Jackson Laboratory) and cortactin- and HS1-KO mice were used at an age of 8-12 weeks. Cortactin- and HS1-KO do not show any obvious phenotype and can reproduce without any problems. Cortactin-KO animals were generated by Cre-LoxP recombination to ablate exon 7 [27, 74]. On the other hand, HS1 KO mice were generated by homologous recombination using two different vectors to delete exon 2 and exon 10. Embryonic Stem cells were electroporated with either of these vectors and successfully transfected HS1 KO colonies were injected in C57BL/6J blastocysts to generate chimeric mice which were crossed to obtain HS1 KO animals [5, 74].These mice have been backcrossed with C57BL/6J WT mice for at least 10 generations. Both cortactin- and HS1-KO animals show no obvious differences in phenotype and reproduction. Of note, only HS1-KO animals appear to gain more weight than WT or cortactin-KO mice around week 12. Mice were housed in a new animal barrier facility at Cinvestav under specific pathogen-free conditions. The Animal Care and Use Committees of Cinvestav (Mexico) has approved all animal experiments.

Mice genotyping

Animals were genotyped by extracting genomic DNA from a small piece of the tail. Tails were digested in 500 µl of lysis buffer (50mM KCl, 50mM Tris-HCl pH 8.0, 2.5 mM EDTA, 0.45% NP-40, 0.45% Tween-20) supplemented with 2.5 µl proteinase K (20mg/ml, Invitrogen). Samples were incubated at 55°C in a Thermomixer (Eppendorf) with agitation 600-8000 rpm over night. Next day the samples were heated to 95°C during 10 minutes to inactivate the proteinase K. Subsequently, all samples were centrifuged for 1 min at 13,000 rpm to clear the soluble material and the supernatants containing genomic DNA were used for genotypification. For cortactin the following primers were used: forward 5′-AGGGTCTGACCATCATGTCC-3′ and reverse 5′-GTGCTGTTCATCCACAATGC-3′ yielding a ~400 bp for the deleted (KO) allele and ~900 bp for the WT allele. In the case of HS1 the WT band

corresponds to ~350 bp while the KO contains ~250 bp and the primers sequences used were: forward 5'-GGCATGGATGGCTGCTGGAC-3'; reverse 5'-CCTTCGTCACATGGAATATG-3'; lacZ 5'-CATGCTTGGAACAACGAGCGC-3'. PCRs were done using the following protocol: activation at 95°C for 2 min, 35 cycles including denaturation phase at 95°C for 20 s, annealing at 60°C for 30 s and extension at 72°C for 45 s, followed by a final extension at 72°C for 3 min.

Figure 8. Figure shows typical end-point PCR amplified products for both cortactin- and HS1-KO alleles

CLP-induced sepsis

Male C57BL/6 and C57BL/6 HS1- or CTTN-KO mice within an age range of 8-12 weeks were used for the induction of sepsis by cecal-ligation and puncture. We used a modified protocol as described [55]. Mice were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine i.p. (numbers correspond to images in figure 9), then the abdominal area was shaved (2) and disinfected with ethanol and iodine (3). A ~1.5 cm incision was made in the *linea alba* (4), the cecum was carefully exposed (5), and half of the cecum was ligated using 4-0 sterile silk (6). After ligation, a single puncture was made in the tip of the cecum with a 21 G needle and 0.5 mm of feces were exposed (6). Finally, the cecum was returned to the peritoneal cavity and the wound was closed using silk and disinfected (7). Sham-operated mice not subjected to CLP served as controls.

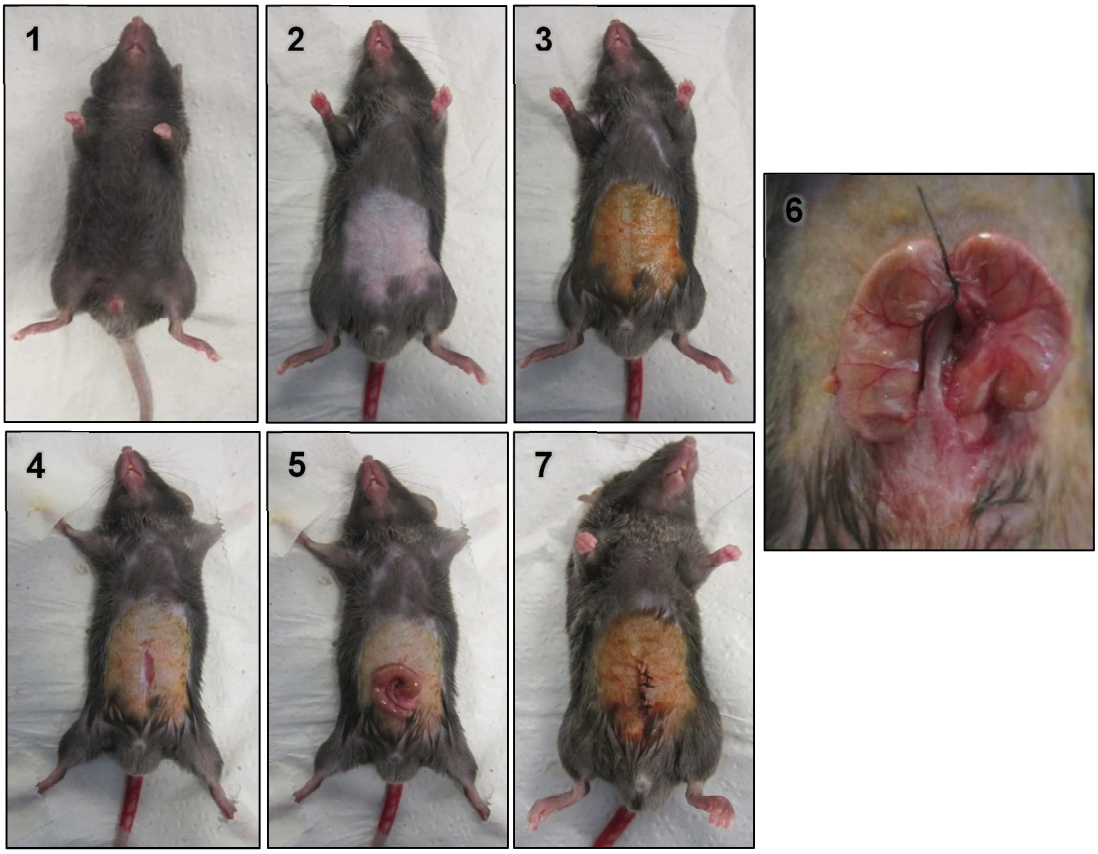

Figure 9. Sequential steps necessary for the induction of peritoneal sepsis by cecal-ligation and puncture.

To help resuscitation and sepsis development, 1 ml of saline was injected subcutaneously in the back of the mice. Twenty-four hours after surgery, mice were anesthetized again and blood was collected from the heart to analyze blood cells and cytokine production, and subsequently animals were sacrificed by anesthesia overdose. All animals were subjected to tissue perfusion by injection of 15-20 ml of PBS through the right ventricle of the heart in order to remove all the blood from the organs.

Survival Curve

After surgeries, animals were placed in their cages with food and water *ad libitum* and monitored for five days. Body weight was measured daily and mice were checked for their general health status and behavior. After five days, surviving animals were sacrificed by anesthesia overdose.

Histology

Twenty-four hours after CLP, mice were sacrificed by anesthesia overdose. For H&E stainings, mice were perfused via the heart's right ventricle with 20 ml of PBS. An incision was made in the lower part of the vena cava to allow fluids to drain. After tissue perfusion, the right lung was ligated from the trachea and cut for analysis of protein and RNA as described below. The left lung was perfused by the trachea using 1 ml of 10% formalin until it was completely inflated and incubated for 5 minutes. Subsequently, the lung was excised from the thoracic cavity and submerged in formalin for 48-72h. Subsequently, tissues were treated for paraffin embedding: first, samples were dehydrated with different alcohol solutions for 1h each, 70% alcohol, 96% alcohol, absolute alcohol, alcohol-Xylol and 100% Xylol. Afterwards, tissues were embedded in Paraffin I from 2-4h and then in Paraffin II for 2h, finally, tissue blocks are placed into paraffin molds previously heated inside an oven for 1 h and left at room temperature to solidify. After this, 5 µm thick tissue sections were cut and transferred to a 40-45°C water bath with gelatin, mounted on glass slides and left to dry for one day. Lung paraffin-cuts were stained with eosin and hematoxylin as follows: tissue sections were deparaffinized following the same alcohols as for paraffin embedding but the order was inverted (without 70% ethanol and the ethanol-xylol mix) and the time was reduced to 5 minutes for xylol and 1 minute for all other alcohols. Afterwards, samples were washed with tap water twice, Harris hematoxylin was added to the tissues and incubated at room temperature for 7 minutes followed by two more washes with tap water. Acidic alcohol was added for 10 seconds and quickly washed off with water two times. Lithium carbonate solution was added for 10 seconds and washed away with water twice. Subsequently, 80% ethanol was added for 1 minute, followed by incubation in eosin for 15 seconds and a series with ethanol and xylol as mentioned before for 1 minute each. Finally, mounting resin was added and samples were observed using a conventional light microscope [29, 75]. A semi-quantitative analysis of the lung was performed by a pathologist in a blinded fashion by

applying a histological score for each of the following criteria: edema, alveolar wall thickening, alveolar hemorrhage, vascular congestion, presence of mucus and infiltration by leukocytes [76, 77].

Intravital microscopy

WT and HS1-KO mice were anesthetized by intraperitoneal injection of 125 mg/kg ketamine hydrochloride (Anesket, PISA, Mex) and 12.5 mg/kg xylazine (Procin, PISA, Mex). Cremaster muscles were prepared for intravital microscopy (as shown in the figure 10) 24 hours after sham or CLP operation [78]. Post-capillary venules with a diameter of 20 – 40 µm were recorded using an intravital upright microscope (Axioscope A1, Carl Zeiss, Germany) with a 40x saline immersion objective WN Achroplan (Carl Zeiss). Rolling and arrested leukocytes were quantified by trans-illumination intravital microscopy and transmigrated cells were quantified by differential interfering contrast (DIC) microscopy. Cells that transmigrated were determined within an area of 100 µm of vessel length over 75 µm on each side of the vessel. Recorded videos and images were analyzed with ImageJ (NIH Bethesda, MD, USA) and ZEN 2 Blue Edition (Carl Zeiss, Germany) software. Rolling flux fraction was calculated as percent of total leukocyte flux. Blood flow center line velocity was calculated by recording 1 minute videos after tail vein injection of 50 µl of sonicated 2 µm red fluorescent Fluospheres beads (ThermoFisher Scienftific).

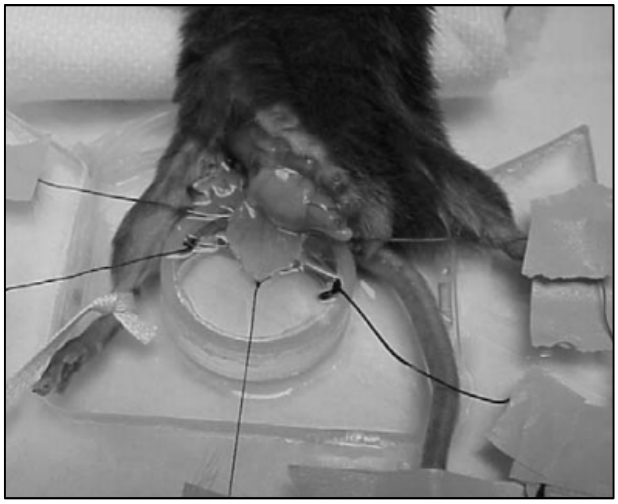

Figure 10. Cremaster muscle exteriorization and fixation for intravital microscopy. The muscle is extended to clearly visualize blood vessels.

qRT-PCR

Total RNA from lung tissues was isolated from five sham and five CLP-induced sepsis WT and KO-HS1 mouse lungs using TRIzol Reagent (Invitrogen, Thermo Fisher Scientific Inc.), quantified on a NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc.), and treated with DNase I, RNase free (Thermo scientific). RNA was reverse transcribed employing oligo-dT and SuperScript II reverse transcriptase (Invitrogen, Thermo Fisher Scientific Inc.), according to the manufacturer's instructions. PCR was carried out in a total volume of 10 µL, containing 5.0 µL Power SYBR Green PCR Master Mix 2X, 100 ng cDNA, and 0.15 µM of each primer in a StepOne™ Real-Time PCR System (Applied Biosystems). Conditions were as follows: activation at 95°C for 10 min, 40 cycles including denaturation at 95°C for 15 s and data acquisition during annealing/extension at 60°C for 60 s. Following the last cycle, the melting curve was generated by heating from 60°C to 95°C in increments of 0.6°C/s. β-actin was used as internal control of amplification. Relative expression was quantified using the $2^{-\Delta\Delta CT}$ method [79]. The following primers were used:

Gene	**Forward 5' – 3'**	**Reverse 5' – 3'**
β-actin	TATCCACCTTCCAGCAGATGT	AGCTCAGTAACAGTCCGCCTA
IL1-β	GCAACTGTTCCTGAACTCAACT	TCTTTTGGGGTCCGTCAACT
IL-6	CCTTCCTACCCCAATTTCCAA	AGATGAATTGGATGGTCTTGGTC
TNF-α	ACGGCATGGATCTCAAAGAC	AGATAGCAAATCGGCTGACG
MCP-1	CCCCAAGAAGGAATGGGTCC	GGTTGTGGAAAAGGTAGTGG

Western blot

Whole lung lysates were obtained by dissecting and cutting the tissues and subsequent blending using a polytron Tissue Master (OMNI International, Georgia, USA) in ice-cold RIPA buffer supplemented with 3x cOmplete™ protease inhibitor cocktail and 1x PhosSTOP® (Roche). Tissue homogenates were centrifuged at 12,000 rpm, 4°C for 30 minutes to clear the lysate from cellular debris and particles, and supernatants were transferred to fresh microtubes and denaturized by adding 5X Laemmli Buffer (250mM Tris-HCl pH 6.8, 10% SDS, 30% glycerol, 5% β-mercapitalethanol) and 0.02% bromophenol blue) and boiling for 10 minutes. Samples were centrifuged for 15 seconds at 12,000 rpm at 4°C to precipitate liquid in the lid, aliquoted and stored at -80°C for later use. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad Laboratories Inc.), subsequently membranes were blocked using TBS-T 0.1% containing 5% BSA (Sigma) for 1hat room temperature. After blocking, membranes were incubated with primary antibodies dissolved in blocking solution at 4°C overnight. After three washes with TBS-T, membranes were incubated with species-specific secondary HRP-coupled antibodies (Santa Cruz) at a 1:5,000 dilution for 2 h at room temperature followed by three more washes with TBS-T. Signals were detected using a ChemiDoc™ Imaging System and SuperSignal® West Pico or SuperSignal® West Femto Chemiluminescent Substrate (Thermo Scientific).

Leukocyte recruitment into the lung

After sacrifice, lungs were perfused with 1x PBS until they turned white. The left lung was excised, transferred into 1.5 ml microtubes and chopped using fine scissors in 1 ml of 37°C pre-warmed PBS containing calcium and magnesium (0.9 mM) and 200 units of collagenase (Sigma, C9891). A small hole was made on the cap tubes using a 21G needle to prevent the solution from becoming hypoxic. Tissues were incubated at 37°C in a water bath for 20 minutes, tubes were vortexed and incubated for 20 minutes more. Then,

the tissue pieces were resuspended using a 1 ml pipette tip with the tip chopped off, and subsequently, samples were incubated for additional 20 minutes. Cell suspensions were passed through a 40 µm cell strainer (Corning) and washed with 5 ml of PBS. Cells were centrifuged at 180xg for 5 minutes and resuspended in 1 ml PBS for flow cytometry analysis.

Flow Cytometry

WT and HS1 KO peripheral blood (PB) cells were obtained by puncture of the right heart ventricle and collected in sterile microtubes containing 0.5 M EDTA as anticoagulant. Leukocytes were counted using a Neubauer chamber and equal number of cells were blocked with TruStain FcX for 20 minutes at room temperature in PBS containing 3% FBS. Neutrophils were detected with Gr-1 (PE-coupled 1:150) and CD11b (PE-coupled 1:150). For staining of lung cell suspensions, anti-mouse L-selectin (APC-coupled 1:50), CD45 (APC/Cy7-coupled 1:100), and rat anti-ICAM-1 antibody that was obtained from the YN1/1.7.4 hybridoma supernatants after growing them for one week in medium without serum. In this case, a secondary goat-anti-mouse (AF488 coupled 1:1000) was employed. Stainings were performed on ice for 30 minutes and then washed with PBS containing 3% FBS. 7-AAD was employed to assess dead cells within all samples. All data acquisition was performed on a BD LSR Fortessa. Data analysis was performed with FlowJo Treestar V10 software.

Analysis of apoptosis

Apoptosis was analyzed by western blot of PARP (uncleaved 116 kDa and cleaved 89 kDa) where the cleaved portion of PARP was normalized to the uncleaved fraction and later to tubulin as loading controls. In a similar way, caspase-3 cleavage was analyzed (uncleaved 35 kDa, cleaved 17 kDa).

Statistics

For comparison of all animal groups, we employed standard unpaired Student T test and unpaired Student T test with Welch's correction when groups

variance was not equal. One-way ANOVA with Bonferroni post-hoc test was used to compare different groups. A value of *$p \leq 0.05$ was considered statistically significant.

Results

We showed that cortactin deficiency leads to decreased levels of active Rap1, a small GTPase that is known to regulate endothelial permeability *in vivo* and *in vitro* [27]. In a more recent publication, and as product of my master and part of my PhD studies, we found that cortactin deficiency is, in addition to Rap1 regulation, associated with increased presence of actin stress fibers in human and murine endothelial cells [28] (Figure 11A), thus contributing to endothelial hyperpermeability via increased actomyosin contractility. Increased contractility led to a destabilization of endothelial intercellular junctions that are connected to the actin cytoskeleton via adaptor molecules such as ZOs and catenins that are known to interact with cortactin [27, 80]. Interestingly, when analyzing molecules involved in actomyosin contractility by western blot and qPCR, we found that cortactin-depleted cells showed an increased amount of total ROCK1 protein levels while myosin light chain kinase (MLCK) remained unchanged. Given its well-known function to induce intracellular contraction of actin stress fibers by phosphorylating MLC2 at serine 19, we also analyzed the phosphorylation state of this protein and found a strong increase in MLC2 phosphorylation (Figure 11B), indicating that increased ROCK1 expression in the absence of cortactin is indeed responsible for increased actin contractility and thus increased vascular permeability. To further prove this idea, we examined if inhibition of actomyosin contractility would restore the increased permeability. Indeed, we found that inhibiting ROCK1 reversed the increased permeability back to levels seen in control endothelial cells. As control, we used the cAMP-elevating endogenous peptide hormone adrenomedullin (ADM) that is known to induce Rap1 and PKA activation, cortical actin formation and thus endothelial barrier stabilization. ROCK1 inhibition almost recovered the effects as strongly as ADM suggesting that increased ROCK1 activity in the absence of cortactin contributes to endothelial dysfunction (Figure 12). Thus, increased ROCK1 levels can be the explanation for the observed increase in vascular permeability without cortactin. These observations prompted us to

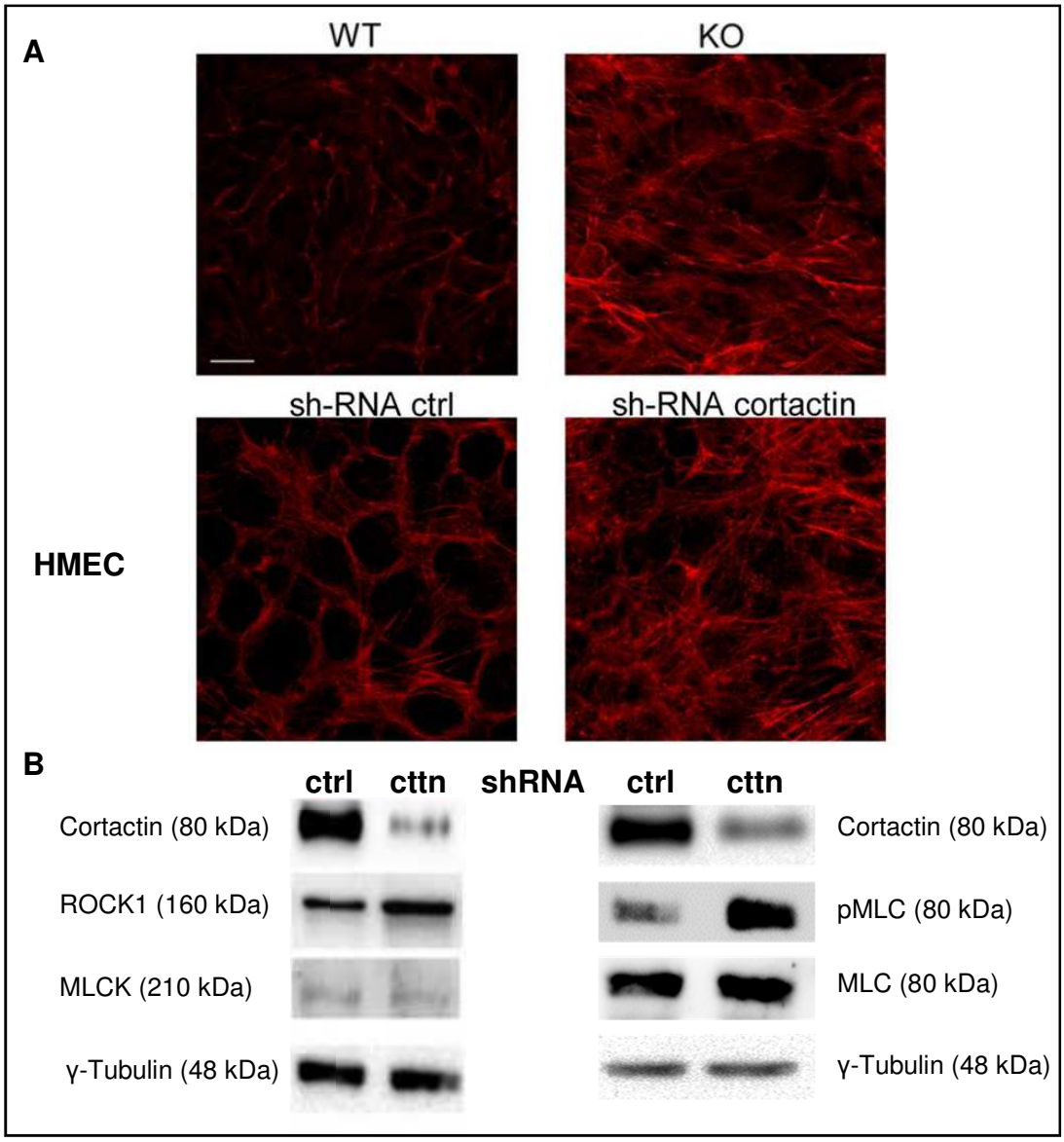

Fig 11. Cortactin deficiency is associated with increased actin stress fibers and increased actomyosin contractility(A) MLEC (murine lung endothelial cells) and HMEC-1 (human microvascular endothelial cells) stained for F-actin using phalloidin (red). **(B)** Representative western blots from HMEC-1 cells transduced with lentiviral particles expressing scrambled (ctrl) or cttn-specific shRNA showing increased ROCK1 expression and MLC phosphorylation. Tubulin served as loading control.

study the role of cortactin in sepsis since endothelial dysfunction and increased vascular permeability are hallmarks of the systemic inflammatory response during sepsis [1, 81, 82].

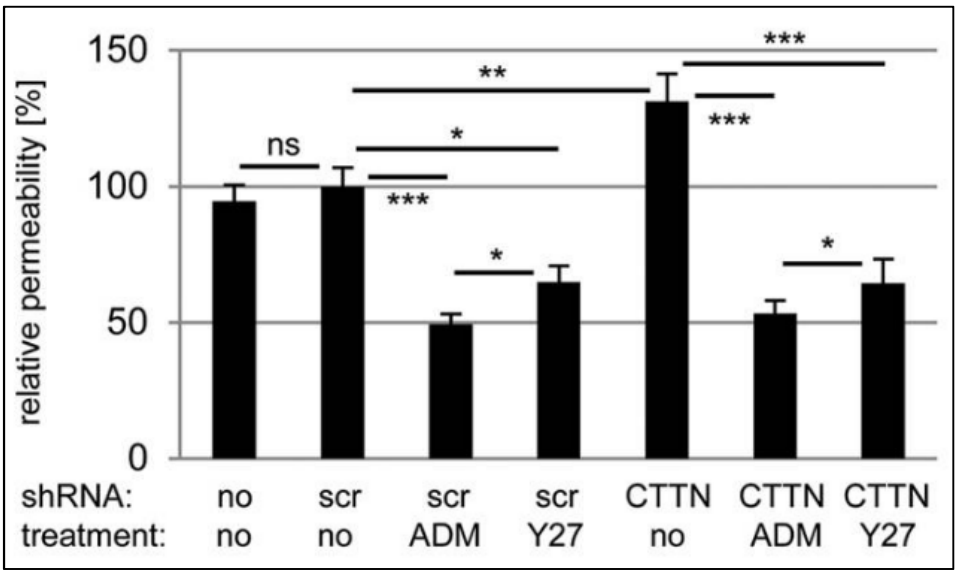

Fig 12. Adrenomedullin treatment and ROCK1 inhibition rescue the increase in vascular permeability induced by cortactin deficiency. HMEC-1 monolayers were treated or not with 100 nM ADM or 10 μM Y-27632. 150 kDa FITC-dextran was added to the upper chamber and incubated for 30 min. Medium from the lower chamber was collected and fluorescence signal intensity measured using a fluorometer. Data are represented as mean +/−SD from three independent experiments and represent relative permeability with control cells expressing scrambled (scr) shRNA set to 100%. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

We speculated that disturbed cortactin-mediated signaling via a Rap1/RhoA/ROCK axis and disturbed actin remodeling contributes to the development of sepsis and sepsis-induced organ failure. This hypothesis is supported by a study showing increased RhoA/ROCK activity in lungs after LPS-induced sepsis [61]. Thus, we investigated molecular mechanisms that allow cortactin or the cortactin homologue in leukocytes HS1 to regulate the expression and activity of ROCK, endothelial hyperpermeability and leukocyte recruitment in cortactin- and HS1-deficient mice using the CLP sepsis model. Additionally, we analyzed if cortactin- or HS1-KO also affects the inflammatory response in general by analyzing the expression profile of pro- and anti-inflammatory cytokines and hormones known to affect vascular barrier integrity and leukocyte recruitment.

Cortactin and HS1 deficiencies improve survival of mice after lethal sepsis induction

Mice were subjected to sham or CLP surgeries and monitored for five days. Body weight was measured daily (data not shown). As expected, all sham-operated mice survived to 100% the whole experiment (Figure 13). However, CLP-operated WT animals showed a mortality of ~40% after 24h, which increased to ~92% after 48h, and only 1 mouse survived for 72h. Surprisingly, cttn-KO mice showed improved survival with only 1 mouse dying after 96h. Similarly, HS1-KO mice also showed improved survival, however, it was not as pronounced as in their cttn-KO counterparts. After 24h, ~78% of the mice survived, after 48h ~40% survived, and one more animal died after 96h, while

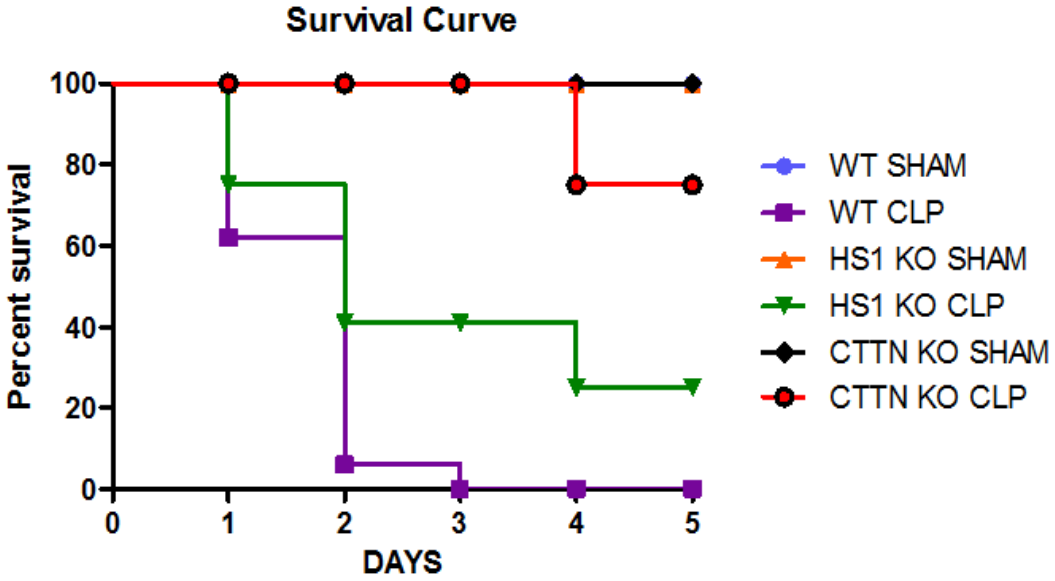

Fig. 13 Five-day survival curve of mice subjected to CLP. Animals were sham or CLP operated and monitored during 5 days to check for survival. WT sham n= 10, WT CLP n=12, CTTN KO SHAM n=1, CTTN KO CLP n=4, HS1 KO SHAM n=9, HS1 KO CLP n=10

3 other mice survived the entire course of the experiment. In the beginning, we expected that the KO animals were going to be more susceptible to a systemic inflammatory response due to the increased permeability in cttn-KO mice, and because of the inefficient transmigration of leukocytes in both HS1- and cttn-KO preventing normal pathogen clearance. However, these data

challenged us to discover the reason for the improved survival of sepsis in the absence of cttn and HS1. Unfortunately, breeding of cttn-KO mice has proven to be challenging, with very few KO offspring so that we had to focus the following experiments on HS1-KO animals.

CLP sepsis upregulates different genes involved in inflammation

To measure the extent of systemic inflammation in our septic mice, synthesis of proinflammatory cytokines was measured. Twenty-four hours after surgeries, animals were anesthetized, blood was collected from the right heart ventricle and placed in sterile tubes containing EDTA as anti-coagulant to obtain plasma samples. Afterwards, the animals were sacrificed with anesthesia overdose and perfused with PBS via the right heart ventricle to clear the lungs of circulating blood. Plasma levels of TNF-α, IL-1β, IL-6 and IL-10 were measured with Milliplex® Magpix according to the manufacturer's instructions (Figure 14). As expected, CLP sepsis induced an increase in plasma concentrations of all the cytokines measured when compared to sham operated animals. No differences between WT and HS1 KO concentrations were observed, except for IL-10, for which HS1-KO CLP animals showed drastically increased levels of this interleukin when compared to WT CLP operated mice. Additionally, to check if CLP-induced sepsis would upregulate pro-inflammatory genes in the lungs, RNA was extracted from these organs and used for cDNA synthesis. We analyzed the mRNA levels of TNF-α, IL-1β, IL-6, KC and MCP-1 by real time PCR. As expected, the mRNA levels of these molecules were significantly upregulated in the lungs of CLP mice after 24 hours, with the exception of IL-1β . However, all of them were even higher in HS1-KO CLP animals (Figure 15). The higher concentrations of these transcripts may be a compensatory response to impaired leukocyte transmigration. We did test IL-10 levels in these animals, and found important upregulation of IL-10 in HS1-KO CLP mice compared to sham operated controls (data not shown). However, we had amplification problems in samples coming from WT animals and therefore these data is not included.

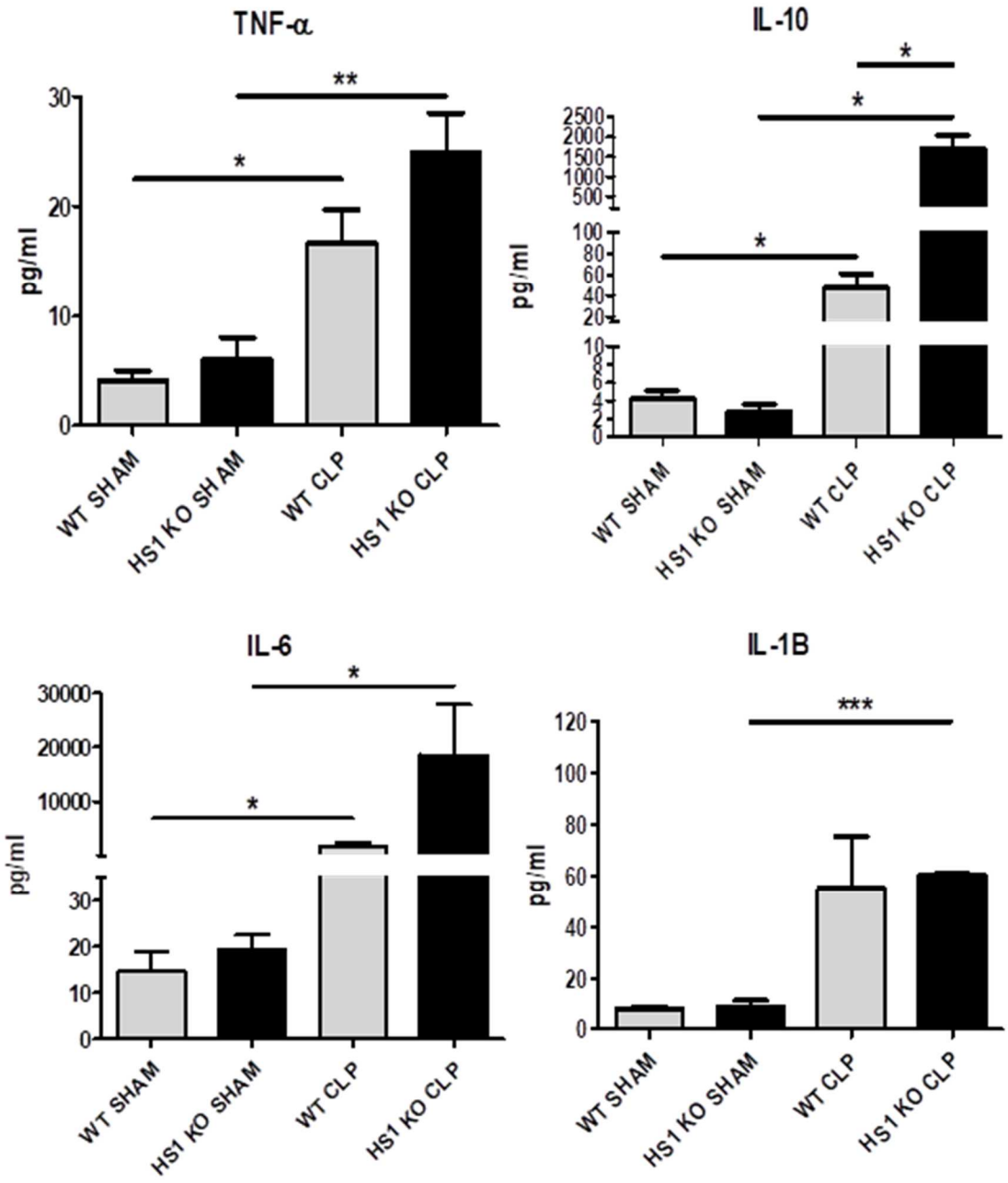

Fig 14. Plasma concentrations of different cytokines. Cytokine secretion was measured into the plasma from at least 3 animals per conditions using Milliplex Magpix kit. Data is represented as mean plus SEM, *p<0.05, ***p<0.01, ***p<0.001

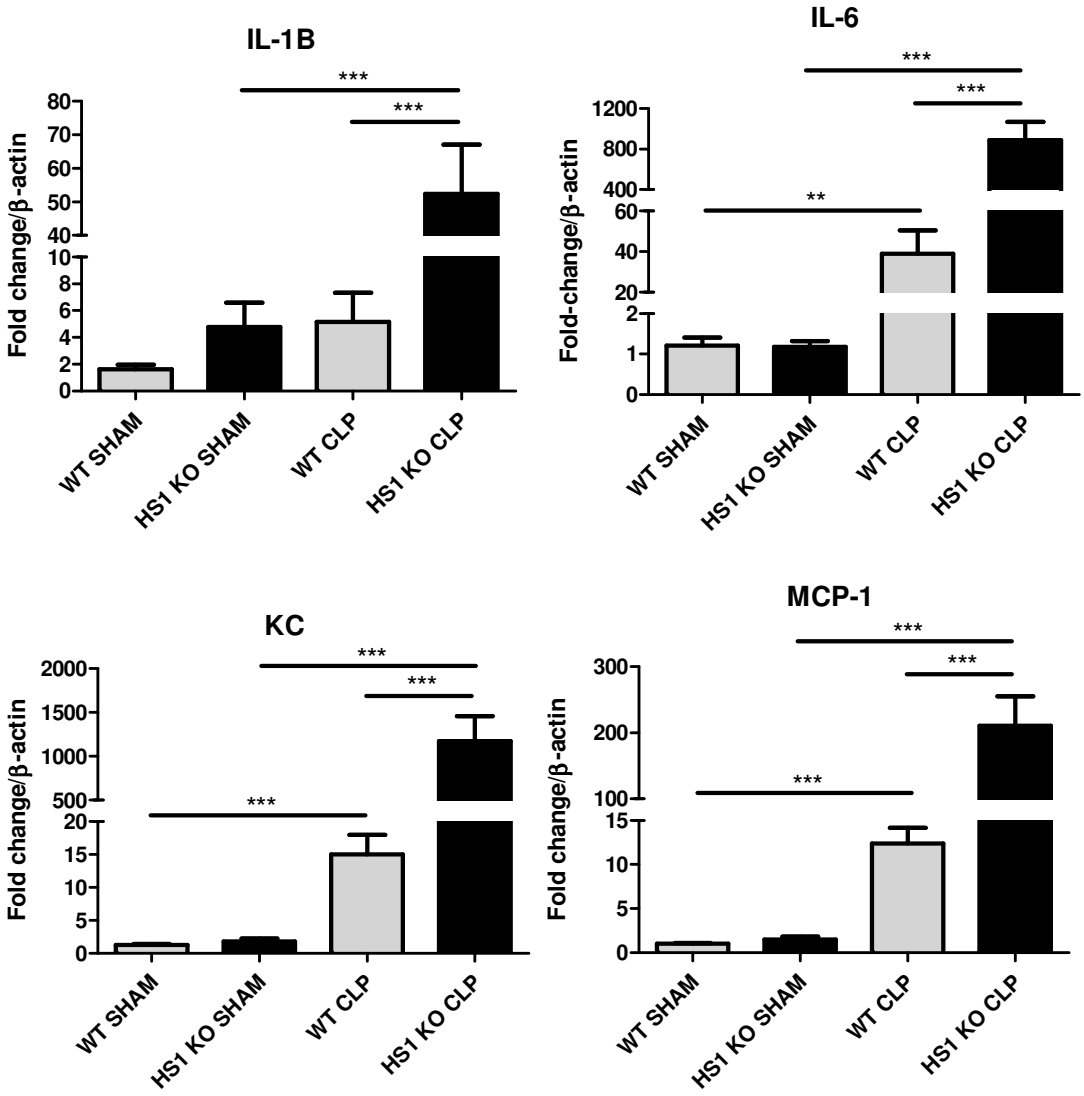

Fig 15. Fold change of mRNA levels from different interleukins and cytokines in the lung after CLP-induced sepsis. mRNA levels of different inflammatory mediators and IL-10 were assessed by real time PCR from the lungs. Data is represented as mean plus SEM n=5, **p<0.01, ***p<0.001

Peritoneal sepsis affects the numbers of WBC and Gr-1 positive in peripheral blood

Several studies have shown that overactivation and excessive recruitment of leukocytes, especially neutrophils are involved in organ tissue damage during sepsis, thus worsening the outcome of patients [83-87]. We speculated that the increased survival of HS1-deficient mice may be in part due to less organ damage as consequence of reduced neutrophil transmigration into affected tissues. To prove this theory, first we decided to analyze the total number of circulating white blood cells (WBC) which were quantified using Turk's solution and a Neubauer chamber. The total number of WBC per microliter were initially lower in HS1-deficient sham operated mice when compared to WT controls (Figure 16A). We expected to observe an increased number of WBCs after sepsis as a normal inflammatory response; however, we found that the total number of WBCs was significantly reduced 24h after CLP in WT animals, indicating that these cells have probably already made their way out of the circulation into different organs. In contrast to what we were expecting, CLP HS1 KO mice showed comparable number of WBC to those observed in their WT counterparts.

To check what was happening to circulating neutrophils, we stained these cells with an anti Gr-1 antibody and analyzed them by flow cytometry and the number of Gr-1 positive cells per microliter was determined (Figure 16B). HS1 KO mice showed a significant reduction of these cells even after sham surgery when compared to WT animals. Moreover, 24h post CLP WT animals showed a reduced number of Gr-1 positive cells, whereas HS1 KO animals did not show any change at all. These results indicate that Gr-1-positive cells may be transmigrating into the tissues in WT animals due to the systemic inflammatory response, which is to be expected and the reason why we see less circulating cells. However, they do not explain why HS1 KO mice have less numbers under both healthy and septic conditions.

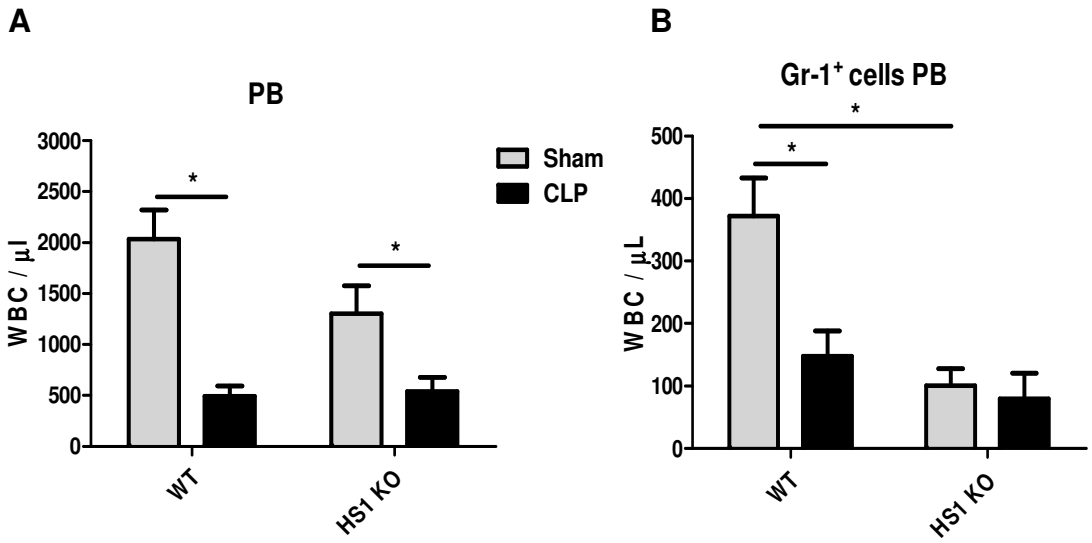

Fig 16. Peripheral blood WBCs and Gr-1+ cells. Total peripheral blood circulating WBCs were quantified using **(A)** Turk's solution on a Neubauer chamber and **(B)** flow cytometry stained for Gr-1 as neutrophil marker. Data is represented as mean plus SEM n=5, *p<0.05

HS1 deficiency protects the lung from CLP-induced lung damage

To analyze if animals had improved survival after CLP in part due to less lung tissue damage, we studied lung histology from WT and HS1 KO animals. To this end, lungs were perfused to clear them from blood and afterwards, 10% formalin was injected via the trachea. The organs were embedded in paraffin, sectioned into 8 µm slices and stained with hematoxylin and eosin. Normal lung histology can be observed in both WT and HS1 KO sham organs, were alveoli structure is not interrupted and appears of normal thickness, bronchi lumen is clear from mucus, vascular vessels are clear from erythrocytes and there are no findings of fibrosis or necrosis (Figure 17A). However, and as expected, lungs from WT CLP mice showed noticeable changes. In general, the normal lung structure was affected by, for example, increased alveoli wall thickness and collapse, edema formation, presence of mucus in the lumen of bronchi, and fibrosis. Despite these changes, we were not able to observe many infiltrated inflammatory cells with this method 24 hours after CLP (Fig. 17B),

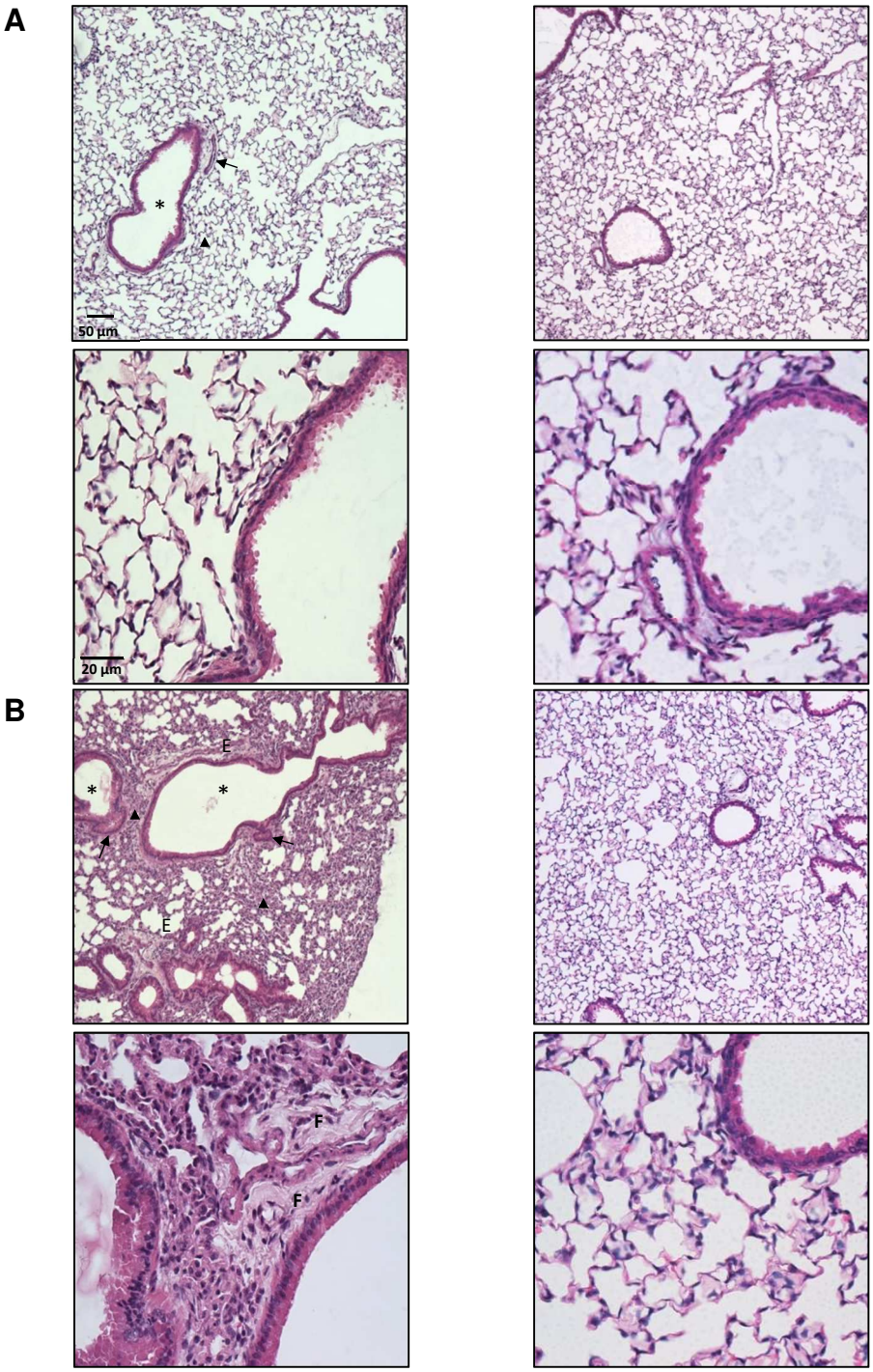

Fig 17. Lung histological micrographs from WT and HS1 KO mice 24h post-CLP. (A) WT (left) and HS1 KO (right) Sham micrographs from H&E stainings at 100X (upper) and 400X (lower) showing general lung normal anatomy (*) Bronchi, (arrowhead) alveoli, (arrow) blood vessel. **(B)** WT (left) and HS1 KO (right) CLP micrographs, (*) mucus at bronchi lumen, (arrow) congested blood vessels, (arrowhead) alveoli wall thickening and alveoli collapse, (E) edema, (F) fibrosis. Lower panel, (F) fibrosis. Experiments consisted of at least 5 animals

probably due to the fact that inflammatory cells did transmigrate early and die soon afterwards by exerting their function. It will be necessary to use a more suitable method to analyze neutrophil infiltration *in situ*. Surprisingly, HS1 deficient mice showed very little to none abnormalities, looking very similar in structure to sham control and with no apparent inflammatory cells, which must likely, is a consequence of the disturbed transmigration in leukocytes from KO animals. Alcian blue stainings of lung paraffin embedded cuts confirmed the presence of mucopolysaccharides in samples coming from WT CLP (Figure 18B) animals not only at bronchi but also at inside alveoli, that is not present in WT sham or either HS1 KO groups (Figure 18A).

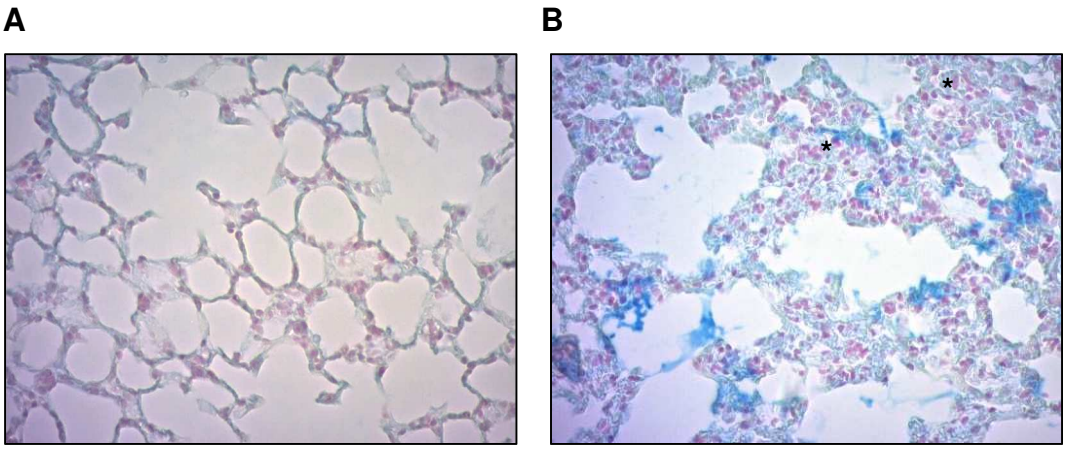

Fig 18. Lung alcian blue stainings from WT and HS1 KO animals. (A) Represents WT sham, HS1 KO sham and HS1 KO CLP animals, were there is a conserved alveolar architecture without mucus presence inside them (upper/lower left panels). **(B)** Is representative for WT CLP mice, showing (*) collapsed alveoli walls with mucus (blue) and vascular congestion (upper/lower right panels)

In addition to this, the lung histological score was quantified in a blinded fashion considering several parameters including fibrosis, necrosis, vascular congestion and edema. Values between 0 and 4 were given for each feature, with 0 meaning no lesions and 4 strongest lesions (Figure 19A). The histological score resulted in an average of 0.2 for WT sham and 0.42 for HS1 KO with no statistical significant difference. However, WT CLP animals had

an average score of 1.73 which was significant when compared to WT sham controls. On the other hand, HS1 KO CLP animals showed an average score of 0.61 and was not statistically significant compared to HS1 KO sham controls. Of note, it was statistically significant when compared to WT CLP indicating that HS1 KO indeed confers a protective effect in septic lungs. As indicator of lung permeability, we analyzed edema formation in the lung separately and observed a significant increase in WT mice subjected to CLP when compared to sham controls. More importantly, this increase was not as prominent in HS1 KO CLP animals, although not significant when compared to WT CLP; while sham controls behaved similarly to the WT sham controls (Figure 19B). Thus, a stabilized vascular barrier leading to less edema formation cannot sufficiently explain the protective effect.

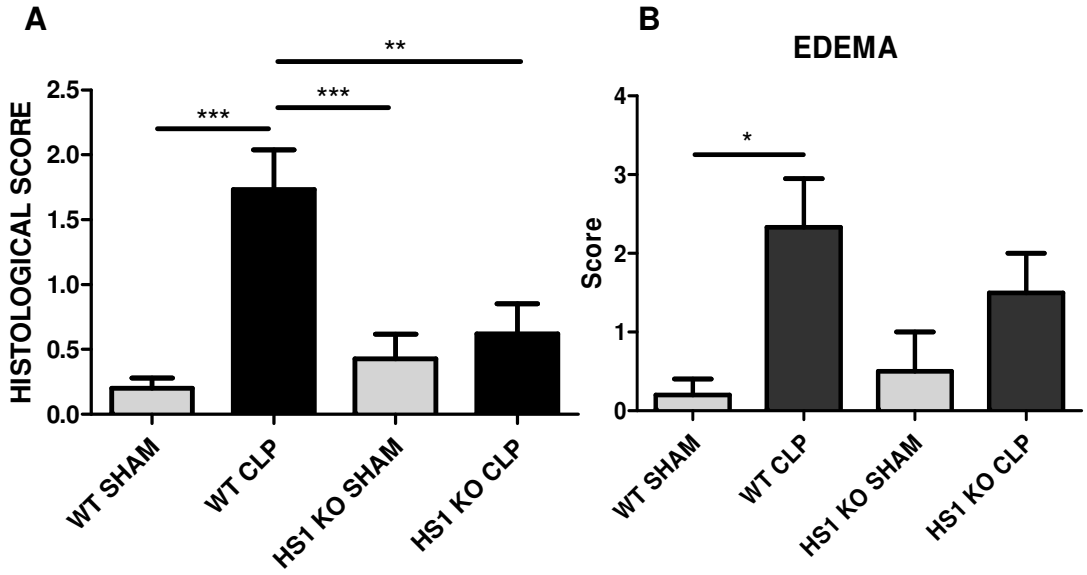

Fig 19. Lung histological and edema scores. (A) Lung scores were determined for 5 animals in each group, where values from 0-4 were given depending on the absence (0) or extended (4) presence of inflammatory changes. **(B)** Edema score. Data is represented as mean plus SEM. **p<0.01, ***p<0.001

Absence of cortactin or HS1 protect the lungs of septic mice from apoptosis

Next, we wanted to analyze the apoptotic state of these tissues to see if reduced cell death in the absence of HS1 could contribute to the observed protective effect during sepsis. To this end, we analyzed whole lung cell lysates by western blot for the DNA-repairing protein PARP and its inactive cleaved form as marker for apoptosis (Figure 20A)**.** The pixel intensity of the western blots was quantified using ImageJ software and used to determine the total amount of cleaved PARP in relation to the uncleaved fraction. We found that CLP increased the cleavage of PARP in the lungs from WT animals (Figure 20B) almost three-fold when compared to the sham controls, a result which is in line with what we observed in the histology. HS1 KO sham showed a similar result as the WT sham animals. Most importantly, HS1 KO CLP cleaved PARP protein levels were comparable to both WT and HS1 KO sham controls and significantly lower than in WT CLP lungs.

For these experiments, we were also able to obtain CTTN-KO animals, so we also measured PARP cleavage in lungs coming of these mice and found very similar results to those obtained from HS1 KO mice, although total and cleaved PARP levels were in general lower in these animals. These results suggested that lungs of both KO animals are protected from apoptosis, which may explain in part preserved lung histology and the protective effect during sepsis.

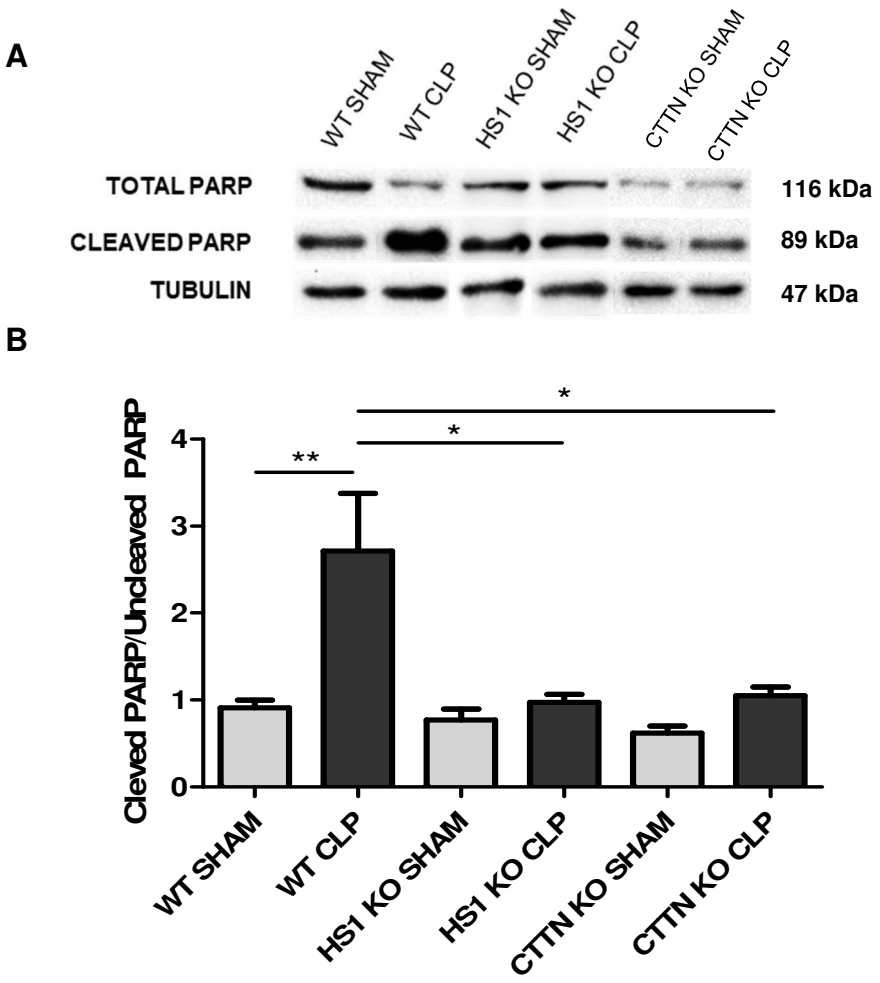

Fig 20. Lung cleaved PARP protein levels normalized to uncleaved PARP. (A) Representative blots for cleaved and uncleaved PARP with proper tubulin loading controls. **(B)** Relative pixel intensity of cleaved PARP normalized to uncleaved PARP. Each condition represents at least 5 animals. Data is represented as mean plus SEM. *p<0.05, **p<0.01, ***p<0.001

HS1-KO deficiency reduces the number of transmigrated and adhered leukocytes in the cremaster muscle after CLP-induced sepsis

To analyze the interactions between endothelial cells and leukocytes and the extent of systemic leukocyte recruitment in our model of sepsis, we conducted *in vivo* intravital microscopy experiments in the cremaster muscle following standard protocols [78, 88]. The cremaster muscle was exteriorized with extreme care to avoid damaging local microcirculation, it was later extended and fixed over a cover slip on a customized acrylic stage to visualize the blood vessels with the microscope. 20-40 µm diameter vessels were used for imaging and quantifications. Representative videos and micrographs from different animals for each condition were taken. WT sham animals showed few to none leukocytes interaction with the vascular endothelium and very few transmigrated leukocytes in the interstitial muscle tissue (Figure 21A). On the other hand, and as expected, WT CLP mice had a significant number of adherent and transmigrated leukocytes (Figure 21B arrows). In contrast to the effects observed in WT animals, neither of the HS1 KO mice showed a significant increase of leukocytes transmigrated into the muscle (Figure 21C and D), confirming what was already published in response to a local, intrascrotal pro-inflammatory stimulus [5]. Our data now show, that leukocyte adhesion and extravasation are also reduced without HS1 in the context of a systemic septic response without an additional local stimulus. Furthermore, during these experiments, the interactions between endothelial cells and leukocytes were recorded to obtain quantifiable data. First, we measured the cell flow, which is determined by how many cells pass through a certain point in the blood vessel in a minute, and we observed a mean of ~31 cells/min in the case of the WT sham controls (Figure 22A), but noticed that HS1 KO animals showed a reduced cell flux, at an average of ~17 cells/min which resulted to be statistically significant. On the other hand, WT CLP animals showed an average of ~10 cells/min, this was to be expected since sepsis reduces blood pressure ultimately affecting blood and

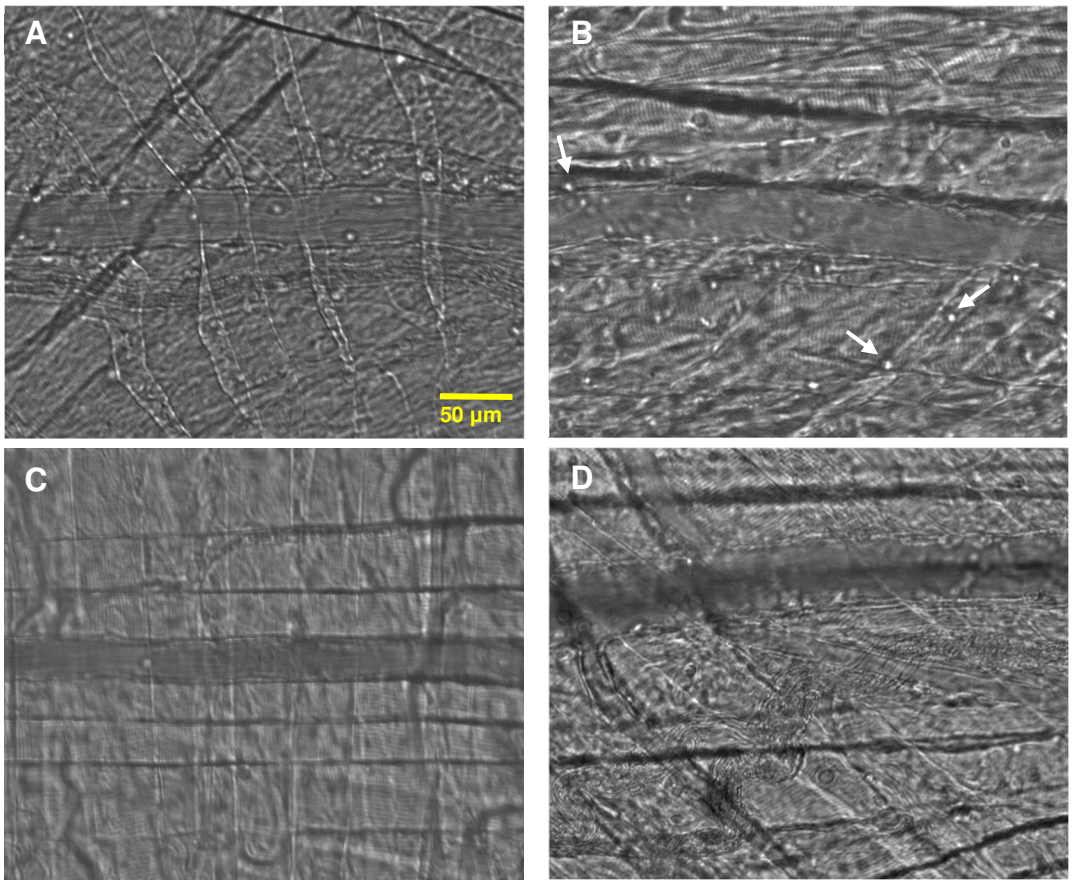

Fig 21. Representative micrographs from cremaster muscle blood vessels and surrounding tissue. Micrographs show a vascular vessel in each picture. **(A)** WT sham controls **(B)** WT CLP **(C)** HS1 KO sham and **(D)** HS1 KO CLP. White arrows point transmigrated leukocytes.

cell flow. However, HS1 KO CLP animals showed even lower cell flux with an average of ~5 cells/min. When analyzing leukocyte rolling velocity (Figure 22B), which is defined by the time cells take to travel 100 µm in the blood vessel, WT sham animals had a mean of 22 µm/sec, CLP animals showed a drastically reduced rolling velocity of around 8 µm/sec, as expected given the fact that inflammatioin induces transition from rolling to slow rolling and then firm adhesion before transmigrating. However, both sham and CLP HS1 KO animals showed an increased and similar rolling velocity, around 28 and 30 µm/sec, respectively, indicating that the transition to slow rolling is disturbed. This process is known to be regulated by activation of LFA-1 which is known

to be disturbed in the absence of HS1 in response to local inflammation, and the same seems to be happening in a systemic inflammatory response [5]. Additionally, we also quantified the number of firmly adherent cells (Figure 22C), where only cells that remained stationary for 30 seconds are counted. While WT animals resulted in ~184 adherent cells/mm^2, CLP WT mice showed a dramatical increased of cells adhesion, with an average of ~443 cells/mm^2. On the other hand, HS1 KO sham mice showed a discrete reduction in cell adhesion, with an average of ~113 cells/mm^2. After CLP, HS1 KO mice did show an increase in cell adhesion ~184 cells/mm^2, which was comparable to the number in WT sham controls, and was significantly lower when compared to WT CLP counts, showing that even after a strong inflammatory stimulus, HS1 KO leukocytes are not arrested efficiently. These results also confirm what has been published in response to a local inflammatory stimulus. Finally, we counted the number of transmigrated cells (Figure 22D), which are already completely outside of the vessels and moving through the adjacent tissues in a determined area. We found that WT sham animals had an average of ~5 transmigrated cells, whereas CLP increased the number of transmigrated cells to ~16, as expected. HS1 KO sham animals showed a similar number (~6) as WT sham controls. By contrast, HS1 KO CLP mice showed a very similar number of transmigrated cells (~8) to those observed in the sham controls, which corresponds to only half of the amount observed in the WT CLP animals. These results clearly show that HS1 deficiency impairs the leukocyte transmigration cascade at different steps, such as rolling, firm adhesion and transmigration, even after sepsis-induced systemic inflammatory response.

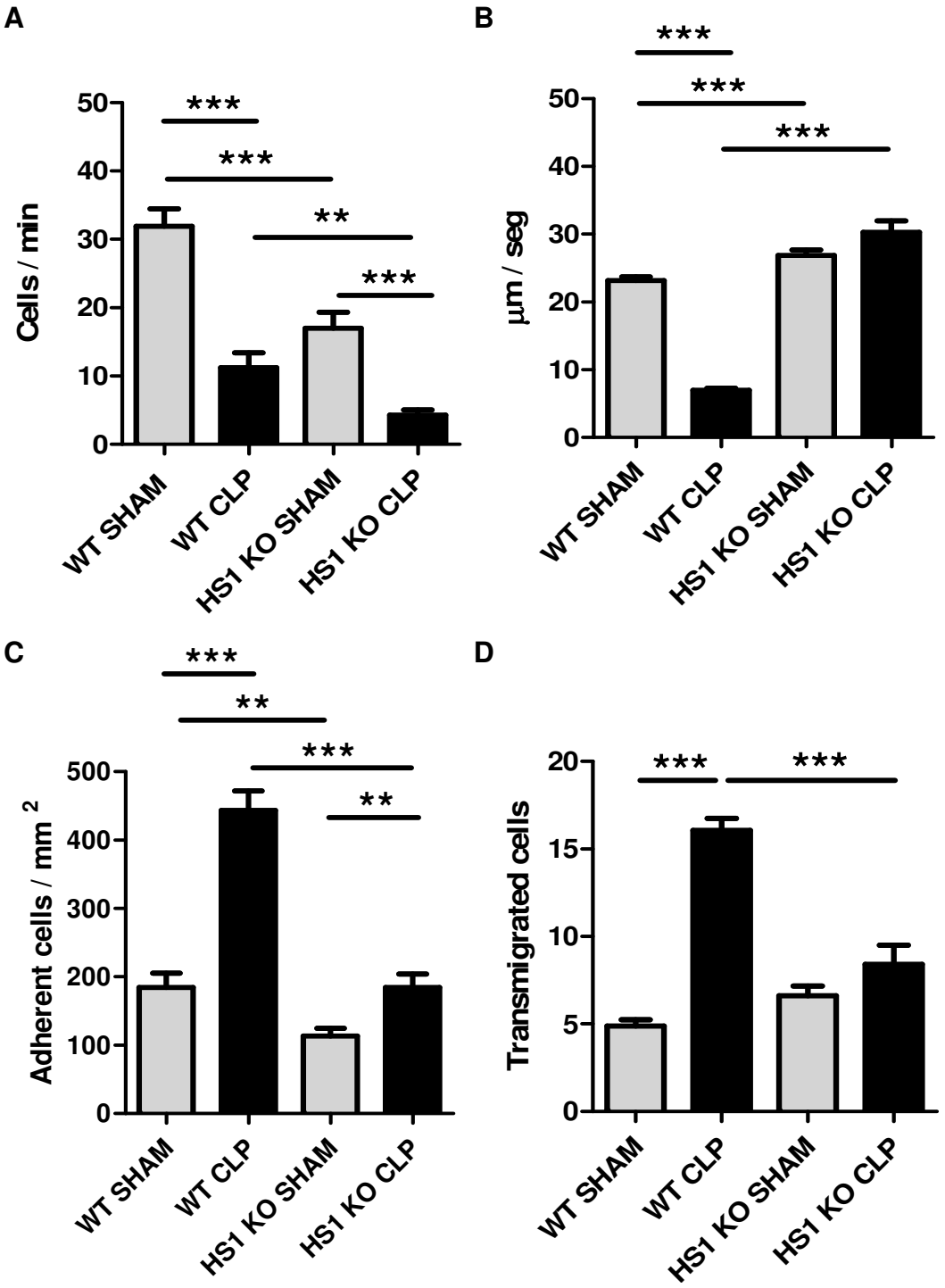

Fig 22. Quantification of different leukocyte-endothelial cells interactions by intravital microscopy of the cremaster muscle. (A) cell flux, **(B)** rolling speed, **(C)** adherent cells and **(D)** transmigrated cells. Each experiment consisted of at least 4 animals. Data is represented as mean plus SEM. **p<0.01, ***p<0.001

Discussion

Sepsis is an important health problem worldwide. Many efforts have been spent to understand this complex condition of systemic inflammation and to find appropriate treatment and therapies to reduce its related high mortality. Although it has been shown that properly timed antibiotics administration is critical to reduce sepsis mortality [89, 90], the remaining dysregulated host inflammatory response is an important aspect in this regard that requires further treatment. Several studies have found attractive potential therapies such as blocking of TNF-α or LPS using antibodies (reviewed in [50, 91]). However, translating these studies from animal models to human clinical trials was unsuccessful. Many reasons could explain this, for example, the use of animal models of sepsis that did not accurately mimic human sepsis, this is in part because these studies have used a single-dose administration of LPS or single-dose inoculation with large number of bacteria, in contrast to human sepsis, which is often of polymicrobial nature. Use of young mice, 8-12 weeks old, that are not treated with antibiotics or fluids therapies, while human sepsis occurs in older subjects that have received any of the previous treatments [50]. Newer promising therapeutic agents are now trying to prevent or heal endothelial cell dysfunction and later organ damage. In this regard, there are studies using different agents to prevent endothelial junction dysfunction which could work as potential therapies for sepsis treatment. For example, treatment with Angiopoietin-1 reduces endothelial apoptosis and blocks VE-cadherin internalization. Slit2N (Slit guidance ligand 2) which prevents microvascular leakage induced by VEGF and stabilizes VE-cadherin at cell junctions via p120-catenin binding [92]. Thus, investigating the potential contribution of molecules involved in actin cytoskeleton and junctional complexes regulation is appealing.

In this project, we analyzed if the actin-binding proteins (ABP) cortactin and HS1 were involved in sepsis-induced organ failure and increased permeability to answer the question whether they could be used as therapeutic targets.

We found that absence of either protein was associated with an increased survival rate of otherwise lethal sepsis induced by cecal-ligation and puncture. These results were a striking surprise for two main reasons: First, we already know that cortactin and HS1 are involved in leukocyte transmigration [5, 27], as absence of either of them resulted in reduced number of transmigrated cells *in vivo* and *in vitro.* Second, cortactin deficiency is additionally associated with increased vascular and epithelial permeability [27-29]. Consequently, these data suggest that lack of any of these ABP will result in overwhelming infection, as leukocytes won't be able to reach and clear pathogens into the infected tissues; and, in the case of cortactin deficiency, increased vascular permeability would probably render animals more susceptible not only to the previous problem, but also due to a higher risk of hypoxia-induced tissue damage due to hypo-perfusion and edema formation. To identify the reasons for these unexpected results we performed a series of biochemical, histochemical and immunological assays. During CLP sepsis it has already been described that bacteria can be found in liver, spleen and blood as soon as 2h post-CLP [93], increasing overtime until 24h [94]. However, we observed surviving animals for one more week after the 5-day survival experiment and in that period, they recovered completely suggesting that, despite their known dysfunction in leukocyte TEM, bacterial infection was not sufficient to kill them, probably because other factors are involved in this process. There is no current evidence that supports the idea of leukocytes phagocytosing and killing bacteria in the bloodstream; however, there is novel and very interesting data showing that erythrocytes can contribute to pathogen clearance in the blood by fixing them through electrical charges and use of oxygen [95]. This could be a potential explanation for pathogen clearance and increased survival of these animals during bacterial sepsis. Although erythro-leukemic cell lines do express HS1 [96], to the best of our knowledge, there is no evidence yet showing functional expression of HS1 in normal erythrocytes. Thus, this could be a topic worth investigating to

determine if HS1 is expressed in these cells and involved in pathogen killing in the blood in septic HS1 KO mice.

We next analyzed the systemic inflammatory state of these animals by measuring the plasma levels of different cytokines such as pro-inflammatory TNF-α, IL-1β, IL-6 and anti-inflammatory IL-10 that all play an important role during sepsis. CLP- subjected WT animals showed an increase in the secretion of these markers when compared to sham controls. HS1 KO sham controls had similar levels to the corresponding WT controls. However, despite the increased survival, HS1 KO CLP animals had a similar secretion profile to that observed in the WT CLP groups with the exception of IL-10 that was expressed at significantly higher levels. Previous studies have demonstrated that IL-10 is critical for sepsis survival. In mice subjected to CLP, administration of IL-10 1h after CLP and every 3h thereafter resulted in increased survival of this experimental group [97], despite the increase in TNF-α serum concentration and lung and liver mRNA. Another study in which the authors used IL-10 KO mice and CLP as sepsis model demonstrated that IL-10 KO mice had increased mortality when compared to IL-10 expressing animals [98]. In the WT control group, serum levels of TNF-α and IL-6 were elevated, but increased further in the samples from IL10 KO mice. Additionally, WT animals showed a maintained increase in IL-10 levels from 5h up to 20h. This study also provided evidence that administration of recombinant IL-10 in these mice 5 h after CLP delayed mortality and improved long-term survival of WT animals and remarkably increased IL-10 deficient mice survival. Finally, a more recent study also using IL-10 KO mice, found protective effects by pre-treating animals 24 hours before CLP with an IL-10 expressing-adenoviral vector [99]. Here, the apoptotic state of lymphoid cells in the thymus was analyzed by caspase-3 cleavage, and they found that caspase-3 activity was increased in IL-10 KO septic mice, and that this effect was reversed after administration of the adenoviral vector. This is in agreement with our data showing that HS1 KO mice had decreased levels of

cleaved PARP during CLP. Interestingly, they also observed that plasma levels of IL-6 were reduced in septic mice treated with the virus. Altogether these data, clearly show the importance of endogenous and exogenous IL-10 to improve survival of septic mice following polymicrobial abdominal sepsis. Consistent with these findings, we also observed increased plasma levels of IL-10 in our WT septic mice, but more importantly, HS1 KO mice had drastically more IL-10 plasma concentrations 24h hours after CLP supporting the idea that these animals are protected from sepsis-induced death because of the anti-inflammatory functions of IL-10 that prevent apoptosis and thus tissue injury.

As already mentioned before, an important player in sepsis lethality is the host response, therefore we analyzed what was happening to leukocytes. We found that after 24h of CLP sepsis the number of circulating leukocytes were reduced indicating efficient transmigration from the blood into inflamed/infected tissues, as expected. The number of peripheral blood leukocytes from HS1 KO mice were reduced in sham operated controls; whereas septic KO mice showed even lower cell numbers compared to septic WT mice. After analyzing cells for Gr-1 expression by flow cytometry, we found that only WT sham animals had a larger number of these cells in peripheral blood which was reduced after 24h of CLP. However, HS1 KO sham animals had less circulating Gr-1 positive cells and didn't change even after sepsis. Previously reported studies have demonstrated that mice KO models for CD18, ICAM-1 and even HS1 [5, 100, 101], all of which also had defects in leukocyte adhesion and transmigration, had increased neutrophil counts. Therefore, the reduced number of circulating neutrophils could be explained by the fact that in our study, mice are subjected to laparotomy surgery which will induce certain degree of inflammation and leukocyte mobilization to the peritoneum even in sham control groups. Such inflammatory stimulus is not present in the studies mentioned above. On the other hand, failure to increase neutrophil numbers even after CLP, is

supported by a previous study in which HS1 depleted CD34+ showed defects in granulocytic differentiation *in vitro* [102], however, this will have to be tested in our experimental groups.

It is very well known that leukocyte activation and efficient transmigration to the sites of inflammation is critical during early stages of sepsis, as it is associated with reduced bacteremia and liver and kidney damage. By contrast, after establishment of sepsis, their presence in the tissues is associated with increased bacterial load and organ damage [103]. We speculated that 24h post-CLP, HS1 KO mice have less lung damage as consequence of reduced leukocyte infiltration and subsequent activation. To prove this theory, we analyzed paraffin embedded lung sections by H&E stainings and found remarkably more damage in tissues of WT CLP mice including edema, hemorrhage, fibrosis, necrosis and presence of mucus, as expected. Of note, septic HS1 KO mice had little to none evidence of lung damage, in a similar way to the respective sham controls. Histopathological scores and analysis of PARP cleavage showed that septic HS1 KO animals were protected from apoptosis in the lung and had no obvious evidence of inflammation. In addition, we could analyze PARP cleavage in a few cortactin KO animals subjected to sham or CLP operations. Importantly, these animals also showed reduced PARP cleavage when compared to their WT counterparts.

We also analyzed leukocyte infiltration in the lungs by flow cytometry and found that both WT and HS1 KO CLP animals had more Gr-1 positive cells infiltrating this tissue than their respective sham controls. However, HS1 KO animals had overall much less infiltrating cells than WT (data not shown). There is evidence showing that reduced leukocyte infiltration in different tissues can be rather beneficial. For example, leukocytes require β1 integrins to reach inflamed tissues [84]. Isolated neutrophils from septic humans and mice showed upregulation of $\alpha_3\beta_1$ integrin (CD49c/CD29) [86]. Administration of a $\alpha_3\beta_1$ (CD49c/CD29) blocking peptide (LXY2) or conditional

downregulation of α_3 integrin subunit in mice resulted in reduced neutrophil infiltration in the lung and reduced mortality after CLP. Additionally, these experimental conditions compromised the ability of neutrophils to secrete TNF-α, IL-6 and IL-10, we didn't analyze the ability of leukocytes to secrete pro-inflammatory mediators. Finally, the authors showed that CD11b was upregulated after $\alpha_3\beta_1$ blockade or depletion, but these changes were not able to compensate the consequences of $\alpha_3\beta_1$ deficiency.

Importantly, we confirmed by intravital microscopy of sham and CLP WT and KO mice that there is indeed impaired leukocyte recruitment in septic WT mice. This is in agreement to a previous study of our lab in collaboration with the Max-Planck Institute in Münster, Germany, which demonstrated that leukocyte infiltration into the CXLC1 stimulated cremaster muscle is impaired [5], although, not under a systemic bacterial infection and inflammatory context.

In summary, our data support the idea that HS1 KO mice are protected from lethal CLP sepsis in part due to by reduced leukocyte infiltration and related tissue damage. We are the first to show such protective effects and improved survival after lethal sepsis induced by CLP in the context of cortactin or HS1 deficiencies that are associated with impaired leukocyte recruitment, reduced tissue apoptosis and consequently better conserved tissue morphology. However, further investigation is required to better characterize the molecular mechanisms that contribute to improved animal survival. Altogether our data suggest cortactin and HS1 as attractive future therapeutic targets for sepsis treatment.

Perspectives

- To analyze the bacterial loads in these animals and to study whether antibiotic treatment would further increase survival in CLP sepsis
- To generate cortactin/HS1 double-KO mice and to analyze if there are additive effects during sepsis
- To analyze if blood pressure is altered by the absence of cortactin or HS1.
- To investigate leukocyte functions such as NETs formation, phagocytosis, and the ability to perform respiratory burst and secrete pro-inflammatory mediators
- To determine the inflammatory response in the peritoneal cavity and potential abscess formation

Publications

REVIEW

Tissue Barriers 1:e26938; 2013; © 2013 Landes Bioscience

Small GTPases of the Ras superfamily regulate intestinal epithelial homeostasis and barrier function via common and unique mechanisms

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Keywords: Rho, Rac, cdc42, Ras, Rap, Arf, inflammatory bowel disease, colitis, tight junction, adherens junction, actin cytoskeleton

Abbreviations: AJ, adherens junction; AJC, apical junctional complex; Arf, ADP-ribosylation factor; Cdc42, cell division cycle 42; DSS, dextran sulfate sodium; EGF, epidermal growth factor; EPEC, enteropathogenic *Escherichia coli*; ERK, extracellular-regulated kinase; FPR, formyl peptide receptor-1; GDP, guanylate-diphosphate; GTP, guanylate-triphosphate; GAP, GTPase-activating protein; GDI, guanine nucleotide dissociation inhibitor; GEF, guanine nucleotide exchange factor; IBD, inflammatory bowel disease; IEC, intestinal epithelial cells; IL, interleukin; JAM, junctional adhesion molecule; JNK, c-jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MDCK, Madine Darby canine kidney; mDia1, mammalian homolog of *Drosophila* diaphanous 1; MEK, MAP/ERK kinase; MLC, myosin light chain; MLCP, myosin light chain phosphatase; mTOR, mammalian target of rapamycin; MVID, microvillous inclusion disease; N-WASP, neural Wiskott-Aldrich syndrome protein; PI3K, phosphatidyl-inositol-3-kinase; Rac1, ras-related C3 botulinum toxin substrate 1; Rap1, ras-related protein 1; Ras, rat sarcoma viral oncogene homolog; RhoA, ras homolog A; ROCK1, Rho kinase 1; TcdA/B, *Clostridium difficile* toxin A/B; TJ, tight junction; TNBS, 2,4,6-trinitrobenzene sulfonic acid; ZO, zonula occludens

Research Article

Experimental Colitis Is Attenuated by Cardioprotective Diet Supplementation That Reduces Oxidative Stress, Inflammation, and Mucosal Damage

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Received 24 October 2015; Accepted 1 December 2015

Academic Editor: Noriko Noguchi

Inflammatory bowel diseases (IBD) such as ulcerative colitis (UC) and Crohn's disease (CD) are multifactorial, relapsing disorders of the gastrointestinal tract. However, the etiology is still poorly understood but involves altered immune responses, epithelial dysfunction, environmental factors, and nutrition. Recently, we have shown that the diet supplement corabion has cardioprotective effects due to reduction of oxidative stress and inflammation. Since oxidative stress and inflammation are also prominent risk factors in IBD, we speculated that corabion also has beneficial effects on experimental colitis. Colitis was induced in male mice by administration of 3.5% (w/v) dextran sulfate sodium (DSS) in drinking water for a period of 3 or 7 days with or without daily gavage feeding of corabion consisting of vitamin C, vitamin E, L-arginine, and eicosapentaenoic and docosahexaenoic acid. We found that corabion administration attenuated DSS-induced colon shortening, tissue damage, and disease activity index during the onset of colitis. Mechanistically, these effects could be explained by reduced neutrophil recruitment, oxidative stress, production of proinflammatory cytokines, and internalization of the junctional proteins ZO-1 and E-cadherin leading to less edema formation. Thus, corabion may be a useful diet supplement for the management of chronic inflammatory intestinal disorders such as IBD.

Review Article 20

The role of actin-binding proteins in the control of endothelial barrier integrity

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Summary
The endothelial barrier of the vasculature is of utmost importance for separating the blood stream from underlying tissues. This barrier is formed by tight and adherens junctions (TJ and AJ) that form intercellular endothelial contacts. TJ and AJ are integral membrane structures that are connected to the actin cytoskeleton via various adaptor molecules. Consequently, the actin cytoskeleton plays a crucial role in regulating the stability of endothelial cell contacts and vascular permeability. While a circumferential cortical actin ring stabilises junctions, the formation of contractile stress fibres, e. g. under inflammatory conditions, can contribute to junction destabilisation. However, the role of actin-binding proteins (ABP) in the control of vascular permeability has long been underestimated. Naturally, ABP regulate permeability via regulation of actin remodelling but some actin-binding molecules can also act independently of actin and control vascular permeability via various signalling mechanisms such as activation of small GTPases. Several studies have recently been published highlighting the importance of actin-binding molecules such as cortactin, ezrin/radixin/moesin, Arp2/3, VASP or WASP for the control of vascular permeability by various mechanisms. These proteins have been described to regulate vascular permeability under various pathophysiological conditions and are thus of clinical relevance as targets for the development of treatment strategies for disorders that are characterised by vascular hyperpermeability such as sepsis. This review highlights recent advances in determining the role of ABP in the control of endothelial cell contacts and vascular permeability.

Keywords
Actin cytoskeleton, cell-cell interactions, endothelial cells, GTPases, inflammation, vascular permeability

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Financial support:
This work was supported by a grant from the Mexican Council for Science and Technology (Conacyt, 179895 to MS).

Received: April 1, 2014
Accepted after major revision: July 1, 2014
Epub ahead of print: August 28, 2014
http://dx.doi.org/10.1160/TH14-04-0298
Thromb Haemost 2015; 113: 20–36

SCIENTIFIC REPORTS

OPEN

Received: 16 November 2015
Accepted: 13 June 2016
Published: 30 June 2016

Loss of cortactin causes endothelial barrier dysfunction via disturbed adrenomedullin secretion and actomyosin contractility

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Changes in vascular permeability occur during inflammation and the actin cytoskeleton plays a crucial role in regulating endothelial cell contacts and permeability. We demonstrated recently that the actin-binding protein cortactin regulates vascular permeability via Rap1. However, it is unknown if the actin cytoskeleton contributes to increased vascular permeability without cortactin. As we consistently observed more actin fibres in cortactin-depleted endothelial cells, we hypothesised that cortactin depletion results in increased stress fibre contractility and endothelial barrier destabilisation. Analysing the contractile machinery, we found increased ROCK1 protein levels in cortactin-depleted endothelium. Concomitantly, myosin light chain phosphorylation was increased while cofilin, mDia and ERM were unaffected. Secretion of the barrier-stabilising hormone adrenomedullin, which activates Rap1 and counteracts actomyosin contractility, was reduced in plasma from cortactin-deficient mice and in supernatants of cortactin-depleted endothelium. Importantly, adrenomedullin administration and ROCK1 inhibition reduced actomyosin contractility and rescued the effect on permeability provoked by cortactin deficiency *in vitro* and *in vivo*. Our data suggest a new role for cortactin in controlling actomyosin contractility with consequences for endothelial barrier integrity.

Tissue Barriers

ISSN: (Print) 2168-8370 (Online) Journal homepage: http://www.tandfonline.com/loi/ktib20

Regulation of endothelial and epithelial barrier functions by peptide hormones of the adrenomedullin family

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To cite this article: Alexander García Ponce, Sandra Chánez Paredes, Karla Fabiola Castro Ochoa & Michael Schnoor (2016): Regulation of endothelial and epithelial barrier functions by peptide hormones of the adrenomedullin family, Tissue Barriers, DOI: 10.1080/21688370.2016.1228439

To link to this article: http://dx.doi.org/10.1080/21688370.2016.1228439

Accepted author version posted online: 25 Aug 2016.
Published online: 25 Aug 2016.

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Cortactin deficiency causes increased RhoA/ROCK1-dependent actomyosin contractility, intestinal epithelial barrier dysfunction, and disproportionately severe DSS-induced colitis

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The intestinal epithelium constitutes a first line of defense of the innate immune system. Epithelial dysfunction is a hallmark of intestinal disorders such as inflammatory bowel diseases (IBDs). The actin cytoskeleton controls epithelial barrier integrity but the function of actin regulators such as cortactin is poorly understood. Given that cortactin controls endothelial permeability, we hypothesized that cortactin is also important for epithelial barrier regulation. We found increased permeability in the colon of cortactin-KO mice that was accompanied by reduced levels of ZO-1, claudin-1, and E-cadherin. By contrast, claudin-2 was upregulated. Cortactin deficiency increased RhoA/ROCK1-dependent actomyosin contractility, and inhibition of ROCK1 rescued the barrier defect. Interestingly, cortactin deficiency caused increased epithelial proliferation without affecting apoptosis. KO mice did not develop spontaneous colitis, but were more susceptible to dextran sulfate sodium colitis and showed severe colon tissue damage and edema formation. KO mice with colitis displayed strong mucus deposition and goblet cell depletion. In healthy human colon tissues, cortactin co-localized with ZO-1 at epithelial cell contacts. In IBDs patients, we observed decreased cortactin levels and loss of co-localization with ZO-1. Thus, cortactin is a master regulator of intestinal epithelial barrier integrity *in vivo* and could serve as a suitable target for pharmacological intervention in IBDs.

INTRODUCTION

The integrity of the epithelial monolayer is critical for healthy intestines. This monolayer constitutes a semi-permeable barrier that is tightly regulated to allow absorption of nutrients and water and at the same time restrict excessive leakage of macromolecules and invasion of bacteria from the gut lumen. Increased epithelial permeability is a characteristic of chronic inflammatory disorders such as inflammatory bowel diseases (IBDs).[1] Epithelial permeability is mostly controlled by tight junctions (TJs),[2] whereas stable cell–cell adhesion depends on adherens junctions (AJs).[3] Together, AJs and TJs form the apical junctional complex that controls epithelial barrier integrity.[4] These junctional structures are composed of transmembrane adhesion receptors that are intracellularly linked to the actin cytoskeleton via adaptor molecules such as zonula occludens (ZO) and catenins.[5,6] It is commonly accepted that proper apical junctional complex functions and thus epithelial barrier integrity in health and disease are strongly dependent on actin dynamics.[4,7] The connection of AJs and TJs to the actin cytoskeleton is further regulated by several actin-binding molecules including cortactin.[8]

Cortactin is a multi-domain protein capable of directly binding actin filaments, other actin regulators such as the Arp2/3 complex and adaptor molecules including ZO-1.[9]

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Received 26 May 2016; accepted 18 December 2016; advance online publication 25 January 2017. doi:10.1038/mi.2016.136

Cell. Mol. Life Sci. (2017) 74:1985–1997
DOI 10.1007/s00018-016-2449-x

Cellular and Molecular Life Sciences

REVIEW

Actin dynamics in the regulation of endothelial barrier functions and neutrophil recruitment during endotoxemia and sepsis

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Received: 19 August 2016 / Revised: 19 December 2016 / Accepted: 21 December 2016 / Published online: 2 February 2017

Abstract Sepsis is a leading cause of death worldwide. Increased vascular permeability is a major hallmark of sepsis. Dynamic alterations in actin fiber formation play an important role in the regulation of endothelial barrier functions and thus vascular permeability. Endothelial integrity requires a delicate balance between the formation of cortical actin filaments that maintain endothelial cell contact stability and the formation of actin stress fibers that generate pulling forces, and thus compromise endothelial cell contact stability. Current research has revealed multiple molecular pathways that regulate actin dynamics and endothelial barrier dysfunction during sepsis. These include intracellular signaling proteins of the small GTPases family (e.g., Rap1, RhoA and Rac1) as well as the molecules that are directly acting on the actomyosin cytoskeleton such as myosin light chain kinase and Rho kinases. Another hallmark of sepsis is an excessive recruitment of neutrophils that also involves changes in the actin cytoskeleton in both endothelial cells and neutrophils. This review focuses on the available evidence about molecules that control actin dynamics and regulate endothelial barrier functions and neutrophil recruitment. We also discuss treatment strategies using pharmaceutical enzyme inhibitors to target excessive vascular permeability and leukocyte recruitment in septic patients.

Keywords Adhesion · Diapedesis · Tight junction · Inflammation · Acute lung injury · Acute respiratory distress syndrome

Introduction

Sepsis is a generalized inflammatory syndrome of the host in response to various infectious stimuli [1]. The most severe form, the septic shock, is accompanied by severe hypotension, impaired microvascular perfusion and organ damage [2]. This may finally lead to multiple organ failure [3]. Surprisingly, sepsis is still not fully recognized as an individual disease entity despite high morbidity and mortality rates in affected patients causing high socio-economic burdens for health care systems worldwide. The incidence

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