CENTRO DE INVESTIGACIÓN Y DE ESTUDIOS AVANZADOS DEL INSTITUTO POLITÉCNICO NACIONAL

UNIDAD ZACATENCO

DEPARTAMENTO DE BIOMEDICINA MOLECULAR

“El papel de cortactina en la arquitectura de las capas que conforman las vénulas poscapilares del músculo cremáster”

T E S I S

Que presenta

Lic. Arturo Armando Valenzuela Padilla

Para obtener el grado

MAESTRO EN CIENCIAS

EN LA ESPECIALIDAD DE

BIOMEDICINA MOLECULAR

Director de tesis

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Ciudad de México Agosto, 2024

CENTER FOR RESEARCH AND ADVANCED STUDIES

OF INSTITUTO POLITECNICO NACIONAL

DEPARTMENT FOR MOLECULAR BIOMEDICINE

“The role of cortactin in the architecture of the layers that form the cremasteric postcapillary venules”

T H E S I S

Presented by

BSc. Arturo Armando Valenzuela Padilla

To obtain the degree

MASTER OF SCIENCE

IN THE SPECIALITY OF

MOLECULAR BIOMEDICINE

Thesis director

Dr. Michael Schnoor

Mexico City August, 2024

AGRADECIMIENTOS

Al Consejo Nacional de Humanidades, Ciencias y Tecnologías (CONAHCYT) por la beca recibida durante el periodo 2022-2024 que me permitió realizar mis estudios de maestría.

Al CINVESTAV por proveerme las herramientas necesarias para mi formación durante la maestría.

Al Dr. Michael Schnoor, por haber creído en mí y darme la oportunidad de trabajar con él. Por sus enseñanzas, paciencia, guía, excelente asesoría y por escucharme en todo momento.

Al Dr. Vianney y Dr. Emmanuel, por su amistad, pláticas, apoyo y la asesoría brindada para el desarrollo de este proyecto de investigación.

A la Dra. Rosaura, por siempre tener tiempo para platicar y reír. Por escucharme, guiarme cuándo más lo necesitaba, por sus enseñanzas y excelente amistad.

Dra. Leticia, al Dr. Miguel, Dr. Nicolás, Dr. Leopoldo y Dr. Marco por su apoyo, consejos y guía durante mis estudios de maestría.

Gracias infinitas a mi familia por su apoyo, consejos, guía, ánimos en todo momento. Y sobre todo por las palabras de aliento.

A Jael y Nadia, por su apoyo, risas, por siempre tener un tiempo para platicar, por guiarme y escucharme. Además, por ser unas excelentes amistades a lo largo de la maestría.

A la Dra. Gwen (la Miss), por su gran amistad. Por siempre haberme apoyado, guiado, escuchado y aconsejado. Sobre todo, por haberme alentado a estudiar un posgrado.

A Gladis, por haberme escuchado, aconsejado y apoyado en todo momento.

A Maru, por su amistad, plática y apoyo en cada momento

A Idaira, aunque está lejos, por haberme apoyado, guiado, aconsejado y enseñado.

A Paola, Sandy y Janeth, por haberme apoyado y ayudado en todo momento.

A Hilda y a mis compañeros de laboratorio por sus enseñanzas.

A Genaro y Pili, por su guía, enseñanzas, consejos y risas.

INDEX

ABBREVATIONS

ABP	Actin-Binding Protein
AJ	Adherent Junctions
α-SMA	Alpha smooth muscle actin
Arp 2/3	Actin related protein 2/3
BM	Basement Membrane
Col IV	Collagen type 4
Cttn	Cortactin
ECs	Endothelial cells
ECM	Extracellular matrix
E-selectin	Endothelial-selectin
F-actin	Filamentous actin
G-act	Globular actin
GAG	Glycosaminoglycans
ICAM-1	Intercellular adhesion molecule 1
IL	Interleukin
ILK	Integrin linked kinase
KO	Knock out
KD	Knock down
Lm	Laminin
LFA-1	Lymphocyte-function associated antigen-1
Mac-1	Macrophage antigen-1
MMP	Metalloproteinase
NE	Neutrophil elastase
Nd-2	Nidogenin-2
NG-2	Neuron-glial antigen-2
NSP	Neutrophil serine protease

PAK	p21-activated kinase
PBS	Phosphate buffer solution
PCV	Postcapillary venules
PDGFR-β	Platelet derived growth factor-β
PECAM-1	Platelet endothelial cell adhesion molecule-1
Per	Perlecan
P-selectin	Platelet-selectin
PSGL-1	P-selectin glycoprotein ligand 1
ROCK 1	Rho kinanse
SPARC	Secreted protein acidic and rich in cysteines
TJ	Tight junctions
TGF-β	Transforming growth factor-β
TNF-α	Tumoral necrosis factor-α
VEGF	Vascular endothelial growth factor
VT	Vesicular trafficking
WT	Wild type
+/-	Heterozygous
-/-	Knock Out

LIST OF FIGURES

RESUMEN

La extravasación de los neutrófilos en un tejido inflamado es esencial para combatir infecciones y, daños endógenos y exógenos. Sin embargo, una infiltración excesiva y desregulada de los neutrófilos se asocia con un daño agudo al tejido. Por lo tanto, una mejor comprensión de los mecanismos que regulan la extravasación de los neutrófilos conducirá a proponer mejores estrategias terapéuticas para enfermedades inflamatorias. Aunque se ha estudiado arduamente la interacción de las células endoteliales de los vasos sanguíneos y los neutrófilos, muy poco se sabe cómo los neutrófilos interactúan con la membrana basal y la capa de pericitos durante la inflamación. Además, se desconocen los mecanismos celulares o moleculares que regulan el rearreglo de la membrana basal y la capa de pericitos durante la inflamación. Cortactina, al ser una proteína que tiene actividad en la regulación de la permeabilidad vascular, en la remodelación del citoesqueleto de actina, en el tráfico vesicular y adhesión celular, de alguna manera podría estar involucrada en el rearreglo de la membrana basal y la capa de pericitos durante la inflamación.

En este estudio empleamos ratones macho C57Bl/6 silvestres, heterocigotos y knock-out de cortactina, y estudiamos la arquitectura de las venas poscapilares del músculo cremáster en condiciones basales e inflamatorias. Analizamos las células endoteliales, la colágena tipo IV, como mayor componente de la membrana basal venular y a los pericitos, usando como marcador la α-actina de músculo liso. Observamos que la cortactina es importante en la formación de las tres capas que componen las venas poscapilares en condiciones basales. Durante la inflamación, la cortactina está involucrada en la remodelación de la Col IV y la capa de los pericitos, disminuyendo el número de regiones de baja expresión (RBE) y aumentando el número de espacios (gaps) entre los pericitos, respectivamente. Además, durante la inflamación, la reducción de cortactina en ratones heterocigotos causa un agrandamiento de las RBE y gaps. En conjunto, observamos que cortactina es importante durante los procesos de remodelación de la Col IV y los pericitos durante la inflamación, y podría tener repercusiones en el reclutamiento de neutrófilos.

ABSTRACT

Neutrophil extravasation into an inflamed tissue is essential to combat infections and endogenous and exogenous damage. However, excessive and unregulated neutrophil infiltration is associated with acute tissue damage. Therefore, a better understanding of the mechanisms that regulate neutrophil extravasation will lead to better therapeutic strategies for inflammatory diseases. Although, the interaction of blood vessel endothelial cells and neutrophils has been extensively studied, very little is known about how neutrophils interact with the basement membrane and pericyte layer during inflammation. Furthermore, the cellular and molecular mechanisms that regulate the rearrangement of the basement membrane and pericyte layer during inflammation are unknown. However, cortactin, a protein shown to regulate vascular permeability, remodeling of the actin cytoskeleton, vesicular trafficking and cell adhesion and migration, is likely also involved in the rearrangement of the basement membrane and the pericyte layer during inflammation.

In this study, we used wild-type, heterozygous and cortactin knock-out male C57Bl/6 mice to study the architecture of postcapillary venules in the cremaster muscle under basal and inflammatory conditions. We analyzed endothelial cells, type IV collagen as major component of the venular basement membrane, and pericytes using α-smooth muscle actin as marker. We observed that cortactin is important in the formation of the three layers that compose the postcapillary venules under basal conditions. During inflammation, cortactin is involved in the remodeling of Col IV and pericytes by decreasing the number of low-expression regions (LERs) and increasing the number of gaps between pericytes, respectively. Furthermore, during inflammation, reduction of cortactin in heterozygous causes enlargement of LERs and gaps. Overall, cortactin is important during inflammation for the remodeling of the basement membrane and the pericyte layer, which could have implications for neutrophil recruitment.

CHAPTER I

I. BACKGROUND

1.1. The inflammatory response

Inflammation is a biological and vital process that occurs in response to damaged cells, irradiation, toxic compounds, and harmful stimuli such as pathogens or injuries [1, 2]. Inflammation starts when the innate immune system recognizes foreign material or invading pathogens. Then, resident immune cells such as macrophages [Mϕ] and dendritic cells [DCs] start producing proinflammatory cytokines and chemokines to attract other immune cells such as neutrophils [Neu], monocytes [Mo], natural killer [NK] cells, mast cells [MCs], eosinophils and basophils [2,3].

Tissue-resident immune cells recognize either *pathogen-associated molecular patterns* (PAMPs), *damage-associated molecular patters* (DAMPs), or *metabolism-associated molecular patterns* (MAMPs) by innate immune receptors known as *pattern recognition receptors* (PRRs) including Toll-like receptors (TLRs), nucleotide oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), C-type lectin receptors (CLRs), and absent in melanoma-2 (AIM-2)-like receptors (ALRs). These receptors trigger downstream signaling pathways inducing the expression of proinflammatory molecules such as TNF-α, IL-1β, -6, -8 and -17, and antimicrobial peptides such as indolicidin, protegrin-1, magainin, abaecin, β-defensin, among others. These molecules are generated as a first line of defense against microorganism while other immune cells arrive at the damage site [3].

The proinflammatory microenvironment generated by tissue resident Mϕ, MCs and DCs leads to the local activation of the vasculature, increased permeability and plasma protein leakage, presentation of chemokines on the endothelial apical surface and neutrophil recruitment. For the inflammatory process to take place and for damage to be resolved, leukocytes, including neutrophils must leave the bloodstream and reach the site of damage. To this end, they must cross three different barriers of postcapillary venule, the endothelial cells (ECs), the basement membrane (BM) and pericytes. After reaching the site of damage, neutrophils carry out their functions, such as

phagocytosis, oxidative burst, and release of antimicrobial peptides and pro-resolving proteins, thus contributing to the resolution of inflammation and tissue repair [4-6].

1.2. Endothelial Cells (ECs)

Vascular endothelial cells form the semi-permeable inner layer of blood vessels including arteries, arterioles, capillaries, venules, and veins that separate blood from the underlying tissue. ECs play a key role in forming the barrier that separates the bloodstream from the surrounding tissues. ECs control the degree of vascular relaxation and constriction, and passage of solutes such as plasma proteins. Additionally, they are important in controlling, platelet adhesion and aggregation, and leukocyte activation, adhesion, and transmigration to the abluminal site during inflammation [7-9]. ECs are anchored to a 60-180 nm thick basal lamina, and together they constitute the intimal layer of blood vessels. The basal lamina is an important component of blood vessels that works as a structural scaffold to support the endothelial layer inside this scaffold, while the outside is covered by smooth muscle cells or pericytes depending on the type of blood vessel [7, 8].

ECs form a monolayer where single cells are joined by junctional adhesive structures [10]. These junctions are essential for the maintenance of endothelial integrity and regulating the passage of molecules and circulating leukocytes. They are comprised of transmembrane protein complexes known as *tight junctions* (TJs) and *adherent junctions* (AJs) [10, 11]. TJs are composed of transmembrane proteins such as claudins, occludin and junctional adhesion molecules (JAM), whereas AJs mostly consist of vascular endothelial cadherin (VE-cadherin) [11] (Figure 1). TJs and AJs are anchored to the adaptor molecules zonular occludens (ZO)-1, -2 and -3, and a-, β- and γ-catenins, respectively, which in turn connect the transmembrane proteins to the cortical actin cytoskeleton and Actin-Binding Proteins (ABP) such as cortactin to stabilize the junctions and thus the entire EC monolayer [10-11].

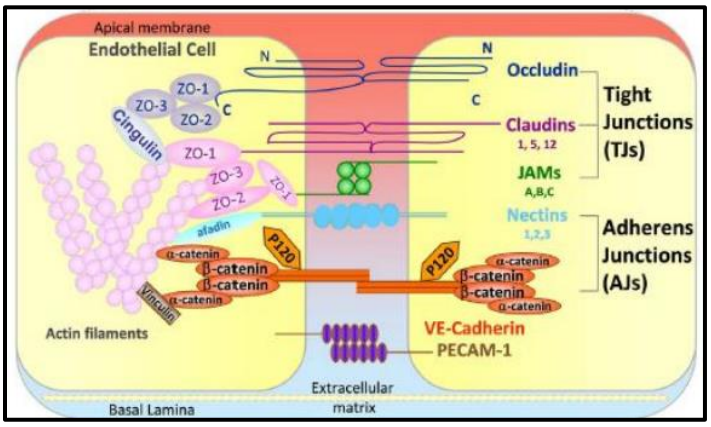

Figure 1. The main transmembrane and adaptor proteins in endothelial cell-cell junctions.

Cell-cell interactions mediated by TJ or AJ and PECAM-1, which is not associated with either type of junction, is shown. Three different claudins and JAMs are reported to be present in endothelial cells (numbers and letters indicated under the protein name). Intracellular scaffolding proteins link transmembrane proteins to the actin cytoskeleton [19].

1.2.1. Functions of EC junctions during neutrophil recruitment

Neutrophils engage in several luminal interactions with ECs, initiating with tethering and rolling along the endothelium, followed by slow rolling, adhesion, crawling and finally transendothelial migration or diapedesis. All these steps are governed by many different molecular interactions [14].

Neutrophil recruitment begins with a local proinflammatory microenvironment, generated by IL-1β, TNF-α, INF-γ, IL-17, IL-18 that are produced and secreted by resident immune cells [10-11]. These molecules activate the ECs and increase the expression of adhesion molecules, mostly intercellular adhesion molecule-1, -2 (ICAM-1, ICAM-2) and vascular adhesion molecule-1 (VCAM-1), E-selectin and P-selectin, and the production and presentation of chemokines on the surface of endothelial cells [12]. Low-affinity interactions of endothelial P- and E-selectins with PSGL-1 (P-selectin glycoprotein ligand-1) and ESL-1 (E-selectin ligand-1) on neutrophils mediate neutrophil capturing onto the activated endothelium followed by neutrophil rolling along the endothelia surface **(Figure 2)**. During rolling, selectin engagement and chemokine recognition by the neutrophil chemokine receptor CXCR2 induce conformational change in the β2 integrins LFA-1 (αLβ2 [CD11a/CD18]) and Mac-1 (αMβ2 [CD11b/CD18]) to enable high-affinity binding to ICAM-1 and ICAM-2 and firm

adhesion. Then, neutrophils reach the site of diapedesis by rearranging their actin cytoskeleton, spreading on the endothelial surface, and intravascular crawling through Mac-1/ICAM-1 interactions [13, 47]. ECs surround the adherent neutrophils with docking structures enriched in ICAM-1, VCAM-1 and actin to guide them to the preferred site of transmigration through the endothelial wall. The formation of these docking structures is regulated by recruitment of adaptor molecules such as cttn, filamin-B, α-actinin, ezrin, radixin and moesin (ERM) and signaling protein such as Rac 1 and Rho G to coordinate the actin remodeling at the apical endothelial surface necessary for protrusion formation [13-17].

Around 80-90% of neutrophils transmigrate preferentially through the paracellular route, i.e. between EC; while the rest transmigrate via the transcellular route, i.e. through an EC **(Figure 2).** During paracellular transmigration, the neutrophils engage in JAM-A, PECAM-1 and CD-99 mediated interactions with ECs to breach the endothelium in a transient and reversible mechanism [15-17]. In addition, internalization of TJs and AJs proteins not required for neutrophil interactions via recycling endosomes is induced by clustering of ICAM-1 that triggers the recruitment and activation of RhoA and Rac1 that control the activity of Src kinase, PYK2 and vascular-endothelial protein tyrosine phosphatase (VE-PTP) at the endothelial cell contacts. This drives the dissociation of VE-PTP from the VE cadherin/catenin complex and Tyr phosphorylation of VE-cadherin and β-catenin by Src kinase and PYK2, leading to the transient loss of VE-cadherin at AJs thus allowing for the crossing of neutrophils through the endothelial cell contacts

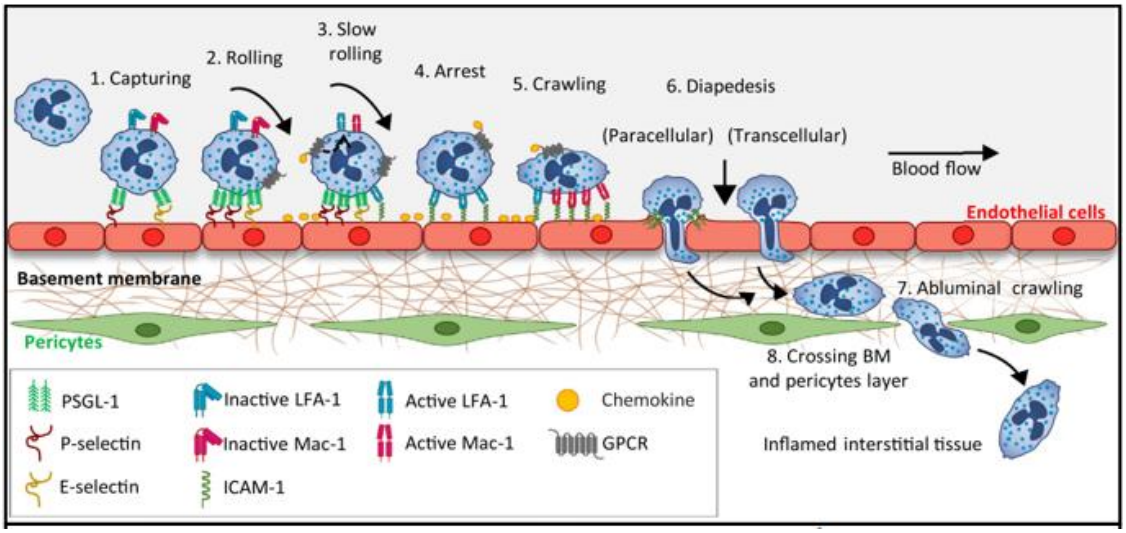

Figure 2. The neutrophil extravasation cascade.

The sequential steps during neutrophil recruitment are shown, as well as the main adhesion molecules that mediate each step of the cascade [11].

1.3. Vascular basement membrane composition, function, and rearrangement during inflammation.

After crossing the endothelium, neutrophils need to cross two other components of the vascular wall, namely the basement membrane (BM) and the pericyte layer, to reach the underlying inflamed tissue [18-23]. The BM is generated during vasculogenesis by endothelial cells and pericytes in response to angiopoietin-1 (Ang-1) and vascular endothelial growth factor (VEGF) [20, 26].

The BM underlies the vascular endothelium to provide support and structure to the vasculature. It consists of two irregular discontinuous thin structural layers. The first one is the basal lamina, and the second one is the lamina reticularis (also known as *reticular lamina*) [22]. The *basal lamina* contains two sublayers, which are the lamina lucida and the lamina densa. The basal lamina is synthesized mostly by ECs, and the lamina reticularis is made mostly by pericytes, but also by fibroblasts. The BM is composed of extracellular matrix (ECM) proteins and is thus the non-cellular component of the venular wall. ECM proteins forming the BM include integrins, laminins (LN), collagen (Col), dystroglycans (Dys), nidogen (Ng), perlecan (Per) fibronectin (FN), hyaluronic acid (HA) and heparin sulfate proteoglycans. The most abundant BM

proteins are LN-10, LN-8, Col IV, Nidogen and Perlecan. LNs, Col IV and FN are interconnected by molecular bridges involving glycoproteins such as Ng-2 and Per. The BM maintains vessel architecture and forms a dynamic environment by presenting RGD domains that serve as ligand for integrin-mediated adhesion and contribute to the regulation of cell behavior, signaling and survival [15, 22, 24].

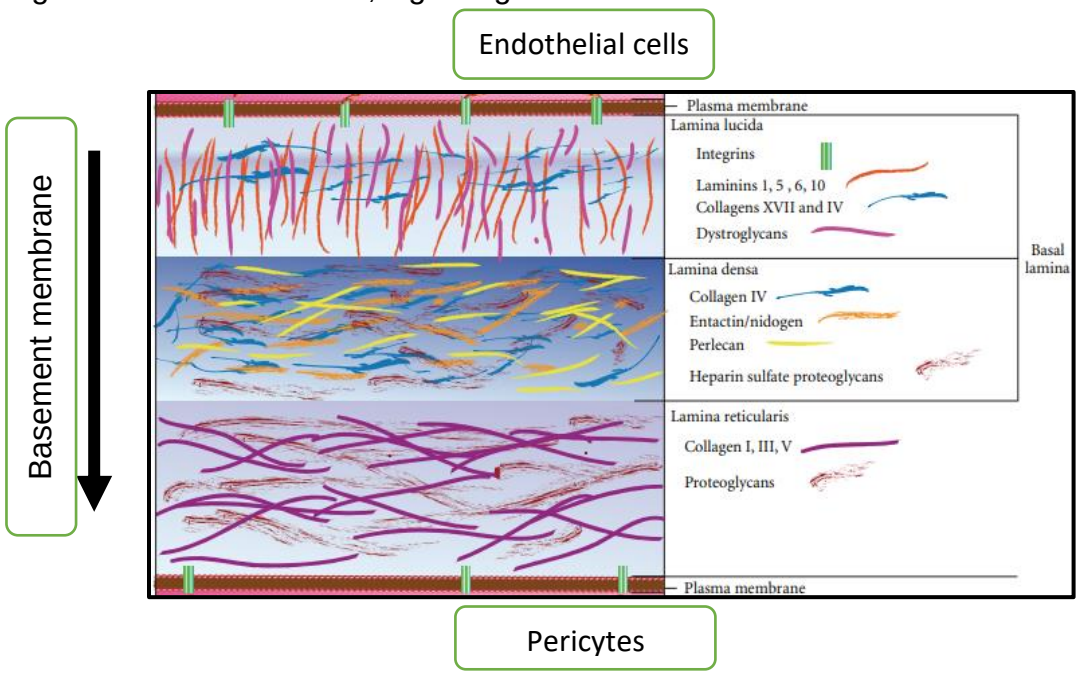

Figure 3. Composition of the venular basement membrane.

The basement membrane underlies the vascular endothelium and consists of two thin structural layers: the basal lamina and the lamina reticularis. The basal lamina is synthesized by endothelial cells whereas the reticular lamina is synthesized by pericytes and other surrounding cells [27]. Proteins forming each layer are shown on the right.

The BM proteins have been well studied in the mouse cremasteric muscle, where they present specific arrangements including *low-expression regions (LERs)* characterized by disruptions in the protein layer **(Figure 4)**. Of note, the sizes and numbers of LERs were different under basal and inflammatory conditions. For example, intrascrotal (i.s) injection of TNF-α in the cremaster muscle induced more and bigger LERs of LN-10 and Per, whereas LERs of Col IV were fewer and bigger during inflammation. These results showed that ECM proteins present differential rearrangements during inflammation via yet unknown mechanisms [22, 48-56]. In addition to BM rearrangement during inflammation, some studies demonstrated that the first neutrophils to transmigrate are covered with either laminin-8 or -10, but not with type IV collagen and

it is believed that this could give an advantage to these neutrophils to migrate faster across the BM [54].

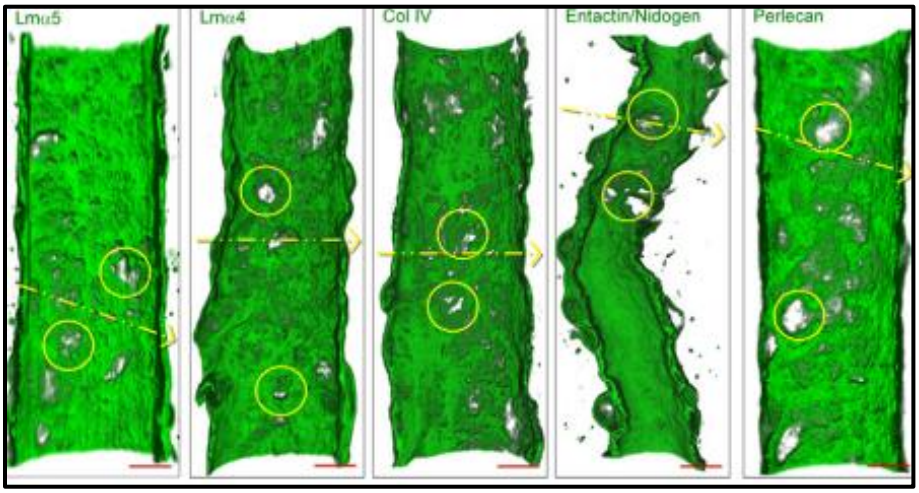

Figure 4. Expression profiles of LERs in the venular basement membrane in mouse cremasteric venules.

LERs (yellow circles) are represented in stainings for LN5 (LN-10), LN4 (LN-8), Col IV, Nidogen and Perlecan [22].

Using confocal intravital microscopy (IVM) of mouse cremaster venules, it has been shown that the rearrangement of the BM during inflammation leads to smaller LERs before the neutrophils reached the basement membrane. What governs this rearrangement prior to the arrival of neutrophils is still unknown [59]. However, neutrophils sense the smaller LERs, preferably LERs of 2-10 μm^2. So far it is not known why neutrophils prefer smaller LERs than larger LERs, but a reason could be the accumulation of receptors or chemoattractants at smaller LERs. Later, LERs increase in size and neutrophils migrate through these LERs towards the pericyte layer [66]. Neutrophils play a role in BM rearrangement, because they release neutrophil elastase (NE), which is the most abundant protease expressed by neutrophils. This serine protease can act on a broad range of substrates, including extracellular matrix (ECM). When NE lacks, neutrophils get trapped within venular walls, mostly at the level of the venular basement membrane (BM) that do not show bigger LERs [61]. After crossing the BM, neutrophils face pericytes as final obstacle before exiting the venules.

1.4. Pericyte functions under basal and inflammatory conditions

Pericytes are contractile cells with a length of 150-200 μm that surround the blood microvasculature. Pericytes exhibit multiple protrusions, and these protrusions generate multiple spaces between them known as gaps [60]. Pericytes fulfill different functions including extracellular matrix synthesis and clearance of toxic metabolites by phagocytosis [26]. Pericytes are morphologically different depending on the vascular bed, and their functions are tissue specific. Venular pericytes are characterized by the expression of platelet-derived growth factor receptor-β (PDGFR-β), the proteoglycan NG-2, and α-smooth muscle actin (α-SMA) that forms actin fibers to provide stability and contractility. CD90 and CD105 (mesenchymal stem cell marker) are used as pericyte markers [25].

Pericytes are anchored to ECs via N-cadherins and connexins. They are attached to the BM through integrins such as $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$ that binds to FN, thus allowing direct communication between BM and pericytes. These cells contribute to vascular support, remodeling, architecture, permeability and metabolite clearance [64]. Under basal condition, the perycites release metabolites, ions and many molecules such as angiopoietin-1, -2, vascular endothelial growth factor (VEGF), platelet-derived growth factor-BB (PDGF-BB) and sphingosine-1 phosphate (S1P), that induce both morphological and functional changes in pericytes during inflammation. For example, Ang-2 induces N-cadherin endocytosis, upregulation of integrins and pericytes migration, whereas PDGF-BB induces new lipid bilayer synthesis, loss of soma and increased number of protrusions, and migration [46, 62-64].

To better understand more about the role of pericytes during inflammation, the impedance (obstruction to alternating current flow) of pericytes obtained from mouse retina was measured after stimulation with TNFα or IL-1β on pericytes. Pericytes showed higher impedance, suggesting that pericytes formed smaller gaps compared to controls [66]. Moreover, it has been shown by IVM that neutrophils interact with

pericytes gaps that can be used as path of least resistance to breach the thin final layer of the venular wall **(Figure 5)** [67].

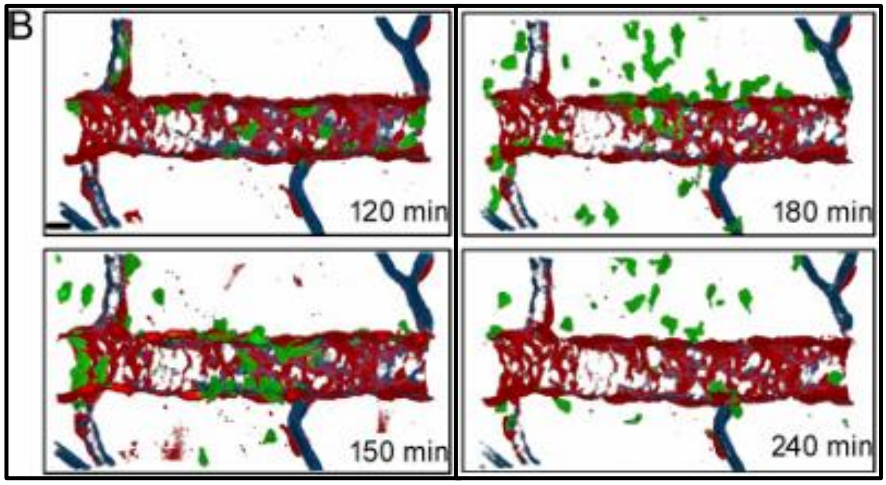

Figure 5 Pericytes gaps enlargement during neutrophil transmigration.

Cremaster muscles of α-SMA-RFPcherry × Lys-EGFP-ki mice exhibiting endogenously labeled pericytes (RFPcherry, red) and neutrophils (EGFP, green) were subjected to EC junctional labeling using a conjugated Alexa Fluor 647 nonblocking anti-PECAM-1 antibody (blue). Reconstructed confocal images of a cremasteric venule are shown over time 120 to 240 minutes after intrascrotal injection of TNF. Bar, 10 µm.

Using confocal IVM of mouse cremaster venules, it has been shown that pericytes present rearrangement during inflammation to produce smaller gaps before the neutrophils reach the pericytes layer. This rearrangement mechanism prior to the arrival of neutrophils is still unknown [59]. However, neutrophils sense the smaller gaps, preferably gaps of 2-10 μm^2. Later, the gaps increase in size just as LERs in the BM, and neutrophils preferentially migrate through these gaps towards the abluminal site [66]. So far it is not known why neutrophils prefer smaller gaps over larger gaps.

Both the BM and pericytes undergo rearrangement to accommodate neutrophils on their way across the PCV towards the inflamed interstitial tissue. However, the cellular and molecular mechanisms that govern these events remain largely unknown. It seems logical to assume that ABP necessary for actin remodeling and thus cell morphology plays a vital role in this process. Studies from our and other laboratories have revealed that the ABP cortactin controls the rearrangement of the actin cytoskeleton, cell migration, vesicular trafficking, ECM secretion, and neutrophil extravasation as described below.

1.5. Cortactin

Branched actin assembly is critical for a variety of cellular processes including cellular protrusion formation, vesicular trafficking and cell motility. Cortactin is an ABP known to stabilize branched actin filaments and to support cell adhesion and migration [25]. Cortactin was first discovered as substrate of Src kinase. It was recognized as an ABP targeting actin structures in the cell cortex and as a molecule that links cytoskeletal organization with signal transduction. Cortactin accumulates in actin-rich lamellipodia formed at the leading edge of migrating cells where it promotes the secretion of ECM, and in invadopodia where it promotes the secretion of ECM-degrading MMPs. Cortactin- deficient mice exist that are viable but show aberrant inflammatory responses as described below [8, 11, 12-17].

Cortactin (cttn) is located on chromosome 11q13.3 in humans (gDNA: from 70, 398, 404 nt to 70, 436, 584 nt), and chromosome 7; 7F5 in mice (gDNA: from 143, 989, 470 nt to 144, 024, 746 nt). The gene generates a 3310 bp mRNA which presents 22 exons and translates into a 550 amino acids protein that is posttranslationally modified by acetylation and phosphorylation that define cttn affinity for actin binding and support of cytoskeletal remodeling. Cttn has been reported to be almost ubiquitously expressed except for some hematopoietic cells including neutrophils that instead express the cortactin homologue HS1[25].

Cttn is organized into functionally distinct domains through which cortactin interacts with a plethora of actin regulatory proteins. It has an N-terminal acidic (NTA) domain that contains a binding site for the Arp2/3 complex, followed by 6.5 tandem repeat regions (37 amino acids each) mediating actin filament binding through the 4th F-actin binding domain. Next is a helical region of unknown functions, which is followed by the central region containing a proline-rich domain abundant in serine, threonine, and tyrosine residues, which are targets for phosphorylation and that mediates binding of cortactin to proteins containing SH2/SH3 domains. Finally, the C-terminal end contains an SH3 domain through which cortactin can interact with many other actin-regulatory proteins, scaffolding proteins, and adaptor proteins such as zonula occludens-1 (ZO-1)

and catenins (-α, -β, -γ) [28]. Given this network of interactions, cortactin serves as scaffold protein that promotes actin polymerization **(Figure 6)** [24-26].

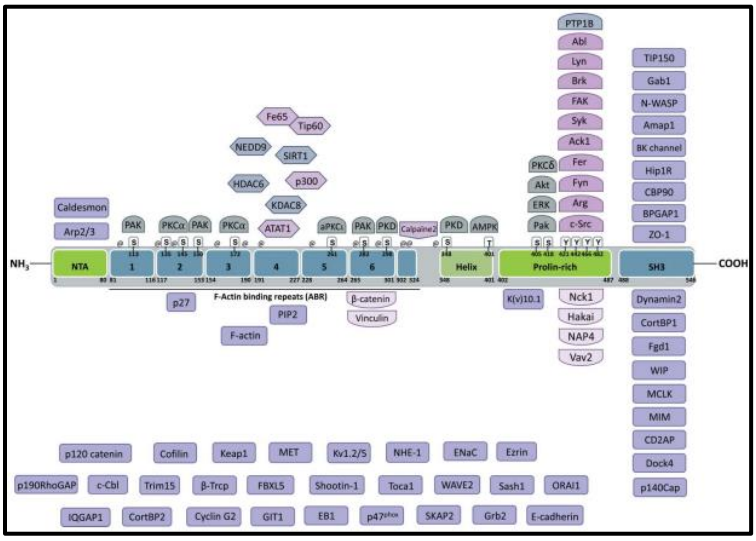

Figure 6. Scheme of cortactin domains showing interactions with other proteins including cytoskeletal regulators.

Shown are the domain organization, post-translational modifications and principal molecules that interact with cortactin depicted in the center [22].

1.5.1. Cortactin functions

Cortactin is involved in regulating vascular permeability and neutrophil trans endothelial migration by controlling actin cytoskeletal remodeling, clustering of adhesion molecules and activation of GTPases. *In vivo* and *in vitro* studies showed that cortactin-deficient mice have increased basal permeability due to reduced basal Rap1 activity and increased ROCK-mediated MLC phosphorylation leading to increased contractile stress fiber formation and junction destabilization that collectively contribute to hyperpermeability [17]. Surprisingly, neutrophils cannot exploit the loose endothelial cell contacts, and, instead, cortactin deficiency reduced neutrophil transmigration *in vivo* and *in vitro* [17]. This reduced neutrophil extravasation was due to defective adhesive interactions of neutrophils with endothelial apical ICAM-1 leading to increased rolling velocity and reduced neutrophil adhesion. In this context, cortactin was required for RhoG-mediated ICAM-1 clustering into ring-like structures surrounding adherent neutrophils **(Figure 7)** [12, 14, 17]. Moreover, phosphorylated cortactin bound directly to E-

selectin to support E-selectin clustering and subsequent leukocyte adhesion onto the endothelial apical surface [68]. Taken together these findings demonstrate that cortactin is required for proper neutrophil-endothelial interactions through regulation of apical adhesion molecule clustering.

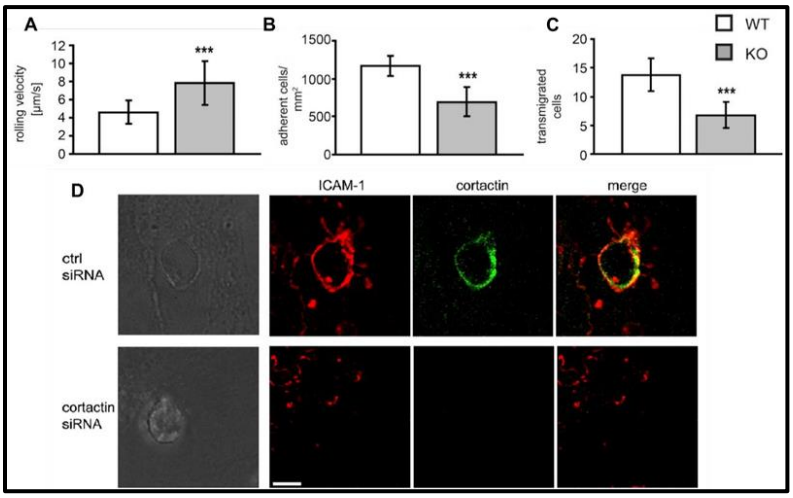

Figure 7. Cortactin modulates neutrophil extravasation.

(A-C) Quantitative analysis of intravital microscopy of mouse cremaster muscle stimulated with TNF-α from WT and KO mice. **(A**) Reduced rolling velocity and **(B)** neutrophil adhesion was observed in the absence of cortactin. **(C)** These defects led to areduced neutrophil transmigration. **(D)** Confocal immunofluorescence of ICAM-1 **(red)** and cortactin **(green)** in co-cultures of WT and cortactin KD HUVEC monolayers with human neutrophils for 20 minutes revealed defective formation of ICAM-1 clusters in the absence of cortactin [17].

Cortactin deficiency also caused damage to the Golgi apparatus cisterns leading to an 80% decrease in vesicular traffic, and aberrant maturation of endosomes [37]. Moreover, cortactin-deficient fibroblasts did not migrate well due to defective secretion of fibronectin that is used to attach the protruding lamellipodia. When these cortactin-deficient fibroblast were plated on a fibronectin-coated surface they could spread and migrate as well as WT cells demonstrating that the defect in migration entirely depends on the reduced fibronectin secretion [30, 44, 45, 47].

However, the roles of cortactin in vesicular trafficking and ECM secretion has so far only been studied in fibroblast. It remains unknown whether cortactin deficiency in ECs and pericytes affects ECM protein secretion and venular BM composition. In addition, it is still unknown whether cortactin is expressed in pericytes and what roles cortactin may play in pericytes in assisting neutrophils to breach the vessel wall.

II. JUSTIFICATION

Neutrophil recruitment into inflamed tissues is a fundamental event during inflammatory responses against cell damage, injury or infection. However, uncontrolled excessive neutrophil extravasation can also contribute to severe tissue damage and the pathogenesis of inflammatory disorders. Anti-inflammatory therapies targeting neutrophil mediated-tissue damage often result in immunosuppression and susceptibility to secondary infections. Therefore, a better understanding of the mechanisms that regulate neutrophil trafficking may lead to improved therapeutic strategies that balance the protective vs. destructive roles of neutrophils. Neutrophil extravasation requires interactions with endothelial cells that need to be tightly controlled to avoid vascular damage while neutrophils pass through the venular wall. Although considerable progress has been made in understanding the neutrophil-ECs adhesive interactions, less is known about the migratory events through BM and pericyte layers to exit the blood vessel. Molecular changes in BM and pericytes occur during neutrophil transmigration but the underlying mechanisms remain unclear and need further exploration. Cortactin is known to control actin cytoskeletal dynamics, vesicle trafficking, and protein secretion. Endothelial cortactin regulates neutrophil-endothelial interactions and neutrophil extravasation. However, it remains to be explored whether cortactin is expressed in pericytes and, if so, how it controls pericyte-neutrophil interactions. Moreover, it remains unknown whether pericyte and ECs cortactin participate in the regulation of BM and LERs.

III. HYPOTHESIS

Cortactin is involved in the regulation of the rearrangement of BM LERs and pericyte gaps during inflammation.

IV.GENERAL AIM

To investigate in cremasteric venules of wild type, heterozygous and cortactin *knock out* mice the architecture of the BM and pericyte layer under basal and inflammatory conditions.

V. PARTICULAR AIMS

1. To characterize LERs in the venular BM of uninflamed and inflamed wild type, heterozygous and cortactin KO cremaster.
2. To characterize the gaps in the venular pericyte layer of uninflamed and inflamed wild type, heterozygous and cortactin KO cremaster.
3. To analyze LERs-gaps colocalization in uninflamed and inflamed wild type, heterozygous and cortactin KO cremaster venules.

CHAPTER 2

2. MATERIAL AND METHODS

Table 1. PBS

1X PBS	138 mM NaCl 3mM KCl 8.1 Mm Na_2HPO_4 1.5 mM KH_2PO_4 pH 7.4

Table 2. Reagents and antibodies

Antibodies	
anti-Collagen type IV	Abcam, #AB6586
anti-PECAM 1	BioLegend, #102502
anti-α-SMA-Cy3	Merck, #C6198-2ML
goat Alexa Fluor 647 anti-rabbit IgG (H+L)	Invitrogen, #A-21245
goat Alexa Fluor 647 anti-mouse IgG (H+L)	Invitrogen, #A-21235

Reagents	
TNF-α	PeproTech, #315-01A
Alexa Fluor 488 Antibody Labeling kit	Invitrogen, #A20181
Fetal Bovine Serum	Biowest, #S1810
96% Ethanol	LGSCIENCE, #ALCOHOL20LGS
Tris-base	ThermoFischer, #17926
HCl	ACS, JT Baker 36.5-38%, #JT9535-05
Taq Polymerase, high GC-rich PCR high perfomance	Promega, #MT10B
$MgCl_2$	Promega, #A351H
Green buffer	Promega, # M891A

Sodium hydroxide	MACRON 97-100%, #7708-10
dNTPs	Thermo Fischer Scientific, #R0191
Ultra-Pure Destilled Water, Nucleases Free	Invitrogen, 10977-015
EDTA	Sigma Aldrich, #E9884-500G
Xylazine	PiSA, # Q-7833-099
Ketamine	PiSA, # Q-7833-028

2.1. Mice

Male C57BL/6 (WT, 8-10 weeks old), $cttn^{+/-}$ (8-10 weeks old), $cttn^{-/-}$ (6 weeks olds) mice were used in this study. These mice were kept in the animal facility at CINVESTAV-IPN under specific pathogen-free conditions. All experiments have been approved by the Institutional Animal Care and Use Committee of Cinvestav. Cortactin KO mice were generated by conditional gene ablation of exon 7 in the *CTTN* gene using the Cre-LoxP recombination method as described previously [33]. In all experiments, mice were anaesthetized by intraperitoneal (i.p.) injection of ketamine hydrochloride (100 mg/kg) and xylazine (10 mg/kg) and euthanized by anesthesia over-dose followed by cervical dislocation.

2.2. Genotyping

Genomic DNA was isolated from WT, $cttn^{+/-}$, $cttn^{-/-}$ mouse tails. 2 mm tail tissues were placed into Eppendorf tubes with 75 µL of NaOH-EDTA (25 mM-0.02 mM, respectively), and vortexed. Afterwards, the samples were heated in a thermoshaker at 97°C for 1 hour. Then, the solution was neutralized with 75 µL 40 mM tris-HCl, pH: 5.5 and centrifuged at 4000 rpm for 3 minutes. The isolated DNA in the supernatant was analyzed by PCR, using a mix containing 5.5 µL nucleases free water, 0.2 µL dNTPs, 2.0 µL green buffer, 1.0 µL MgCl2, 0.1 µL GoTaq Pol (5 units/ µL), 2.0 µL isolated DNA, 0.5 µL primer 1 and 0.5 µL primer 2. Each mouse was genotyped using two sets of primers with the following sequences: (1) 5´-agggtctgaccatcatgtcc-3´ and 5´-gtgctgttcatccacaatgc-3´, and (2) 5´-cggagagctaggctgttagc-3´ and 5´-

cctggaataagtcagccaagc-3´. The proportions previously described to prepare the mix are per set of primers.

The PCR conditions were activation at 95 °C for 2 minutes, followed by 35 cycles of denaturation at 95 °C for 20 seconds, annealing at 59 °C for 30 seconds and extension at 72 °C for 45 seconds, followed by a final extension at 72 °C for 5 minutes. PCR products were analyzed in a 1.5% agarose gel after electrophoresis at 100 mV for 30 minutes.

2.3. *Ex vivo c*remaster muscle staining

2 µg of anti-Col IV and 4 µg of anti-PECAM-1 were diluted separately in 300 µL 1x PBS. 300 µL PBS containing anti-Col IV antibody was administered by instrascrotal (i.s) injection in mice for 3 hours before starting the experiment. Two hours after anti-Col IV administration, 300 µl PBS containing the anti-PECAM-1 antibody was administered by intrascrotal injection for 1 hour. Then, the scrotum was cut with surgical scissors, both cremaster muscles were exposed, and the tissues were surgically removed. Tissues were fixed and permeabilized for 20 minutes in 96% ethanol at -20 °C, then washed with excessive cold 1x PBS. Then, whole tissues were transferred into an Eppendorf tube and incubated in 100 µL of sterile 1x PBS containing 10% FBS and conjugated α-SMA-Cy3 (1:500 dilution) primary antibody and incubated overnight at 4°C. The next day, after washing the tissues, cremaster muscles were incubated with goat anti-rabbit AF 647 (1:1000 dilution) for 3 hours at room temperature (RT) to label the anti-Col IV antibody. Finally, the cremasters were washed three times using 1x PBS for 15 minutes each at RT and mounted on glass cover slides with PBS.

The anti-PECAM-1 antibody was conjugated to AF-488 fluorophore using commercial labeling kits (Thermo Fischer Scientific, USA). Briefly, in 1 mL of deionized water, Component B® reagent and 1M sodium bicarbonate were dissolved. Then, in 100 µL of this solution 100 µg of anti-PECAM-1 antibody was mixed with Alexa Fluor 488® (AF 488) dye for 1 hour at room temperature in the dark. The purification column was filled with 1.5 mL of resin and centrifuged at 1100x g for 3 minutes. Finally, the anti-PECAM

1 AF 488® solution was added to the column and centrifuged again at 1100 x g for 5 minutes to collect the labeled antibody.

2.3.1. Induction of inflammation

In anesthetized mice, cremaster muscle inflammation was induced by i.s injection of 300 ng of mouse TNF-α in 400 µL sterilized 1x PBS for 4 hours. And one hour after TNF-α was administrated, the procedure described above was carried out to label the proteins of interest.

2.4. Confocal microscopy analysis of cremaster muscles

The samples were visualized using a Leica TCS SP8 laser-scanning confocal microscope with a 40x oil-immersion objective (Numerical Aperture [NA] 1.0). 40 Z-stack images of postcapillary venules (within a diameter of 20–40 µm) were captured at a resolution of 1,024 × 1,024 pixels in the x × y × z plane, corresponding to a voxel size of ~0.26 × ~0.26 × ~1/0.5 µm, respectively. Confocal gain, offsets, and laser power were first set on simple stained tissue before being applied for analysis of triple-stained tissues (Col IV, PECAM-1 and α-SMA). Those settings were applied to all conditions. Resulting confocal images were analyzed using the 3D reconstruction software Imaris-Bit Plane (Oxford Instruments, UK) version 10.1. Mean Fluorescence Intensity (MFI) and LERs-gaps quantifications were done using Fiji-ImageJ (National Institutes of Health) software version 1.8.0.

2.4.1. Image analysis

To quantify the number and size of LERs and gaps, confocal images were reconstructed in 3D and split in half *in silico* along the longitudinal vessel axis using ImageJ. Resulting image sections of half vessels were transformed into grayscale intensity. Then, the threshold was determined according to immunofluorescence intensity to invert the signal and detect uncolored LERs and gaps. To determine the MFI in both BM and pericyte layers, ten representative images per mouse were obtained with a total of 30 images per group. These images were obtained from mice under basal and inflammatory conditions. Next, from each of the images, the Col IV

and a-SMA signals were split. Subsequently, the contour of the PCVs was delimited in each image and the background was eliminated. Afterwards, a threshold was determined per group of mice and applied to all images of each group to obtain the real fluoresce signal for Col IV and α-SMA. All analyzed images were 213 μm long to homogenize all information to the same PCV length for representative measurements.

2.7. Statical analysis

Statistical analysis was performed using Student's t-test for comparison of two groups, or two-way analysis of variance (ANOVA) with Bonferroni's multiple comparisons test for comparison of more than two groups using Prism, GraphPad software version 8.1.2. Data of different experiments were presented as means ± standard error of the mean (SEM). A p-value ≤ 0.001 was considered statistically significant.

CHAPTER III

3. RESULTS

While morphological changes in the ECs of PCV and neutrophil-EC interaction have been well studied, little es known about the changes occurring in the BM and pericytes that the neutrophil must also breach [48]. Some studies have demonstrated that the BM and pericytes undergo rearrangements under inflammatory conditions to support neutrophil transmigration to the abluminal site [48-50]. However, the underlying mechanisms driving these rearrangements remain unclear. Given the role of cortactin in the secretion of proteins and cell adhesion and migration, we hypothesized that loss of cortactin affects the morphology of both BM and pericyte layer. To label the BM, we chose collagen type IV (Col IV) because it comprises up to 60% of the BM protein content [51]. To label the pericytes, we chose α-SMA, a well-known marker protein of pericytes [69]. BM and pericyte layer were studies in PCVs of 20-40 µm diameter, because neutrophil transmigrate preferentially through these PCVs [17].

3.1. The architecture of PCVs is different between WT and cttn$^{+/-}$ mice under basal condition.

First, we investigated the structure of the Col IV layer in PCVs of C57Bl/6 (WT) and cttn$^{+/-}$ male mice under basal condition. PECAM-1 in WT PCVs was correctly distributed at intracellular junctions as expected **(Figure 8, A)** [17, 25]. Col IV showed an irregular distribution with different intensities of the signal across the PCVs and distinct sizes and shapes of LERs **(Figure 8, A)**. Analyzing cremasteric PCVs of cttn$^{+/-}$ male mice **(Figure 8, B)** under basal condition, we observed marked differences in comparison to WT PCVs. First, in the ECs, PECAM-1 was not homogenously distributed at the junctions, with significant signal detection in the cytoplasm, suggesting internalization. We also, observed a slightly elongated EC morphology, as previously reported [17]. Second, Col IV distribution was even more heterogenous with sites of strong Col IV accumulation, and with more and bigger LERs, compared to WT PCVs.

Moreover, under basal conditions in WT PCVs the pericytes presented an irregular distribution showing the characteristic elongated morphology of pericytes **(Figure 8, A)**

[53]. The pericyte layer presented numerous gaps with distinct sizes and shapes as reported previously [22, 24,48, 52, 54-55]. Analyzing cremasteric PCVs of cttn$^{+/-}$ male mice **(Figure 8, B)**, we did not observe marked differences in comparison to WT PCVs. The pericytes did not show such an irregular distribution as the BM layer, but apparently had more and bigger gaps. Taken together, these data, suggest that cortactin plays an important role in the architecture of PCVs architecture, especially for the ECs and BM layers, with an apparently less important role for the pericyte layer under basal conditions.

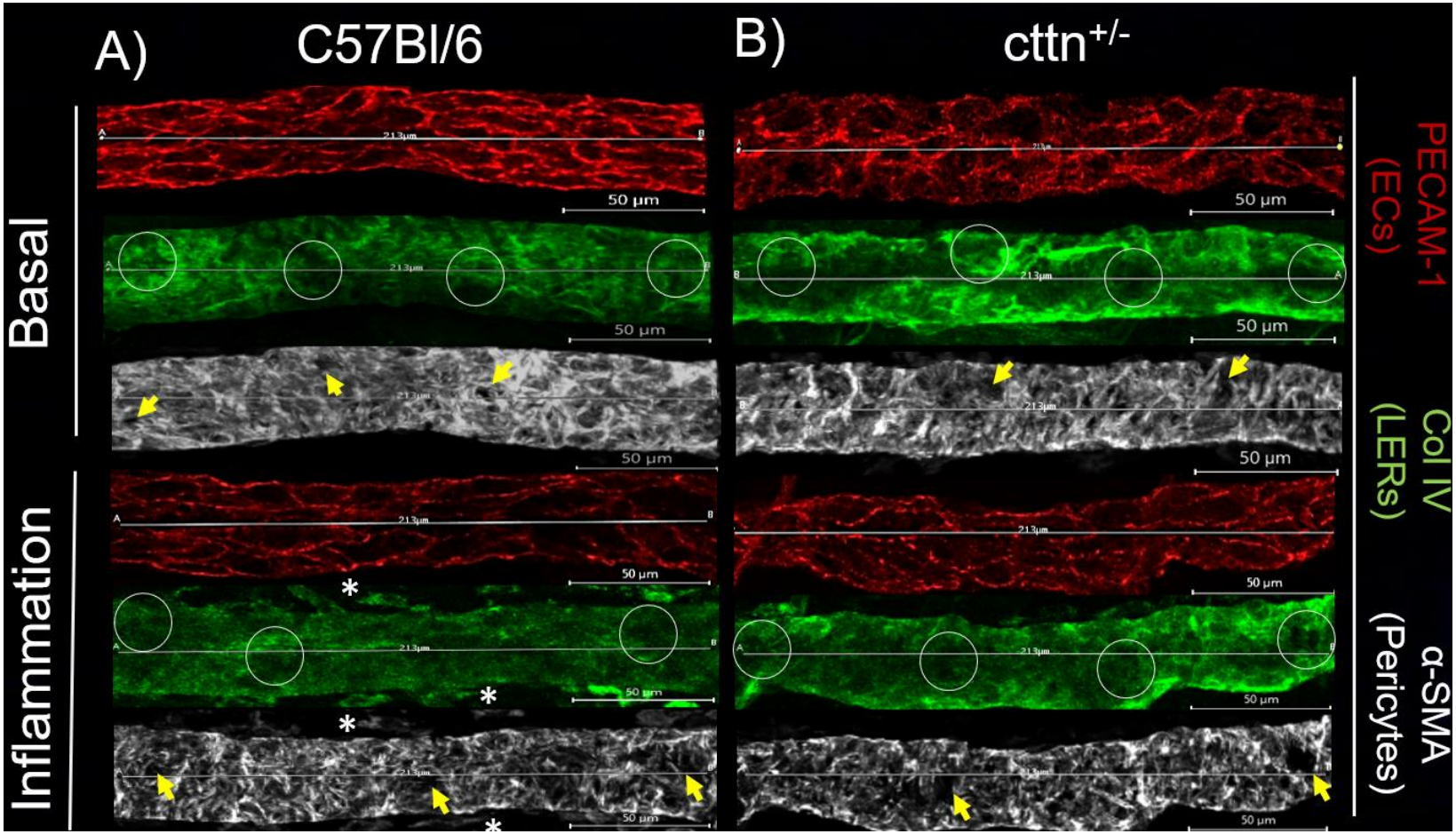

Figure 8. Collagen type IV and the pericyte layer change under inflammatory conditions.

Representative 3D-reconstruction of confocal images of longitudinal cremasteric post-capillary venules (PCVs) immunostained for endothelial cells (PECAM-1, red), collagen type IV (Col IV, green) to reveal low-expression regions (LERs) and α-smooth muscle actin (α-SMA) labeling pericyte layer (white) in **(A)** C57Bl/6 (WT) male mice and **(B)** cttn $^{+/-}$ male mice. The upper panel shows basal conditions and the lower panel inflammatory conditions induced by intrascrotal injection (i.s) of 300 ng TNF-α in 400 µL 1x PBS for 4 hours. Images show 213 µm long of entire PCVs. White circles indicate LERs, yellow arrows indicate gaps, white asterisks indicate unknown perivascular cells. n=3, bar= 50 µm.

3.2. The architecture of PCVs is different between WT and cttn$^{+/-}$ mice under inflammatory condition.

It has been shown that different proinflammatory stimuli generate rearrangements in the PCV layers [54-56]. Even though numerous studies have reported that cortactin plays a significant role in ECs during inflammation, it has not been reported whether this molecule has any effect on BM or pericytes during inflammation.

To investigate this, we analyzed the architecture of PCVs after TNF-α stimulation. We observed that under inflammatory conditions in WT PCVs, PECAM-1 was irregularly distributed at intracellular junctions, as expected **(Figure 8, A)** [25]. Col IV along the PCV showed an irregular distribution, with a clear decrease in the intensity of the Col IV signal. Furthermore, the number of LERs increased and they had different shapes and sizes, compared to WT male mice under basal conditions [22, 24,48, 52, 54-55]. We also noticed perivascular cells of unknown origin of different sizes and morphologies that were positive for Col IV **(Figure 8, A).**

Analyzing inflamed cremasteric PCVs of cttn$^{+/-}$ male mice, we observed marked differences compared to inflamed WT PCVs **(Figure 8, B).** First, PECAM-1 signal was discontinuous at cell junctions and some signal was observed in the cytoplasm, suggesting PECAM-1 internalization and loss of barrier integrity. Also, an elongated ECs morphology was obvious as previously reported [17]. Second, Col IV distribution in inflamed cttn$^{+/-}$ PCVs was different to Col IV in inflamed WT mice, with a higher Col IV intensity and bigger gaps than inflamed WT mice.

On the other hand, in inflamed PCVs in cttn$^{+/-}$ male mice we observed decreased and even more discontinuous PECAM-1 signal in ECs compared to uninflamed PCVs in cttn$^{+/-}$ male mice **(Figure 8, B)**. Col IV distribution in inflamed cttn$^{+/-}$ was different to Col IV in basal cttn$^{+/-}$ male mice, with lower Col IV signal intensity, but overall-similar numbers of LERs of similar sizes.

Additionally, it has been shown that different proinflammatory stimuli generate rearrangements of pericytes [54-56]. Even though numerous studies have reported that

cortactin plays a role during inflammation, it has not been reported whether this molecule has any effect on pericyte layer rearrangement during inflammation.

Pericytes in inflamed PCVs in WT male mice also presented irregular distribution with elongated morphology, but we observed marked rearrangement of the pericyte layer during inflammation with more and larger gaps that had different sizes and shapes, similar to what has been previously reported **(Figure 8, A)** [25]. We also noticed a decrease in the α-SMA signal, which has not been reported before **(Figure 8, A)**. On the other hand, comparing pericyte layer in inflamed cttn$^{+/-}$ male mice **(Figure 8, B)** to inflamed WT male mice, we observed increased number of gaps, which were bigger and larger, with different sizes and shapes. We also noticed a decrease in the α-SMA signal **(Figure 8, B).** Finally, comparing inflamed cttn$^{+/-}$ male mice to basal cttn$^{+/-}$ male mice, we noticed that inflamed cttn$^{+/-}$ male mice still presented elongated pericyte morphology, but with more and larger gaps of different shapes compared to basal cttn$^{+/-}$ male mice **(Figure 8, B)**. Also, we still noticed α-SMA signal reduction. All these data show that cortactin plays a role in the rearrangements of the PCV layers during inflammation.

3.3. The architecture of collagen type IV and pericyte layer in cttn$^{-/-}$ mice under basal condition

Given the role of cortactin in the secretion of proteins [35], cell adhesion [30] and migration [16], we hypothesized that complete loss of cortactin affects the morphology of both BM and pericyte layer even more than in PCVs of cortactin heterozygous male mice. We investigated the structure of the BM and the pericyte layer in cortactin KO mice under basal conditions, and we observed loss of the correct distribution of PECAM-1 at the intercellular junctions that did not even allow a clear determination of ECs morphology **(Figure 9).** This is in line with previous data showing loss of vascular barrier integrity in cortactin-deficient mice [17]. Col IV presented a heterogeneous distribution without the high expression regions that we observed in basal cttn$^{+/-}$ mice. Furthermore, we observed that Col IV presented filamentous structures, that have previously not

reported in *in vivo* models. We also noted an increase in LERs, with a larger size compared to basal WT and cttn$^{+/-}$ mice.

The pericyte layer also presented a more irregular distribution than the basal cttn$^{+/-}$ pericyte layer, with changes in cellular morphology, a clear decrease in the α-SMA signal and increase in the number of gaps, which were even larger than in basal cttn$^{+/-}$ mice. It is important to note here that these data are derived from only one KO mouse. We are currently waiting for more KO mice to complete our analysis. But these preliminary data suggest that complete lack of cortactin affects the correct formation of all three PCV layers even more than the 50% reduction in heterozygous mice.

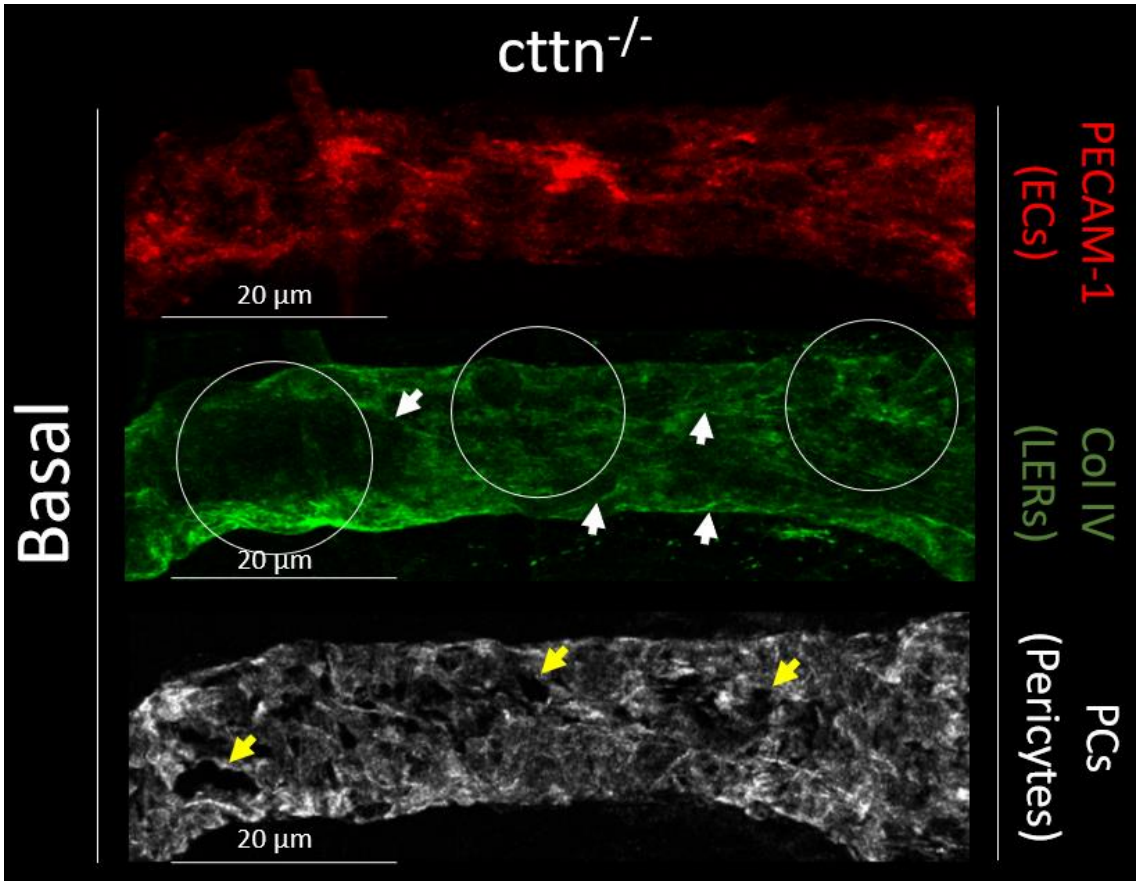

Figure 9. Collagen type IV and the pericyte cell layer under basal condition in a cortactin-deficient cremaster.

Representative 3D-reconstruction of confocal images of entire longitudinal cremasteric post-capillary venules immunostained for endothelial cells (PECAM-, red), collagen type IV (Col IV, green) to reveal low-expression regions (LERs) and α-smooth muscle actin (α-SMA) labeling pericytes (white) in 6-week-old cttn$^{-/-}$ male mice under basal condition. Images show 130 µm long venules. White circles indicate LERs, yellow arrows indicate gaps, white arrows indicate filamentous Col IV, n=1, bar= 20 µm.

3.4. LERs and gaps do not colocalize with EC junctions under basal or inflammatory conditions.

Given that neutrophils preferentially migrate across EC junctions, LERs and gaps [17, 55, 56, 66], we wondered whether junctions, LERs and gaps colocalize, and whether their localization depends on cortactin. To investigate, we performed a more in-depth analysis of the immunofluorescence images shown in **Figure 8, A-B**. From each PCV of each mouse, a random region with a length of 15 micrometers was selected and the colocalization of LERs and gaps over intercellular junctions of the ECs were compared under basal and inflammatory conditions.

In basal WT PCVs, LERs of Col IV did not colocalize with EC junctions, neither under basal nor inflammatory conditions **(Figure 10, A).** Similarly, LERs and gaps were also not located at the same position under basal conditions. However, colocalization of LERs and gaps increased during inflammation partly owed to their increase in size. These data confirm previous reports [55, 56, 66]. Next, we analyzed colocalization of junctions, LERs and gaps in PCVs of $cttn^{+/-}$ male mice under basal and inflammatory conditions. We observed here that the larger LERs colocalized with EC junctions, but that smaller LERs only marginally coincided with junctions under both basal and inflammatory conditions **(Figure 10, B),** which was also true for the colocalization of LERs and gaps. Comparing Col IV and pericytes, a considerable increase in the number of gaps was observed, with different sizes and shapes, but few LERs were located under gaps. These data indicate that the decrease of cortactin expression affects the number and size of LERs and gaps, but not necessarily their colocalization in PCVs.

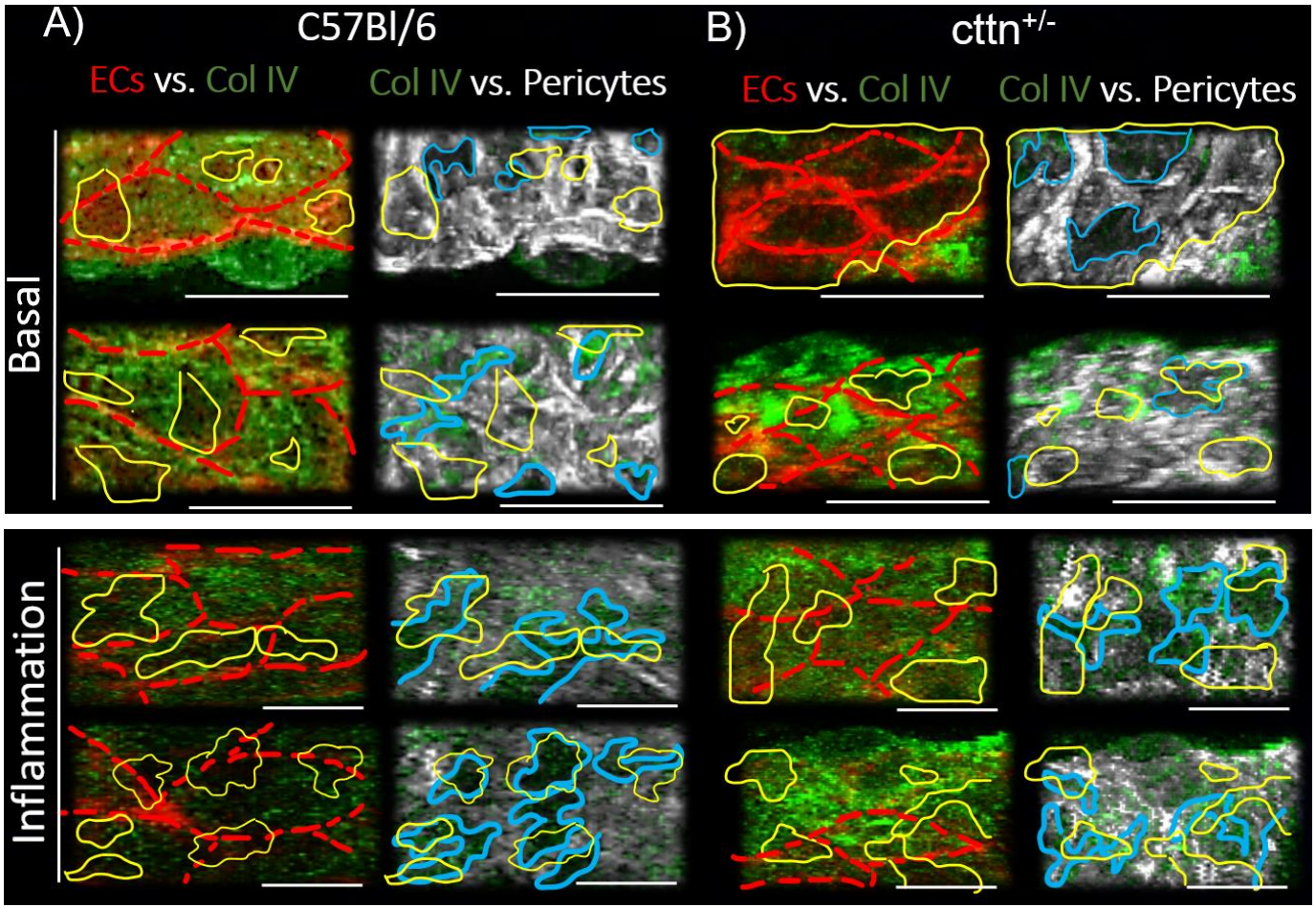

Figure 10. Loss of cortactin induces changes in Col IV and pericytes under basal and inflammatory conditions.

Cremaster muscle inflammation was induced by intrascrotal injection of 300 ng TNF-α in 400 µL 1x PBS for 4 hours. **(A)** C57Bl/6 (WT) male mice and **(B)** cttn$^{+/-}$ male mice under basal condition **(upper panel)** and inflammatory conditions **(lower panel)**. Images represent digital zooms of the images from figure 8. Tissues were immunostained for PECAM-1 (ECs, red), Col IV (green) and α-SMA (pericytes, white). Red dotted lines indicate ECs junctions. Yellow lines indicate LERs. Blue lines indicate gaps. Bar: 15 µm.

3.5. Col IV is reduced and the number of LERs decreased during inflammation.

Given that Col IV showed rearrangement during inflammation [55-60], we wondered whether this rearrangement is mediated by degradation of Col IV. To investigate what happens to Col IV during inflammation in PCVs, we determined the MFI of the Col IV signal in both WT and cttn$^{+/-}$ mice, under basal and inflammatory conditions. We observed that under both basal and inflammatory conditions, cttn$^{+/-}$ mice presented an increase in Col IV MFI compared to basal WT mice **(Figure 11, A)**. Inflamed WT cremaster PCVs demonstrated a clear decrease in Col IV MFI compared to basal WT PCVs, a finding that has not been reported previously in *in vivo* models. On the other hand, inflamed cttn$^{+/-}$ male mice did not show a decrease in Col IV MFI compared to basal cttn$^{+/-}$ male mice **(Figure 11, A)**. These data indicate that cortactin has a function during the degradation of Col IV during inflammation.

Given that Col IV showed rearrangement and decreased signal during inflammation, we wondered whether this is related to the number of LERs. To analyze this, from the images mentioned above, we quantified the number of LERs in WT and cttn$^{+/-}$ mice under basal and inflammatory conditions. We observed under basal condition that WT male mice presented an average of 130 LERs, whereas cttn$^{+/-}$ male mice presented an average of 320 LERs **(Figure 11, B)**. On the other hand, during inflammation, WT male mice showed a clear decrease in the number of LERs, with an average number of 89 LERs, compared to basal WT male mice similar to what was reported previously [56, 57]. By contrast, inflamed cttn$^{+/-}$ male mice did not show a statistically significant decrease in the number of LERs compared to the basal cttn$^{+/-}$ male mice, but the number was still significantly higher compared to inflamed WT mice **(Figure 11, B)**. These data indicate that cortactin is important for the amount of Col IV and the number of Col IV LERs in under both basal and inflammatory conditions.

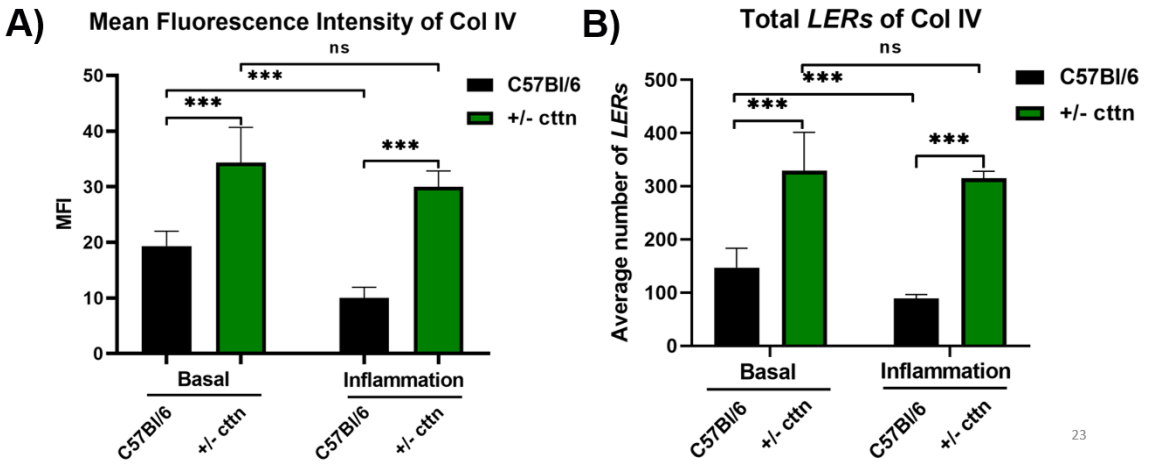

Figure 11. The MFI and the number of LERs in Collagen type IV decreased in PCVs during inflammation.

Cremaster muscles were subjected to local inflammation by intrascrotal administration of 300 ng of TNF-α in 400 µL 1x PBS for 4 hours. From Figure 8, representative 3D-reconstruction of confocal images of longitudinal cremasteric post-capillary venules (PCVs) immunostained for Col IV were selected to quantify **(A)** Mean Fluorescence Intensity (MFI) and to reveal **(B)** the number of LERs in the Col IV layer under basal and inflammatory conditions in post-capillary venules of 20-40 µm diameter and 213 µm long. Data are represented as means ± SEM, n=3 mice/group; 10 PCVs per mouse. ***p<0.001, ns= non-significant.

3.6. α-SMA is reduced in pericytes, and the number of gaps increased during inflammation.

Given that pericytes showed rearrangement during inflammation [67], we wondered whether this rearrangement depends on changes in α-SMA leading to an altered number of pericyte gaps. To test this idea, we analyzed the MFI of the α-SMA signal in both WT and cttn$^{+/-}$ mice under basal and inflammatory conditions. We observed that under both basal and inflammatory conditions, cttn$^{+/-}$ mice presented an increase in MFI compared to WT male mice **(Figure 11, A)**. However, during inflammation, WT male mice demonstrated a significant decrease in α-SMA MFI compared to basal WT male mice, a finding that has not been reported previously in *in vivo* models. On the other hand, inflamed cttn$^{+/-}$ male mice did not show a significant decrease in α-SMA MFI compared to basal cttn$^{+/-}$ male mice **(Figure 11, A)**. These data indicate that cortactin regulates the amount of α-SMA in pericytes under basal and inflammatory conditions.

Given the previous data, we wondered next whether these phenomena are related to the number of gaps. To analyze this, from the images mentioned above, we quantified the number of gaps in WT and cttn$^{+/-}$ mice under basal and inflammatory conditions. We observed that under basal condition, WT male mice presented an average of 185 gaps, whereas the cttn$^{+/-}$ male mice presented an average of 730 gaps **(Figure 11, B)**. On the other hand, during inflammation, WT male mice showed a clear increase in the number of gaps with an average number of 579 gaps similar to what has been reported previously [67]. Inflamed cttn$^{+/-}$ male mice did not show a significant increase in gaps compared to basal cttn$^{+/-}$ male mice, with an average number of 759 gaps **(Figure 11, B)**. These data indicate that cortactin is important for the rearrangement of pericytes gaps in PCVs.

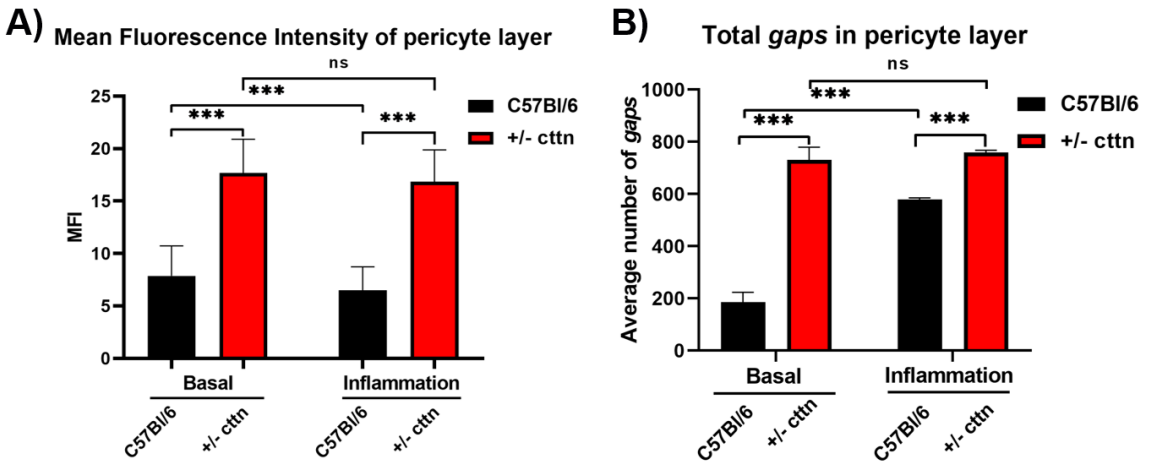

Figure 12. The MFI of α-SMA and the number of gaps in pericyte layer increased in PCVs during inflammation.

Cremaster muscles were subjected to local inflammation by intrascrotal administration of 300 ng of TNF-α in 400 µL 1x PBS for 4 hours. From Figure 8, most representative 3D-reconstruction of confocal images of longitudinal cremasteric post-capillary venules (PCVs) immunostained for α-SMA were selected to quantify **(A)** Mean Fluorescence Intensity (MFI) and to reveal **(B)** the number of gaps in the pericyte layer under basal and inflammatory conditions in post-capillary venules of 20-40 µm diameter and 213 µm long. Data are represented as means ± SEM, n=3 mice/group; 10 PCVs per mouse. ***p<0.001, ns= non-significant.

3.7. Col IV rearranges into larger LERs during inflammation

Given that Col IV showed rearrangement during inflammation, a decrease in MFI and a decrease in the number of LERs during inflammation, me wondered what sizes of LERs are the most abundant. To investigate this, we analyzed the average

number of LERs by size in μm^2 in both WT and $cttn^{+/-}$ mice under basal and inflammatory conditions.

We observed that during basal conditions, the most abundant LERs were those of 1-3 μm^2, both in WT and $cttn^{+/-}$ male mice **(Figure 12, A),** data that coincided with previous reports. The number of LERs of 1-3 μm^2 was significantly higher in $cttn^{+/-}$ mice. On the other hand, during inflammation the WT male mice presented rearrangement of smaller LERs towards larger LERs, with the most abundant being those with a size of 3-5 μm^2 **(Figure 12, B)** similar to previously reported data [61, 66]. Finally, during inflammation the $cttn^{+/-}$ male mice did not present a significant rearrangement towards larger LERs, with the most abundant LERs still being those with a size of 2-3 μm^2 **(Figure 12, B)**. These data clearly indicate that cortactin is involved in the rearrangement of Col IV into larger LERs during inflammation.

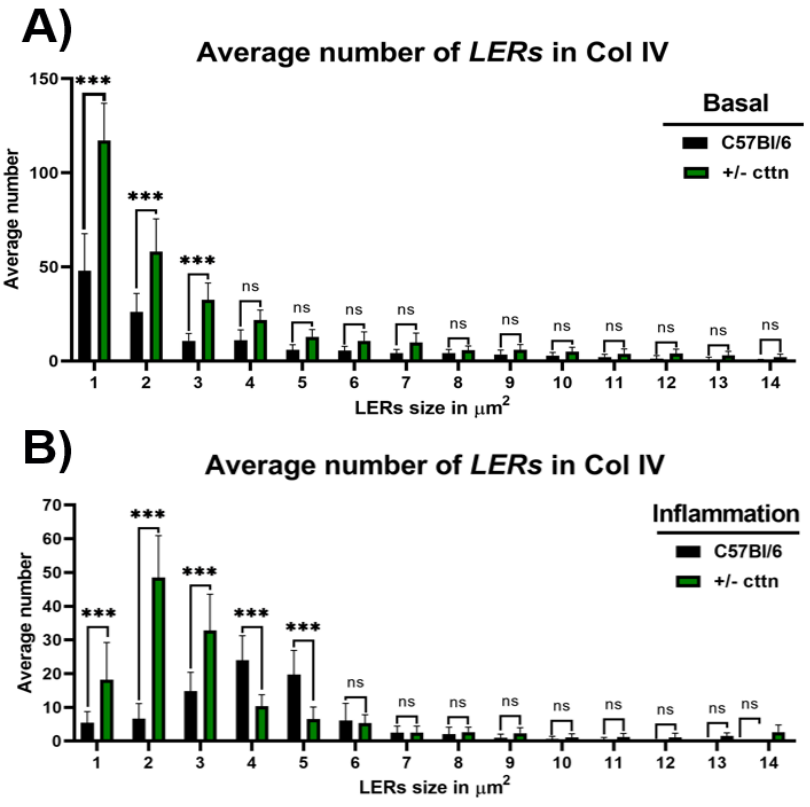

Figure 13. Col IV rearranges into bigger LERs during inflammation

Cremaster muscles were subjected to local inflammation by intrascrotal administration of 300 ng of TNF-α in 400 µL 1x PBS for 4 hours. From representative 3D-reconstruction of confocal images of longitudinal cremasteric post-capillary venules (PCVs) immunostained for Col IV were selected to quantify the number of LERs by different sizes in μm^2 **(A)** under basal condition and **(B)** inflammatory conditions in post-capillary venules of 20-40 µm diameter and 213 µm long. Data are represented as means ± SEM, n=3 mice/group; 10 PCVs per mouse. ***p<0.001, ns= non-significant.

3.8. Pericytes rearrange into larger gaps during inflammation.

Given that pericytes showed rearrangement during inflammation [64, 67], a decrease in α-SMA MFI and an increase in the number of gaps during inflammation, we wondered what sizes of gaps are the most abundant. To investigate this, we analyzed the average number of gaps by size in μm^2 in both WT and $cttn^{+/-}$ mice under basal and inflammatory conditions.

We observed that under basal conditions, in WT male mice, the most frequent gaps were those of size 1-4 μm^2 **(Figure 13, A),** similar to previously reported data. Interestingly, in the cttn+/- male mice, the most frequent gaps were those with a size of 1-7 μm^2 **(Figure 13, A).** The number of gaps in this size range was significantly higher in $cttn^{+/-}$ mice. On the other hand, during inflammation, in WT male mice, the most abundant gaps were those of 1-7 μm^2, with peaks of 4-5 μm^2 **(Figure 13, B)** similar to previously reported data [67]. On the other hand, the cttn +/- male mice did not presented this significant rearrangement into larger gaps, with the most abundant gaps still being those with a size of 1-3 μm^2 **(Figure 13, B)**. These findings indicate that cortactin is involved in the remodeling of the pericyte layer into larger gaps during inflammation.

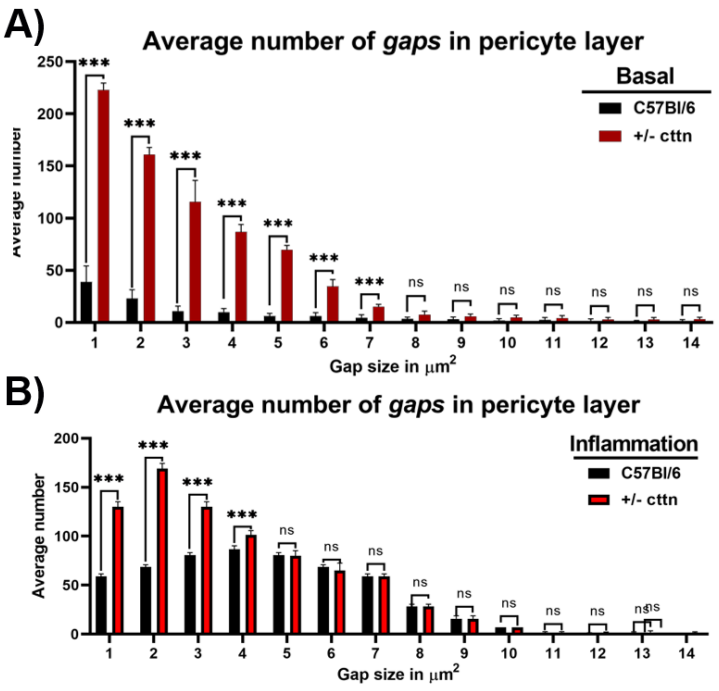

Figure 14. Gaps in pericytes layers rearrange into bigger gaps during inflammation. Cremaster muscles were subjected to local inflammation by intrascrotal administration of 300 ng of TNF-α in 400 µL 1x PBS for 4 hours. From representative 3D-reconstruction of confocal images of longitudinal cremasteric post-capillary venules (PCVs) immunostained for pericytes were selected to quantify the number of gaps by different sizes in μm^2 **(A)** under basal condition and **(B)** inflammatory conditions in post-capillary venules of 20-40 µm diameter and 213 µm long. Data are represented as means ± SEM, n=3 mice/group; 10 PCVs per mouse. ***p<0.001, ns= non-significant.

CHAPTER IV

DISSCUSION

PCVs are composed of endothelial cells, the venular basement membrane (BM), and perivascular cells known as pericytes [12, 17]. Together, they maintain blood vessel homeostasis, and when homeostasis is lost, an inflammatory response begins [17, 18, 21, 25]. Neutrophils are the first cells to reach the site of damage, extravasating through the intercellular junctions of the ECs, towards the BM, and then the pericyte layer, towards the affected interstitial tissue, where they carry out their effector functions, to prevent the spreading of the infection or damage with the goal to restore tissue homeostasis. [12, 17, 18, 25].

Much progress has been made in understanding the cellular and molecular mechanisms that govern neutrophil extravasation, but most studies have focused on studying the events that regulate transendothelial migration. Other studies have focused on studying the molecular mechanisms regulating neutrophil breaching of BM and pericytes during inflammation, but the cellular or molecular mechanisms that regulate these steps including rearrangement of the BM and the pericyte layer still remain elusive [35-37, 60].

The venular BM is composed of Lm-8, -10, Per, Nd-2 and Col IV [51]. Under basal conditions, these proteins present an irregular distribution along blood vessels with multiple low-expression regions (LERs) [69]. To date, the cellular or molecular events that generate these LERs and their function under basal conditions are unknown. However, several studies have demonstrated that, during inflammation, LERs of each protein vary in size and number. To date, the mechanisms that cause size changes of LERs are unknown. Interestingly, the number of total LERs in Lm-8, -10, and Per increase during inflammation, whereas LERs in Nd-2 and Col IV decrease [51-69]. These data indicate that each BM protein undergoes different rearrangements during inflammation.

To study the BM rearrangement during inflammation in more detail, we stained cremasteric PCVs for Col IV and observed that basal WT male mice presented an

irregular distribution of Col IV, with an average number of 130 LERs in the region of interest in each PCV, with most LERs of sizes of 1-3 μm^2 similar to previously reported data [52]. Inflamed WT male mice presented a decrease in the number of LERs, with an average of 89 LERs of sizes of 4-6 μm^2. These data confirm the reduction of Col IV LERs and the rearrangement to smaller Col IV LERs during inflammation. Possible explanation could be (1) an increased production of Col IV leading to the filling of larger LERs and elimination of smaller LERs; (2) an increase in vesicular trafficking leading to more secretion of MMPs to generate bigger LERs, or (3) endocytosis of Col IV in smaller LERs to generate larger LERs. More studies are needed to better understand the mechanisms of LER remodeling. It is worth highlighting that this rearrangement is important, because neutrophils migrate over Col IV, searching for LERs of sizes of 4-6 μm^2, where they preferentially transmigrate towards the pericyte layer [52]. It is tempting to speculate that the preference for LERs of sizes of 4-6 μm^2 is due to a spatial presentation of ligands needed for neutrophil adhesion and crawling. However, this idea requires further investigation.

Another observation regarding the rearrangement of Col IV in inflamed WT male mice was a significant reduction of Col IV. This reduction of Col IV protein in the BM layer during inflammation is a result that has not yet been reported before in vivo. Therefore, it will be interesting to study a possible correlation between the recruitment of neutrophils and the degradation of Col IV at different times during inflammation. Also, inflammation was induced for 4 hours. This is because at the fourth hour a minimal number of neutrophils are transmigrating, according to previous reports [17, 52, 66]. Therefore, it was expected to find changes in the structure of Col IV. Interestingly, Col IV has not been studied at different time points during inflammation, and it will be important to know when degradation begins and when the original architecture is restored. Another reason for the reduction of Col IV could be the activity of neutrophil-derived elastase. This molecule has several targets in the extracellular matrix and one of them is Col IV [72]. A previous study showed that elastase-deficient neutrophils, apart from being arrested in the BM,

do not rearrange the BM [61]. Therefore, it will be interesting to inhibit the elastase activity and observe the changes in Col IV at different times.

As cortactin is a molecule that is involved in the regulation of vascular permeability, vesicular trafficking, and neutrophil extravasation, it seems logical to assume that it regulates BM rearrangements during inflammation to facilitate neutrophil passage. Analyzing cttn$^{+/-}$ male mice, we observed under basal conditions an aberrant distribution of Col IV with sites of Col IV accumulation and an average number of more than 300 LERs mostly of a size of 1-3 μm^2. On the other hand, inflamed cttn$^{+/-}$ male mice presented an aberrant distribution of Col IV, with a non-significant decrease in Col IV, and a similar average number around 300 LERs, mostly 1-3 μm^2 in size, with no statistical differences compared to the basal cttn$^{+/-}$ male mice. Additionally, in basal cttn$^{+/-}$ male mice we observed regions where there is accumulation of Col IV under basal conditions. We did not expect this result since cortactin is necessary for efficient vesicular trafficking and thus protein secretion. Reduction of cortactin should therefore be accompanied by reduced Col IV and an increase in LERs number in PCVs. On the other hand, Col IV accumulation could be attributed to the fact that the lack of cortactin generates an increase un vascular permeability [17] that would allow insoluble Col IV from the circulation [71] to be deposited in clusters on postcapillary venules without being distributed efficiently. Also, the small changes observed in inflamed cttn$^{+/-}$ male mice, might be due to altered MMP-mediated degradation as cortactin also regulates MMP secretion. Thus, it will be interesting to perform these experiments in the presence of MMP inhibitors to analyze cortactin-dependent and MMP-mediated effects on BM rearrangement.

We wanted to confirm the data from cortactin heterozygous male mice in cortactin KO mice, but, unfortunately, we did not obtain enough KO male mice to make solid conclusions. Preliminary data from one male mice, showed a loss of the correct distribution of PECAM-1 at the cellular junctions, in line with previous reports [17]. Col IV presented a more heterogenous distribution and more LERs than basal cttn$^{+/-}$ male mice. Interestingly, we did not observe Col IV cluster depositions as in

basal cttn$^{+/-}$ male mice, but what we observed were thin filaments of Col IV, data that has not been previously reported. Currently, we do not have an explanation for this observation. We will complete these studies in the future to study the role of cortactin in Col IV distribution, LERs formations and rearrangement under basal and inflammatory conditions.

Pericytes are also able to produce BM proteins and MMPs and can therefore be involved in BM architecture regulation [42, 46, 50]. Of note, unpublished data from our laboratory demonstrated that pericytes do express cortactin. Thus, absence of cortactin in pericytes could have consequences for the distribution of BM proteins, the formation of LERs, and pericyte morphology. Previous reports have mentioned that postcapillary pericytes, under basal conditions, present an irregular distribution, with elongated morphology, and with several protrusions, which when in contact with other pericytes generate gaps of different sizes and shapes, with an average number of 200 gaps in PCVs, the most frequent being those with a size of 1-3 μm^2. Under inflammatory conditions, it has been reported that pericytes undergo structural rearrangement, with an increase in the number of total gaps, with an average number of 590 gaps and a shift towards larger gaps of 3-6 μm^2 [42, 53, 67]. However, the cellular and molecular mechanisms that induce this increase in the number of gaps during inflammation are unknown.

To investigate the role of pericytes during inflammation, we selected α-SMA, as specific marker of these cells. First, we characterized pericytes in basal WT male mice. We observed that these cells have an irregular distribution, with elongated morphology, presenting gaps of different sizes and shapes with an average number of 185 gaps, the most frequent being those with sizes of 1-7 μm^2. On the other hand, in the inflamed WT male mice, PCVs also presented an irregular distribution, with elongated morphology and an increase in gaps of different sizes and shapes. According to the quantification, inflamed PCVs had an average of 579 gaps, the most frequent being those with a size of 3-6 μm^2. These data show that inflammation indeed induces pericyte rearrangement. Although the underlying mechanisms are still unknown, possible explanations may include a decrease in

the number of protrusions in the pericytes that generate an increase in the number of gaps, and a possible change in migration of pericytes, or pericyte numbers within the PCVs that change gap numbers and sizes. In this regard, the average number of postcapillary pericytes in the cremaster muscle has not been reported so far. To answer this question, vascular microsurgery to obtain PCV segments followed by enzymatic digestion to disaggregate the PCVs and determine pericyte numbers could be performed. Such experiments would allow the quantification of potential changes in pericyte numbers over time during inflammation. Notably, neutrophils search for gaps of 4-6 μm^2, where they preferentially exit the venule [66]. We believe that the preference for gaps between sizes of 4-6 μm^2 could be due to a preferable arrangement of receptors such as ICAM-1 that are then more accessible to neutrophils to support neutrophil interactions with pericytes [56, 67]. This way, pericytes could be guiding neutrophils towards the exit points via gaps of certain sizes.

Another observation regarding the rearrangement of the pericyte layer in inflamed WT male mice was a significant reduction in the α-SMA signal. This reduction of α-SMA has not yet been reported in vivo. Therefore, it is of interest to further study the correlation between the recruitment of neutrophils and the decrease in α-SMA at different times during the inflammatory response.

Analyzing the role of cortactin in pericytes, we characterized the pericyte layer in basal $cttn^{+/-}$ male mice. We observed that the pericyte layer presented an irregular distribution, with elongated morphology as in basal WT male mice. Interestingly, we observed more and larger gaps compared to basal WT male mice, with a total average of 730 gaps, with the most frequent gaps showing sizes of 3-6 μm^2. We also observed a decrease in α-SMA signal, similar to basal WT pericytes. On the other hand, characterizing the pericyte layer in inflamed $cttn^{+/-}$ male mice, we observed a less disorganized distribution, with more and larger gaps compared to basal $cttn^{+/-}$ male mice, with 759 gaps on average, with most frequent gaps of sizes from 1-4 μm^2. Also, we observed a stronger decrease in α-SMA signal compared to basal $cttn^{+/-}$ male mice. According to our observations, in the basal $cttn^{+/-}$ male

mice, the arrangement that the pericyte layer undergo could be due to a decrease in the number of PCV pericytes, a phenomenon that may have started since the vascular development of the mouse, or due to a shortening of protrusions or a decrease in the number of protrusions. Therefore, quantification of pericytes as mentioned above is an important experiment to perform in the future. Another experimental strategy to determine pericyte protrusions is to obtain a pure primary culture of pericytes from both WT and cttn$^{+/-}$ mice and compare morphological changes over time during inflammation by confocal microscopy. A cortactin-dependent change in the number of pericytes or the number of pericyte protrusions and thus the available surface can have important consequences for the interaction of neutrophils with pericytes on their way across the venular wall, because it can affect the available receptors for neutrophil adhesion.

Given that cortactin is involved in ICAM-1 clustering in ECs [17], it is tempting to speculate that cortactin also regulates ICAM-1 functions in pericytes and by doing so controls the stability of neutrophil pericyte adhesive interactions. Thus, partial or complete loss of cortactin in pericytes could destabilize these interactions and affect neutrophil exit. Further studies are needed to analyze ICAM-1 distribution in pericytes and how it affects neutrophil-pericyte interactions and neutrophil-breaching of the pericyte layer.

Our data provide evidence that cortactin plays an important role in the distribution and rearrangement of the BM and pericyte layer during inflammation. Another question we wanted to answer was whether these cortactin-dependent rearrangements contribute to the relocalization of LERs and gaps in closer proximity with each other and with EC junctions to reduce the path neutrophil must follow between the layers. To analyze this, we selected the most representative images of both WT and cttn$^{+/-}$ mice under basal and inflammatory conditions and selected random regions to compare the location of EC junctions, LERs and gaps. We found that neither under basal nor inflammatory conditions in both WT and cttn$^{+/-}$ mice LERs and gaps exactly colocalized with intercellular junctions of the ECs. However, the different sizes and shapes resulting from inflammatory LERs,

and gaps rearrangement caused a partial overlap with intercellular junctions. However, whether this is only owed to the size changes is currently inconclusive and requires further investigation. Together, we conclude that cortactin affects the number and shapes of LERs and gaps, but not necessarily their colocalization.

CONCLUSION

This work demonstrated for the first time that cortactin is important for the formation of the BM and the pericyte layer. Furthermore, cortactin plays an important role during inflammation in cremasteric PCVs by regulating the rearrangement of Col IV and the pericyte layer. As LERs and gaps are important for neutrophil transmigration, targeting cortactin to manipulate these rearrangements could help to regulate vascular homeostasis and neutrophil recruitment in inflammatory diseases. However, more research is still required to better understand the cellular and molecular mechanisms that govern these cortactin-mediated rearrangements in the BM and pericyte layer.

PERSPECTIVES

1. Perform PECAM-1, Col IV and a-SMA staining in cortactin KO mice and quantify the number of LERs and gaps and analyze the most abundant LERs and gaps according to size.

2. Analyze the decrease in Col IV and a-SMA after different times of TNF-α treatment and correlate the MFI with the recruitment of neutrophils, in WT, $cttn^{+/-}$ y $cttn^{-/-}$ male mice.

3. Quantify protein reduction of Col IV and a-SMA in PCVs after different times TNF-α treatment in WT, $cttn^{+/-}$ y $cttn^{-/-}$ male mice.

4. Analyze the degradation of Col IV after inhibition of MMPs in WT, $cttn^{+/-}$ y $cttn^{-/-}$ male mice.

5. Inhibit vesicular trafficking during inflammation and analyze the architecture of PCVs in WT, $cttn^{+/-}$ y $cttn^{-/-}$ male mice.

6. Characterize other BM proteins such as Nd-2 and Lm-10 proteins under basal and inflammatory conditions in WT, $cttn^{+/-}$ and $cttn^{-/-}$ mice.

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