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“El papel de la proteína de unión a actina HS1 en la colitis experimental”

T E S I S

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M.C. KARLA FABIOLA CASTRO OCHOA

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Director de la Tesis: Dr. Michael Schnoor

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"The role of the actin-binding protein HS1 in experimental colitis"

T H E S I S

Presented by

M.Sc. KARLA FABIOLA CASTRO OCHOA

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1. ABSTRACT

Intestinal epithelial barrier dysfunction has been associated with the pathogenesis of ulcerative colitis (UC). During the active phase of UC, neutrophils are recruited to the lamina propria for bacterial clearance and resolution of inflammation. However, excessive neutrophil extravasation to the lamina propria and subsequent transepithelial migration (TEpM) towards the intestinal lumen disrupts the intestinal epithelial barrier leading to increased intestinal permeability, bacterial translocation and an exacerbated immune response triggering colitis symptoms. Neutrophils require actin cytoskeletal rearrangements to extravasate, navigate through the interstitium and cross the epithelium to reach the intestinal lumen. These rearrangements are regulated by actin-binding proteins (ABP). Hematopoietic cell-specific lyn substrate 1 (HS1) is an ABP exclusively expressed in hematopoietic cells that regulates Arp2/3-dependent chemotaxis in different leukocytes including neutrophils. Given that HS1 regulates neutrophil extravasation, we hypothesized that HS1 deficiency could modulate neutrophil numbers in the mucosa during colitis, and thus protect against neutrophil-inflicted colon tissue damage. To investigate how the absence of HS1 influences the development of experimental colitis, HS1 knock-out (KO) and littermate wild-type (WT) mice were treated with 5% dextran sulfate sodium (DSS) for five and seven days. Development of DSS-colitis in KO mice was significantly less severe compared to WT mice especially after day five. Histological analysis revealed that intestinal tissue integrity was better preserved in KO colons. Neutrophil recruitment to the mucosa and transepithelial migration into the colon lumen were significantly reduced in KO mice. Moreover, KO mice showed less internalization of junction proteins and oxidative stress. Consequently, intestinal permeability *in vivo* was also reduced in the absence of HS1. In conclusion, absence of HS1 reduces neutrophil recruitment to the lamina propria and decreases TEpM, thus preventing intestinal barrier dysfunction and ameliorating colitis development.

RESUMEN

La disfunción de la barrera epitelial intestinal ha estado asociada con la patogénesis de la colitis ulcerosa (CU). Durante la fase activa de CU, neutrófilos son reclutados a la lámina propria para eliminación de bacterias y resolución de la inflamación. Sin embargo, la extravasación masiva de neutrófilos a la lámina propria y la subsecuente migración transepithelial (TEpM) hacia el lumen intestinal afecta la barrera epitelial intestinal al desregular las uniones intercelulares lo cual aumenta la permeabilidad intestinal, conduciendo a la translocación bacteriana y eventualmente a una respuesta inmunológica exacerbada desencadenando los síntomas de colitis. Los neutrófilos requieren rearreglos del citoesqueleto para la extravasación, navegar por el intersticio y a través del epitelio para llegar al lumen intestinal. Estos rearreglos están regulados por proteínas de unión a actina (ABP). “Hematopoietic cell-specific lyn substrate 1” (HS1) es una ABP exclusivamente expresada en células hematopoyéticas que regula la quimiotaxis dependiente de Arp2/3 en diferentes leucocitos incluyendo los neutrófilos. Dado que HS1 regula la extravasación de neutrófilos, hipotetizamos que la deficiencia de HS1 podría estar modulando el número de neutrófilos en la mucosa durante colitis y, por lo tanto, protegiendo del daño infligido por los neutrófilos al tejido del colon. Para investigar cómo la ausencia de HS1 influye el desarrollo de la colitis experimental, ratones knock-out (KO) de HS1 y wild-type (WT) de la misma camada, fueron tratados con 5% dextrano sulfato de sodio (DSS) por cinco y siete días. El desarrollo de la colitis-DSS en los ratones KO fue significativamente menos severo comparado con el WT especialmente después del día cinco. Análisis histológicos revelaron que la integridad del tejido intestinal se mantuvo mejor preservada en los colones KO. El reclutamiento de neutrófilos a la mucosa y la migración transepithelial hacia el lumen del colon se redujeron significativamente en los ratones KO. Además, los ratones KO mostraron menor internalización de proteínas de unión y estrés oxidativo. En consecuencia, la permeabilidad intestinal in vivo también se redujo en ausencia de HS1. En conclusión, la ausencia de HS1 reduce el reclutamiento de neutrófilos hacia la lámina propria y disminuye la TEpM, evitando así la disfunción de la barrera intestinal y atenuando el desarrollo de la colitis.

2. INTRODUCTION

2.1 Inflammatory Bowel Diseases

Inflammatory bowel diseases (IBD) are characterized by chronic and recurrent inflammation of the gastro-intestinal tract. The incidence of IBD is constantly increasing affecting millions of people worldwide [1, 2]. The most common manifestations are ulcerative colitis (UC) and Crohn's disease (CD). Patients with these disorders commonly present weight loss, abdominal pain, diarrhea and intestinal bleeding [3]. UC is characterized as a confined inflammation of the mucosa and affects mainly the rectum and parts of the colon in an uninterrupted manner, whereas CD causes transmural inflammation, affects any part of the gastrointestinal tract in a discontinuous pattern, and is associated with complications like granulomas, fistulas and strictures [4, 5].

The etiology of these diseases remains incompletely understood; however, various factors are implicated in the pathogenesis of IBD, such as genetic susceptibility, environmental and microbiological factors, defects of the epithelial barrier and dysregulation of innate and adaptive immune responses [3, 6]. In both UC and CD, the intestinal epithelial barrier is affected causing increased permeability that leads to a chronic and recurrent inflammation partly due to an uncontrolled immune response to the antigens of the intestinal microbiota [4]. Therefore, therapies for IBD aim to restore the intestinal barrier and mitigate the exacerbated immune response. Treatment protocols for IBD are determined according to clinical progression of the disease and parts of the gastrointestinal tract affected [7]. Since IBD is mainly diagnosed during an active phase of the disease, patients begin with a combination of corticosteroids treatment often in combination with the antioxidative and anti-inflammatory drug 5-aminosalicylate. During active IBD, patients are also treated with immunosuppressives such as azathioprine. However, if these therapies are not sufficient to induce and maintain remission, biological therapies such as anti-TNF antibodies are introduced [8]. If patients do not respond to all these therapeutic options, surgical removal of the affected parts is indicated. Recent studies have

shown beneficial effects of nutritional supplements to improve intestinal barrier functions and delay progression of experimental colitis so that they my serve as additional therapeutic approach [9].

2.2 The Intestinal barrier

The intestinal barrier prevents direct contact between luminal contents and the interstitium, but allows the transit of water, electrolytes and nutrients [10] (Figure 1). The barrier is formed by a mucus layer, the epithelium and the lamina propria. The intestinal mucosal surface is in contact with millions of dietary and environmental antigens as well as commensal bacteria. Thus, maintenance of intestinal barrier functions is critical for preserving intestinal homeostasis.

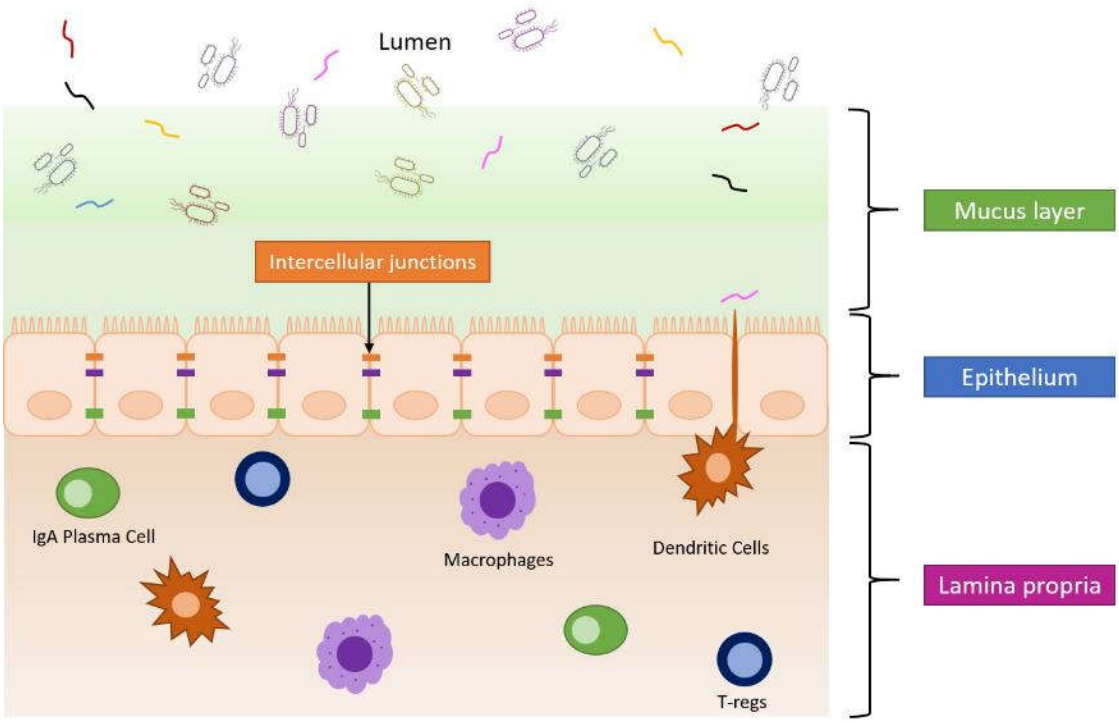

Figure 1. Components of the intestinal barrier. The mucus layer formed by mucins prevents the contact of bacteria with the epithelium and is divided in an outer and inner layer. The outer layer is more soluble, and some bacteria can penetrate it. On the other hand, the dense inner layer is resistant to bacterial penetration. A monolayer of tightly joined epithelial cells forms the epithelium, followed by the lamina propria, a thin layer of connective tissue harboring numerous immune cells. Image adapted from [11].

2.2.1 The mucus layer

The mucus layer is the first obstacle preventing the contact of large molecules and bacteria with the epithelium. It is composed of glycoproteins mucins that are secreted by specialized epithelial cells termed goblet cells. The colon mucus layer is formed by two different strata made of Mucin 2 (MUC2). In the inner layer, MUC2 is densely packed; but further away from the epithelium it becomes soluble and loose due to proteolytic cleavage, which does not disrupt the function of MUC2, but instead allows it to expand. Even though the molecular mechanisms of this process are not known, it seems that endogenous host proteases are responsible for this cleavage since germ-free mice also present a loose mucus layer [12]. Mice deficient for MUC2 developed spontaneous intestinal inflammation. The epithelial cells of these mice were distorted and flattened and the architecture of the lamina propria was lost. Moreover, the proinflammatory cytokines TNF-α and IL-1β were upregulated in their colons [13]. MUC2 KO mice did not produce compensatory mucins and therefore completely lacked a mucus layer. Thus, bacteria were in constant direct contact with the epithelium, and in some cases bacteria penetrated epithelial cells, which likely explains the development of spontaneous colitis and high susceptibility to colorectal cancer development [14].

2.2.2 The intestinal epithelium

The central component of the intestinal barrier is the epithelium, a monolayer of epithelial cells that originate from pluripotent stem cells located at the bottom of intestinal crypts. Epithelial cells are connected by intercellular junctions, which help maintain barrier integrity and regulate the passage of ions, water and molecules [15].

The junctional complexes that contribute to barrier function are: tight junctions (TJ), adherens junctions (AJ) and desmosomes (Figure 2). TJ are the most apical intercellular junctions and maintain the barrier sealed while regulating permeability to ions, water and nutrients, also called gate function. Another function is

segregation of membrane proteins and lipids from the apical surface to those in the basolateral membrane to maintain cell polarity, denominated fence function [16, 17]. TJ are formed by transmembrane proteins such as claudins, occludins and junction adhesion molecules (JAM), which are linked to the actin cytoskeleton by interactions with intracellular TJ adaptor proteins such as the *Zonula Occludens* (ZO).

AJ contribute to maintain adhesive force and proximity between adjacent epithelial cells. They are composed of the transmembrane proteins cadherins with E-cadherin being mainly expressed in the intestinal epithelium. E-cadherin directly interacts with the adaptor proteins p120-catenin and β-catenin. Meanwhile, β-catenin interacts with α-catenin, which connects the E-cadherin/catenin complex to the perijunctional actomyosin ring [10, 18, 19].

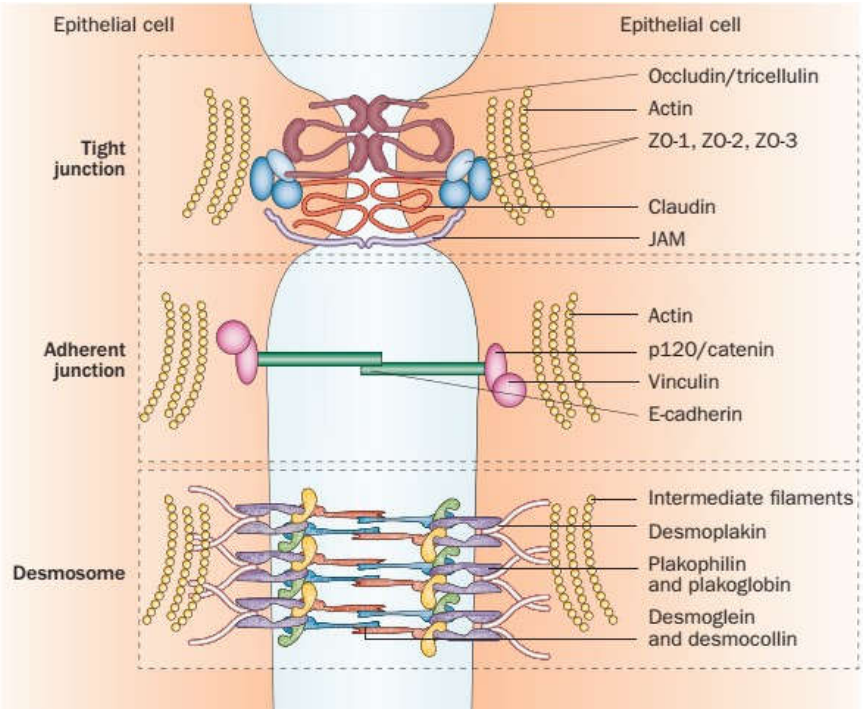

Figure 2. Molecular components of intercellular junctions. Intercellular junctions maintain epithelial cell polarity and permeability by controlled dynamic interactions between the diverse integral and peripheral proteins that compose these complexes [20].

Desmosomes provide strong intercellular adhesion, maintain cellular proximity and are also a site of intercellular communication. They are formed by the

transmembrane proteins desmocollin and desmoglein which in turn bind to plakoglobin forming the desmosomal plaque. Desmoplakin links this plaque to the intermediate filaments, providing the mechanical strength to maintain tissue integrity [21].

2.2.3 Lamina propria

Located beneath the epithelium is the lamina propria, a thin layer of loose connective tissue that contains blood and lymphatic vessels and cells of the immune system such as dendritic cells (DC), macrophages (mϕ), B-cells differentiated into plasmatic cells, regulatory T-cells (Tregs) and a low number of neutrophils. Resident immune cells and the epithelium together, maintain intestinal homeostasis [10, 22].

2.3 Intestinal homeostasis

The gastrointestinal tract is in constant contact with antigens, however, under healthy conditions, a delicate balance between the luminal microbiota, the epithelium and the resident immune system is maintained termed intestinal homeostasis [23]. To prevent an overreaction towards luminal bacteria and antigens, different mechanisms guarantee immune tolerance and intestinal homeostasis. This requires strict immune surveillance, and constant interaction and communication between intestinal epithelial cells, intestinal microbes and local cells of the immune system (Figure 3).

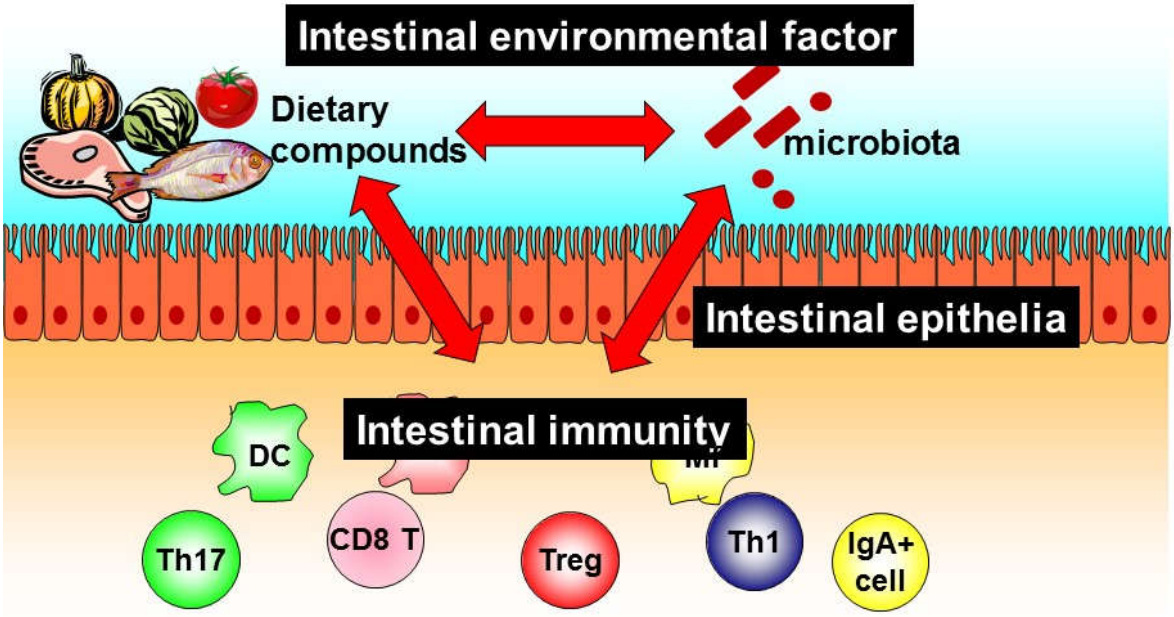

Figure 3. Intestinal homeostasis. To preserve intestinal homeostasis and thus a healthy colon, well-regulated interactions between the intestinal luminal environment, the epithelium and the intestinal immune system is necessary [24].

Epithelial cells contribute to the maintenance of intestinal homeostasis by secreting antimicrobial proteins such as defensins, cathelicidins and C-type lectins. Some of these antimicrobial peptides are expressed constitutively, others in response to the activation of pattern recognition receptors (PPRs) such as nucleotide-binding oligomerization domain-containing protein 2 (NOD2) and Toll-like receptors (TLRs) [25]. Specialized epithelial cells denominated M (Microfold) cells capture soluble antigens and transport them to the lamina propria to be sampled by residing DC or to be phagocytosed by mϕ [26].

DC are sentinel cells dispersed throughout the lamina propria and in lymphoid structures that can take up antigens through different routes. DC sample antigens transported by M cells, but they can also sample antigens directly from the intestinal lumen by protruding dendrites through the epithelial intercellular space, thus contributing directly to immune surveillance. Primed DC migrate to gut-associated

lymphoid tissue (GALT) and present antigens to naive T-cells. Primed T-cells in response express gut homing receptors including α4β7 integrin and CCR9, migrate to the lamina propria and reside there as long-lived effector memory T-cells to mount rapid and efficient immune responses to known pathogenic insults [27].

Intestinal mϕ are phagocytic cells residing in the lamina propria beneath the epithelium that contribute to tissue remodeling and help in senescent and apoptotic cell clearance. They produce PGE2 that stimulates the proliferation of epithelial progenitors at the intestinal crypts, thereby regulating the integrity of the epithelial barrier. Moreover, these phagocytes do not elicit a proinflammatory response when they encounter bacteria or their products, but constitutively produce IL-10 and low levels of TNF-α [28].

In the colon, B-cells are antibody-secreting cells differentiated as IgA-producing plasma cells [29]. Locally secreted IgA binds to polymeric Ig receptor (pIgR) expressed at the basolateral side of epithelial cells and is transported to the gut lumen [30]. Once in the lumen, IgA can prevent adhesion of commensal bacteria to epithelial cells by blocking bacterial epitopes, and reduce bacteria mobility by unspecific binding [31].

Tregs maintain immune tolerance by preventing abnormal responses to commensal bacteria, mainly by producing the anti-inflammatory cytokine IL-10 and transforming growth factor beta 1 (TGF-β), which inhibits Th1 responses and inflammatory T-cell differentiation [32].

2.4 Intestinal inflammation during IBD: Role of neutrophils

IBD is associated with intestinal barrier dysfunction [33]. Defects in the different components of the intestinal barrier allow the contact of luminal commensal bacteria with the epithelium, as well as bacterial translocation to the lamina propria due to increased epithelial permeability triggering a sustained inflammatory response. Epithelial cells become activated and release alarmins such as IL-25 that activate immune cells and initiate an immune response [34]. At the site of inflammation,

resident cells such as macrophages and dendritic cells are activated by pathogen-associated molecular patterns (PAMPs) and damage-associated molecular pattern molecules (DAMPs). In response, they release chemokines such as CXCL1 to induce the recruitment of neutrophils [35].

2.4.1 Migration into colon mucosa and lumen

To reach the site of inflammation, neutrophils migrate from the bloodstream following a complex, multistep process involving adhesive interactions of neutrophils with the vascular endothelium such as tethering, rolling, arrest, crawling and transmigration through the venular wall [36]. Once extravasated, neutrophils migrate through the interstitial tissue to reach the lamina propria. Some neutrophils even cross the intestinal epithelium and remain at the apical side of epithelial cells, where they phagocytose invading pathogens and finally detach and enter the intestinal lumen [37, 38].

Like transendothelial migration, neutrophil transepithelial migration (TEpM) is a multistep process involving sequential interactions with epithelial cells [39] (Figure 4). This process occurs in three stages: adhesion, migration and postmigration. Migrating neutrophils first interact with the basolateral surface of the epithelium in a Mac1 (CD11b/CD18)-dependent fashion. Basolaterally expressed epithelial ligands have not been identified, but proteoglycans containing fucose sugar moieties expressed on the epithelial surface have been implicated as ligands for the neutrophil β2-integrin Mac1. Following adhesion, neutrophils migrate through the lateral paracellular space exploiting interactions between CD47 (expressed on both neutrophils and epithelial cells) and signal regulatory protein α (Sirpα) expressed on neutrophils. Additionally, epithelial-expressed CAR binds to JAM-like molecule (JAM-L) expressed on neutrophils [40]. Once neutrophils reach the apical surface, CD11b/CD18 binds to ICAM-1, which is expressed at the apical surface of epithelial cells [41]. While ICAM-1 promotes retention of neutrophils at the apical side of epithelial cells, epithelial CD44v6 and CD55 mediate detachment of neutrophils into the intestinal lumen [42, 43]. A possible ligand for CD55 is the neutrophil receptor

CD97, however, the exact molecular mechanisms of neutrophil detachment still have to be elucidated [44].

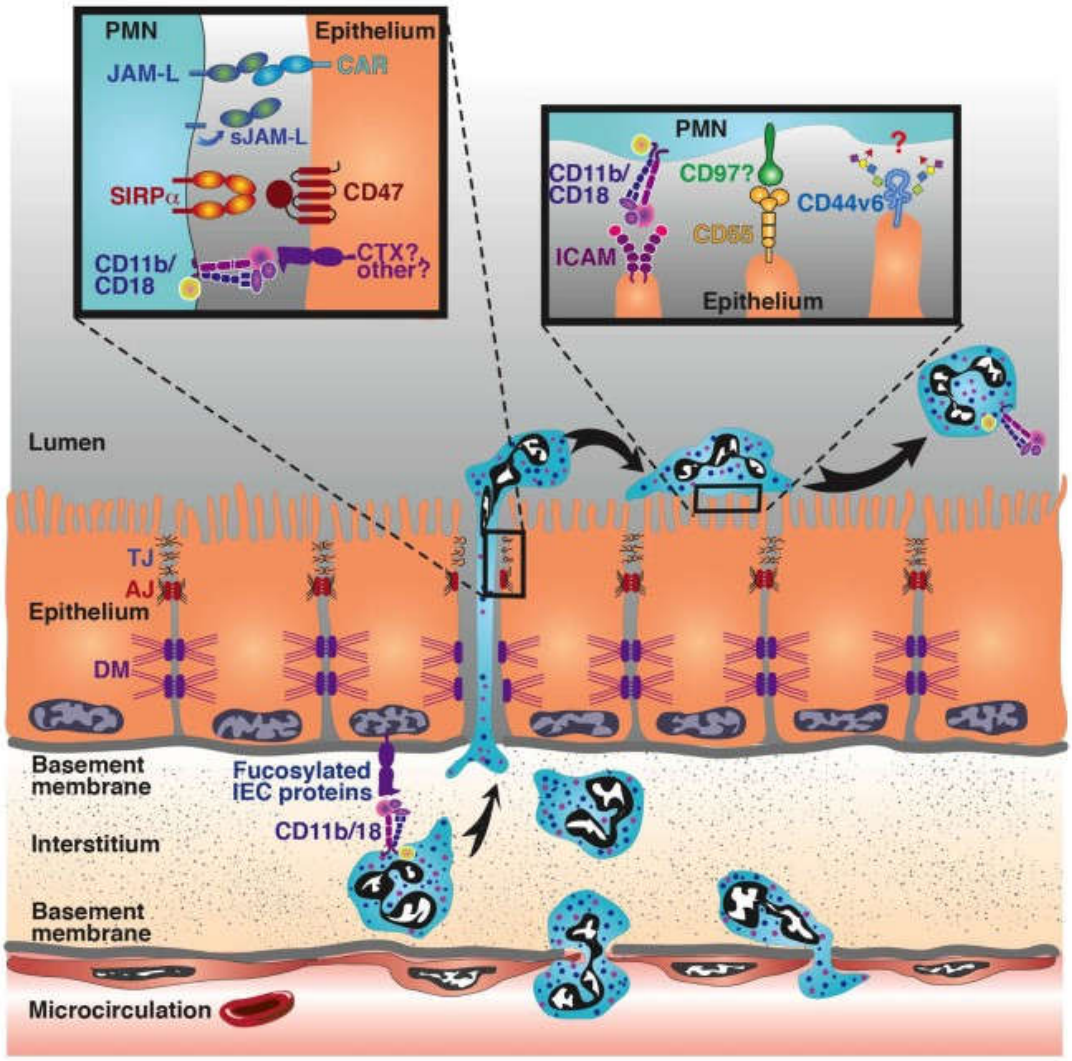

Figure 4. Stages of neutrophil migration from the bloodstream across the vascular endothelium followed by migration through the interstitium to reach the lamina propria and eventually neutrophil transepithelial migration [37]. The involved receptor-ligand pairs in each step are depicted.

2.4.2 The dual roles of neutrophils in IBD

Following migration to the site of inflammation, neutrophils exert different functions that aid in bacterial clearance such as phagocytosis, production of reactive oxygen species (ROS), release of lytic enzymes, and neutrophil extracellular traps (NETs) [45]. Once the infection is contained, neutrophils also aid in wound healing and resolution of inflammation by releasing the enzyme 5-lypoxigenase, which is involved in the production of beneficial molecules like lipoxin A4 and resolvin E1 that inhibit neutrophil recruitment and transepithelial migration [46, 47] and protectin D1 that promotes phagocytosis of apoptotic neutrophils [48].

If barrier function is not restored the inflammatory response becomes uncontrolled and leads to the development of chronic inflammation [33]. The release of alarmins by intestinal epithelial cells continues and more neutrophils are recruited to the colon mucosa [34]. Massive recruitment of neutrophils and activation during inflammation induce important changes in tissue integrity. For instance, high levels and prolonged exposure to ROS induce DNA damage, lipid peroxidation and protein oxidation, which damages the surrounding tissue, especially the epithelium resulting in more damage to the epithelial barrier [49].

While little neutrophil transepithelial migration is beneficial for inflammation resolution and does not disrupt barrier function, massive uncontrolled interaction between neutrophils with the basolateral side of the epithelium increases epithelial permeability in a myosin light-chain kinase (MLCK)-dependent manner without disrupting intercellular junctions [50]. However, neutrophil-derived proteases such as elastase cleave E-cadherin during TEpM to destabilize the apical junction complex leading to excessive epithelial permeability [51]. Moreover, during the early stage of TEpM, neutrophil-derived serine proteases such as elastase and proteinase-3 activate epithelial protease-activated receptors (PAR) 1 and 2 to increase epithelial barrier permeability, however, the exact mechanism is still unknown [52].

After massive TEpM, neutrophils accumulate in intestinal crypts forming abscesses which are a classic feature of active IBD. These neutrophils in crypt abscesses are

exposed to bacteria and diverse antigens resulting in their hyperactivation triggering crypt dysplasia and edema formation as a consequence of the release of adenosine monophosphate (AMP). AMP is converted to adenosine that binds to the epithelial A2B receptor stimulating electrogenic chloride transport and passive water flux, which is the main cause of diarrhea [39] (Figure 5).

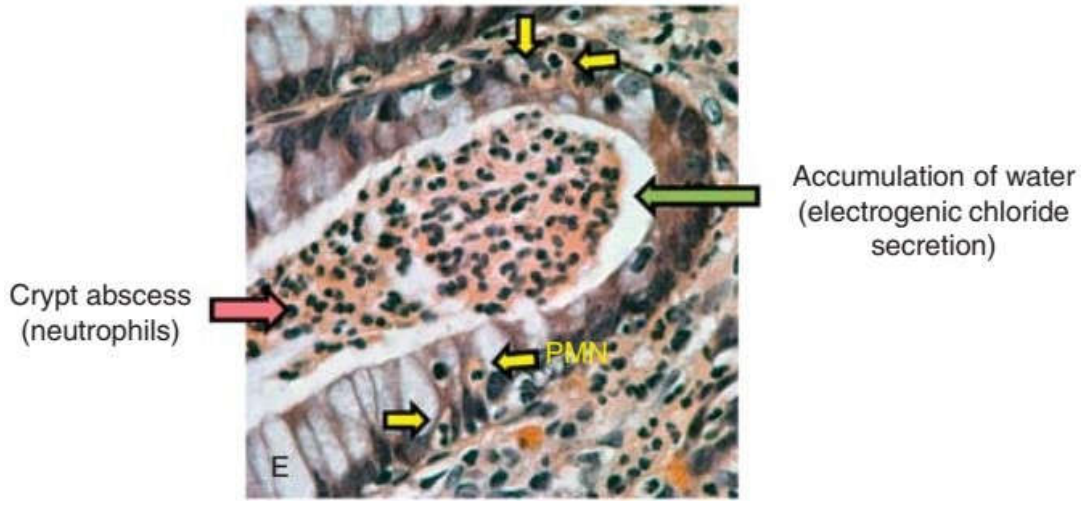

Figure 5. Transmigrating neutrophils (yellow arrows) crossing the epithelium to form a crypt abscess (red arrow). Edema (green arrow) is the result of stimulated electrogenic chloride transport and passive water flux in response to neutrophil-released adenosine monophosphate. Image adapted from [39].

Overall, neutrophils are important to combat infections, promote resolution of inflammation and wound healing, but they contribute to tissue damage if recruitment and activation is not controlled properly. Disruption of intestinal barrier functions due to excessive neutrophil TEpM allows the translocation of more luminal antigens exacerbating the immune response turning into a vicious feedback loop. Thus, it is important to better understand the mechanisms governing neutrophil recruitment to the mucosa and to find ways to reduce it during chronic inflammation.

2.5 Hematopoietic cell-specific lyn substrate 1 (HS1)

In order to (trans)-migrate, neutrophils require extensive cytoskeletal rearrangements regulated by ABP. HS1 is a multi-domain ABP specifically expressed in hematopoietic cells [53]. Its gene was mapped to human chromosome 3q13 [54] and chromosome 16 in mouse. HS1 has an N-terminal acidic (NTA) domain that binds the actin-related protein 2/3 (Arp2/3) complex and acts as a nucleation-promoting factor (NPF) [55]. This domain is followed by a 3.5 tandem repeats region and a coiled-coil region that bind to F-actin. The central region has a domain rich in prolines containing targets for phosphorylations, which regulate HS1 functions. The C-terminus contains an SH3 domain that can bind proteins like dynamin 2 and the Wiskott-Aldrich syndrome protein (WASP)/WASP-interacting protein (WIP) complex [56, 57] (Figure 6). HS1 is involved in the regulation of several processes in immune cells such as migration [58], chemotaxis [59, 60], cytotoxicity [59], antigen uptake and presentation [61], and receptor signaling [62] as described below.

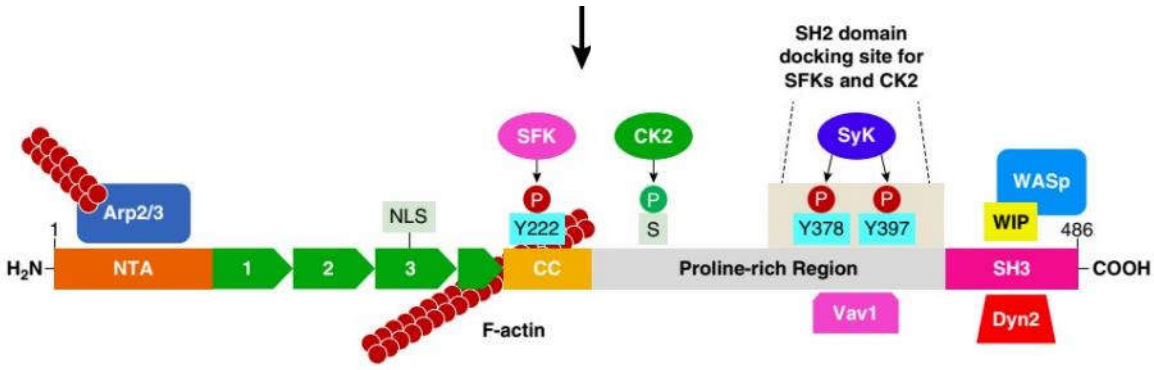

Figure 6. Domain organization of HS1. Abbreviations in alphabetical order: CC, coiled-coil region; CK2, casein kinase 2; Dyn2, Dynamin 2; F-actin, filamentous actin; NLS, nuclear localization signal; NTA, N-terminal acidic domain; SFKs, Src family kinases; SH2, SRC Homology 2 Domain; SH3, SRC Homology 3 Domain; Syk, spleen tyrosine kinase; VAV1, Vav Guanine Nucleotide Exchange Factor 1 ;WASP, Wiskott–Aldrich syndrome protein; WIP, WASP-interacting protein. Interaction partners are also depicted. Image adapted from [63].

2.5.1 HS1 role in immune cells

2.5.1.1 T-cells

The formation of the immunological synapse (IS) is essential for recognition of antigen-presenting cells (APCs) by T-cells. Upon TCR engagement, HS1 becomes phosphorylated and regulates actin filament assembly for the stabilization of the IS. During this process, HS1 becomes phosphorylated by Lck, Fyn, Lyn, ZAP70 and Abl [64-66]. These phosphorylations are required for the binding of HS1 to other proteins such as Vav1, PLCy1 and p85, and thus IS formation [65]. Actin dynamics during IS formation influence IL-2 production, a key cytokine for T-cell activation and proliferation [67]. HS1-deficient T-cells show significantly reduced IL-2 production and defective Ca^{2+} signaling [68]. T-cell chemotaxis is also affected in the absence of HS1. When T-cells are exposed to CXCL12, HS1 becomes phosphorylated allowing the formation of a SH2-domain docking site, where the protein non-catalytic region of tyrosine kinase (Nck) binds to HS1 to regulate actin polymerization during chemotaxis. In the absence of either HS1 or Nck, or both together, T-cells show reduced chemotaxis towards a CXCL12 gradient [69].

2.5.1.2 B-cells

Cross-linking of B-cell antigen receptors (BCR) induces B-cell activation [70]. As consequence, kinases such as Lyn and Spleen tyrosine kinase (Syk) associate with HS1 and phosphorylate it in a sequential manner. First, Syk phosphorylates the sites Y378 and Y397 [71], then Lyn can associate with HS1 for further phosphorylation [72]. Once HS1 is hyperphosphorylated, it translocates to the nucleus where it is thought to regulate B-cell apoptosis [71]. In the absence of HS1, B-cells present defective BCR-mediated apoptosis [73]. Unlike T-cells, absence of HS1 does not seem to affect B-cell chemotactic migration towards a CXCL12 gradient but influences spontaneous migration [74].

2.5.1.3 Natural killer cells

In natural killer (NK) cells, HS1 is essential for many functions. During the formation of the lytic synapse, HS1 localizes at the cell-cell contact site and becomes phosphorylated. The cytolytic capacity of NK cells depends on HS1 phosphorylation at Y387 and is significantly reduced in HS1 knock-down NK cells [59]. Activation of adhesion molecules such as LFA-1 and ICAM-1 is required in transendothelial migration (TEM) of NK cells. In the absence of HS1, the conversion of LFA-1 from low to the high affinity form is impaired and reduces binding to ICAM-1. Moreover, NK cell chemotaxis is impaired in HS1-depleted cells due to reduced recruitment and activation of Vav1, Cdc42 and Rac1 [59]. Phosphorylation of HS1 is required in all these processes, with Y397 phosphorylation participating in most of them, whereas Y378 is only required for NK cell chemotaxis [58, 59]. Transendothelial migration of NK cells is also affected in the absence of HS1, however only transcellular migration is partially reduced in comparison to WT NK cells [58].

2.5.1.4 Dendritic dells

Dendritic cells from HS1-KO mice show deficiencies in podosome array organization. Interestingly, HS1 deficiency increases migration velocity, but persistent directional migration during chemotaxis is reduced [56]. Furthermore, HS1 has been found to be necessary for the uptake, processing and presentation of protein antigens, but only if these antigens are taken up by receptor-mediated endocytosis [61].

2.5.1.5 Neutrophils

HS1 has been found to localize at the cell periphery and relocalize to the leading edge of fMLP-stimulated primary neutrophils [60]. In HS1-deficient PLB-985 cells, random motility is not affected, whereas chemotaxis is impaired and requires phosphorylation of Y222, Y378 and Y397 for interaction with the Arp2/3 complex. Moreover, activation of Rac2 and its GEF Vav1 induced by fMLP stimulation is reduced in HS1-deficient PLB-985 cells since HS1 interacts directly with Vav1 when

HS1 is phosphorylated in Y378 and Y397. [60]. Additionally, transendothelial migration of neutrophils is reduced in HS1-KO mice due to defects in firm adhesion as a consequence of defective chemokine-induced LFA-1 activation regulated by Rap1 and Rac1 [75]. The role of HS1 in neutrophil functions has also been analyzed *in-vitro*. HS1-deficient neutrophils are less efficient in phagocyting opsonized zymosan, but not non-opsonized zymosan suggesting that HS1 regulates FcR-mediated phagocytosis. Interestingly, absence of HS1 does not impair ROS production (unpublished data from our group).

2.5.2 HS1 in inflammatory disorders

Although much information has been published in recent years about the role of HS1 in immune cells *in vitro*, little is known about the effects of HS1 deficiency during inflammation *in vivo*.

2.5.2.1 Peritonitis

Peritonitis is an inflammation of the peritoneum, a thin semipermeable membrane that lines the abdominal cavity. This condition occurs when bacteria reach the peritoneum due to contamination by gastrointestinal or genitourinary microorganisms triggering an immune response to control the infection [76]. Resident immune cells are the first to respond leading to the recruitment of numerous neutrophils to eliminate pathogens and induce inflammation resolution [77]. In a murine model of peritonitis, absence of HS1 resulted in significantly reduced neutrophil recruitment to the peritoneal cavity (Figure 7) [75]. The β2-integrin LFA-1 (CD11a/CD18) is essential for neutrophil transendothelial migration to the peritoneum [78]. However, no significant difference was observed between LFA-1 surface levels of HS1-deficient and WT neutrophils; and LFA-1 activation was not analyzed. Instead, activation of the small GTPases Rap1 and Rac1, required for efficient LFA-1 activation, was significantly reduced without HS1 and binding to soluble ICAM-1 was as well deficient.

Without a proper anti-inflammatory response for pathogen clearance and resolution of inflammation, peritonitis can lead to sepsis [76].

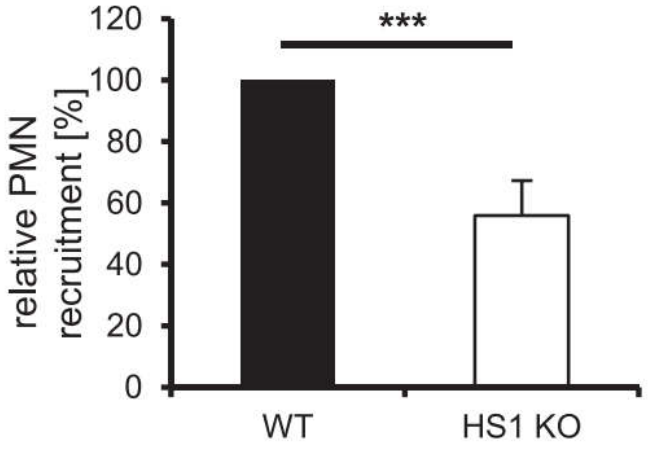

Figure 7. Neutrophil recruitment to the peritoneum in WT and HS1 KO mice, 4 h after 3% thioglycolate injection. Results are displayed as means ± SD from 8 WT and 7 HS1 KO mice. Image taken from [75].

2.5.2.2 Sepsis

Sepsis is the consequence of a dysregulated immune response to a severe infection, characterized by systemic inflammation and tissue injury leading to multiorgan failure and death [79]. The lung is one of the organs mainly affected by sepsis [80]. Like in UC, neutrophils contribute to lung tissue damage during sepsis. Activated neutrophils form clusters inside the pulmonary vasculature, thus blocking blood circulation and leading to the formation of necrotic tissue. Additionally, neutrophils undergo apoptosis and release reactive oxygen species (ROS), NETs and elastase further damaging the surrounding tissue [81].

In our group, we have found that HS1 deficiency improves survival in a murine model of lethal sepsis. More specifically, sepsis-induced lung damage characterized by edema formation, blood vessel congestion, alveoli wall thickening, alveoli collapse and fibrosis was ameliorated in HS1-KO mice compared to WT (unpublished data).

Moreover, we observed by intravital microscopy of the cremaster muscle that leukocyte adhesion and extravasation was reduced in septic cremaster venules of HS1-KO mice compared to septic WT mice in the absence of a local inflammatory

stimulus (Figure 8). Thus, reduced neutrophil recruitment in the absence of HS1 contributes to tissue protection during inflammatory diseases.

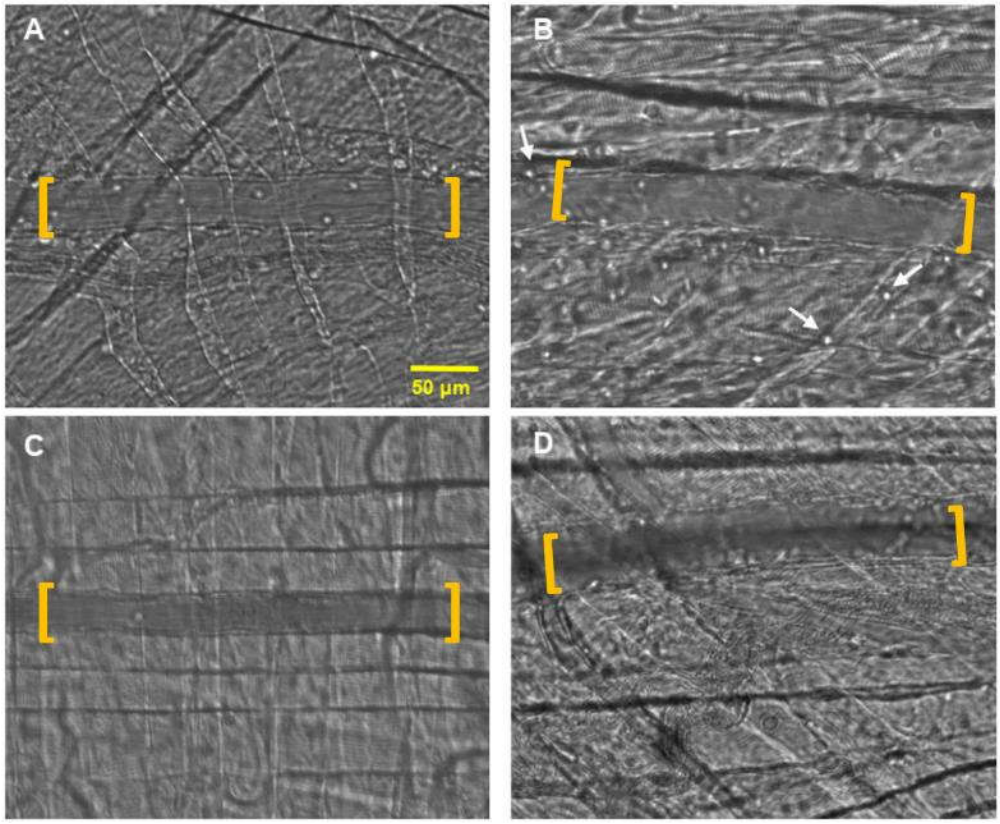

Figure 8. Representative images from cremaster muscle blood vessels. Images show a blood vessel enclosed in yellow brackets. Arrows point at transmigrated neutrophils. (A) WT sham controls (B) WT CLP (C) HS1 KO sham and (D) HS1 KO CLP.

Clearly, neutrophils play an important role in the pathophysiology of inflammatory disorders such as peritonitis and sepsis, and neutrophil recruitment is reduced in the absence of HS1. However, the role of HS1 in IBD pathogenesis remains elusive. Thus, it will be important to determine whether the absence of HS1 modulates neutrophil recruitment, transepithelial migration and disease severity in an experimental colitis model.

3. PROBLEM STATEMENT AND JUSTIFICATION

During chronic intestinal inflammation, exacerbated neutrophil infiltration into the lamina propria occurs. While controlled neutrophil recruitment is beneficial to combat infection and inflammation, excessive neutrophil presence leads to an overwhelming release of reactive oxygen species (ROS), myeloperoxidase (MPO), metalloproteases (MMP-8 and MMP-9), elastase and other effector proteins causing mucosal injury and increased barrier permeability. HS1 is an ABP involved in neutrophil chemotaxis and its deficiency reduces neutrophil recruitment to the sites of inflammation. Thus, it will be important to study the effects of HS1 deficiency on neutrophil recruitment to the lamina propria and transepithelial migration during colitis.

4. HYPOTHESIS

HS1 is involved in the development of intestinal inflammation by regulating neutrophil recruitment and transepithelial migration that in turn affects intestinal epithelial barrier functions.

5. GENERAL OBJECTIVE

To investigate the role of HS1 in neutrophil recruitment and the regulation of intestinal epithelial barrier functions during experimental colitis.

6. PARTICULAR OBJECTIVES

1. To determine the susceptibility of HS1-KO mice to experimental colitis.
2. To analyze tissue morphology and oxidative stress during colitis in HS1-KO mice
3. To evaluate intestinal epithelial barrier functions in HS1-KO mice during basal and colitic conditions.
4. To investigate the effects of HS1 deficiency on neutrophil recruitment and transepithelial migration in vivo.
5. To characterize the expression and localization of HS1 in tissue biopsies of UC patients.

7. MATERIALS AND METHODS

7.1 Materials and reagents

Material/Reagent	Company
Dextran Sulfate Sodium (M.W. 40,000)	Carbosynth
ColoScreen	Helena Laboratories
Ketamine	PiSA Agropecuaria
Xylazine	PiSA Agropecuaria
Histosette (Embedding cassette)	Simport
Liquid paraffin	Paraplast
Plastic cubes, square mold, size: 22x22x22 mm	Electron Microscopy Sciences
Tissue-tek. (O.C.T.)	Sakura Finetek
Absolute xylene	J.T. Baker
Eosin-Y	J.T. Baker
Harris hematoxylin	Sigma-Aldrich
Lithium carbonate	Sigma-Aldrich
Synthetic resin	Poly Mont
Hydrochloric acid 1 M	J.T. Baker
10% formaldehyde	J.T. Baker
FD4 – FITC-Dextran (3000-5000 dalton)	Sigma-Aldrich
BD Microtainer® blood collection tubes	BD
Absolute ethanol	J.T. Baker
Tween 20	Sigma-Aldrich
Bovine Serum Albumin	Sigma-Aldrich
Dihydroethidium	ThermoFisher Scientific
ProLong® Gold	ThermoFisher Scientific
DC Protein Assay	BioRad
Nitrocellulose membrane, pore 0.45 µm	BioRad

SuperSignal® West Pico	ThermoFisher Scientific
SuperSignal® West Femto	ThermoFisher Scientific
TNF-α	PeproTech
IFN-γ	PeproTech
fMLP	Sigma-Aldrich
EDTA	Sigma-Aldrich
DTT	Fisher BioReagents
Paraformaldehyde	Millipore Sigma
Fetal Bovine Serum	By-productos
Collagenase C2139	Sigma-Aldrich
DNase I	Roche
Non-sterile CellTrics filters 50 µm	Sysmex
Falcon Cell Strainers 100 µm	Corning Life Sciences

7.1.1 Buffers

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

PBS	138 mM NaCl 3 mM KCl 8.1 mM Na2HPO4 1.5 mM KH2PO4
PBS-T (1L)	100 ml 10x PBS 0.05% Tween20
Ca/Mg Free HBSS	5.3 mM KCl 0.44 mM KH2PO4 138 mM NaCl 4 mM NaHCO3 0.30 mM Na2HPO4

	5.6 mM Glucose
IF Blocking buffer	PBS-T 2% BSA
RIPA	50 mM TrisHCl pH7.4 150 mM NaCl 2 mM EDTA 1% NP-40 0.1% SDS
5X SDS Loading Buffer	250 mM Tris-HCl pH6.8 10%SDS 30% glycerol 5% β-mercaptoethanol 0.02% bromophenol blue
SDS-page Buffer	25 mM Tris 192 mM glycine 0.1% SDS pH 8.3
Transfer Buffer	20% methanol 25 mM Tris 192 mM glycine pH 8.3
TBS	150 mM NaCl 10 mM Tris pH 8.0
TBS-T (1L)	100ml 10X TBS 0.1% Tween20
Blocking Buffer	TBS-T 5% Skim milk

7.1.2 Antibodies

Primary antibodies:

Antigen	Source	Company	Catalog number
HS1 (D5A9)-rodent	Rabbit	Cell-signaling	#3892
HS1 (D83A8)-human	Rabbit	Cell-signaling	#3890
ZO-1	Rabbit	Invitrogen	#40-2200
Claudin 1	Rabbit	Thermo Fisher Scientific	#71-7800
Claudin 2	Rabbit	Thermo Fisher Scientific	# PA5-13334
γ-Tubulin	Mouse	Sigma-Aldrich	#T6557
Gr-1	Rat	Hybridoma	

Secondary antibodies:

Antigen	Source	Company	Catalog number
anti-mouse IgG-HRP	Goat	Santa Cruz	sc-2005
anti-rabbit IgG-HRP	Goat	Santa Cruz	Sc-2004
Alexa Fluor 488 anti-rabbit IgG (H+L)	Goat	Invitrogen	A11008
Alexa Fluor 568 anti-rabbit IgG (H+L)	Goat	Invitrogen	A11061

7.1.3 Equipment

Equipment	Company
Infinite 200 PRO Microplate reader	Tecan Life Sciences
SPE Confocal Microscope	Leica
Cryostat	Leica
Orbital Shaker-Incubator	Daigger
Polytron homogenizer	OMNI International Tissue Master 125
ChemiDoc MP System	BioRad

BD FACSCanto II	BD Biosciences
TCS SPE Confocal Microscope	Leica

7.2 Animals

HS1-KO mice were kindly donated by Dr. Klemens Rottner, TU Braunschweig, Germany [73]. Male HS1-KO and WT littermates on a C57Bl/6 genetic background, aged 6-8 weeks, weighing 20-25g were obtained from the barrier-type animal facility at CINVESTAV. They were provided with a standard pellet diet and water ad libitum. All animal experiments have been approved by the institutional animal care and use committee of Cinvestav.

7.3 Methods

7.3.1 Induction of colitis

Acute colitis was induced by administration of 5.0% w/v DSS in drinking water *ad libitum* for 5 or 7 days. Control animals received normal drinking water.

7.3.2 Assessment of disease severity

Evaluation of the clinical course of colitis development was determined using a disease activity index consisting of three parameters: weight loss (calculated as the difference in percent between baseline weight and actual weight), stool consistency (determined by observing a fresh stool sample according to the criteria in table 2) and intestinal bleeding (occult blood was detected using the guaiac slide test ColoScreen) [82]. For each parameter, a score of 0-4 was given, the maximum disease index is 12 (Table 2).

Table 2. Disease activity index score system.

SCORE	WEIGHT LOSS %	STOOL CONSISTENCY	INTESTINAL BLEEDING
0	None	Normal	None
1	1 – 5		
2	5 – 10	Pasty stools	Occult bleeding
3	10 – 20		
4	>20	Diarrhea	Gross Bleeding

7.3.3 Tissue collection

Animals were euthanized by cervical dislocation. An abdominal midline incision was made to remove the colon; and its weight and length were recorded. Then, the colon was carefully flushed with chilled PBS to remove feces and prepared for subsequent experiments:

For histopathological analysis, the colon was opened longitudinally and rolled to form “Swiss rolls” [83]. The roll was placed inside an embedding cassette and submerged in 5 ml of 10% formaldehyde for 48 h at room temperature (RT) for fixation. Subsequently, the fixed tissue was immersed in liquid paraffin in plastic cubes and stored at RT until further use.

For immunofluorescence staining, the colon was rolled to form a “Swiss roll” and placed inside an aluminum cup (~1.5 cm^3) filled with optimal cutting temperature compound (OTC) and frozen at -80°C until further use.

For protein isolation, ~100mg of colon sample was placed inside a tube, snap-frozen in liquid nitrogen and stored at -80°C until further use.

7.3.4 Histopathological analysis

Paraffin sections were stained with hematoxylin and eosin using standard protocols [84]. Briefly, colon samples were cut using a microtome at a thickness of 5 µm and then mounted on glass slides. Samples were deparaffinized and then incubated with Harris hematoxylin, followed by lithium carbonate and Eosin-Y. Synthetic resin was applied to the colon tissue samples and then they were covered with coverslips. Images were taken using a bright-field microscope with 40x magnification to determine tissue damage.

Histological inflammation score was determined by a pathologist in a blinded fashion considering the inflammatory cell infiltrate and intestinal architecture (Table 3) [85]. The score of both parameters were added and represented as the total score.

Table 3. Calculation of histological inflammation score [85].

INFLAMMATORY CELL INFILTRATE			**INTESTINAL ARCHITECTURE**		
Severity	**Extent**	**Score 1**	**Epithelial changes**	**Mucosal architecture**	**Score 2**
Mild	Mucosa	1	Focal erosions		1
Moderate	Mucosa and submucosa	2	Erosions	± Focal ulcerations	2
Marked	Transmural	3		Extended ulcerations ± granulation tissue ± pseudopolyps	3
			Total score: Sum of scores 1 and 2		0-6

7.3.5 FITC-Dextran permeability assay

Healthy and colitic mice were starved for 6 h, gavaged with 150 µL/mouse of 80 mg/mL 3000-5000 Dalton FITC-dextran in sterile 1x PBS [86]. Blood was collected by cardiac puncture 4 h post-gavage and kept in BD Microtainer plasma separating tubes for 30 minutes in the dark at RT for plasma separation. Then tubes were

centrifuged for 5 min at 10,000 rcf at 20°C. Plasma was transferred to another tube and FITC-dextran fluorescence was determined after diluting samples 1:10 with PBS 1X and placing them in a 96-well plate in triplicates. Relative fluorescence units were measured using a Tecan spectrophotometer at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. To determine relative fluorescent units, PBS blank fluorescence was subtracted from samples. Permeability is expressed as relative fluorescence units between the experimental groups being compared.

7.3.6 Immunofluorescence (IF)

Frozen tissue sections (8 µm thick) were mounted on glass slides, fixed and permeabilized with 96% ethanol for 30 minutes at -20°C, rinsed with PBS 1X, and blocked overnight with PBS-Tween 0.01% containing 2% BSA. Primary antibodies were incubated overnight at 4°C, slides were rinsed again with PBS-0.01% Tween and MilliQ water, and then incubated with species-specific secondary antibodies labelled with the fluorochrome Alexa Fluor 488 or 568 at RT for 2 hours. Slides were preserved using ProLong Gold Antifade Mounting Medium containing DAPI and analyzed on a Leica TCS SPE confocal microscope with 40x and 63x magnification.

7.3.7 Tissue lysate preparation for western blot

Approximately 100mg of tissue or isolated mouse intestinal epithelial cells were homogenized using a Polytron homogenizer in 500 µl of ice cold RIPA, then samples were centrifuged for 30 min at 15,000 rpm at 4°C. Supernatants were transferred to fresh tubes and 1X SDS loading buffer was added to the sample and boiled at 100°C for 5 min.

7.3.8 Western blot

Tissue lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes (pore size 0.45 µm). Membranes were blocked with TBS-0.1% Tween containing 5% skim milk for 1 hour at RT, followed by overnight incubation with primary antibodies at 4°C. After incubation, membranes were washed and incubated with species-specific secondary antibodies labelled with HRP, incubated with Super-West Pico substrate and observed on a ChemiDoc device (BioRad).

7.3.9 Fluorescence microscopy of oxidative stress

Fresh colon cryosections mounted on glass slides were incubated with 5 µM dihydroethidium (DHE, Life Technologies, Grand Island, NY) in water at 37°C for 30 min in the dark as previously described [87]. Fluorescence of oxidized ethidium was determined on a Leica TCS SPE confocal microscope with 40x magnification.

7.3.10 Leukocyte transepithelial migration *in vivo*

Mice were treated by intraperitoneal injection of TNF-α (100 ng; Peprotech, Mexico) and IFN-γ (100 ng; Peprotech, Mexico) in 200 µl of sterile 0.9% saline solution or only 200 µl of sterile 0.9% saline solution [88]. 24hrs after treatment, mice were anesthetized with ketamine/xylazine (100 mg/kg and 13 mg/kg of body weight, respectively) in 0.9% saline solution via intraperitoneal injection. A laparotomy was performed to exteriorize the caecum and proximal colon. Fecal content was removed by making a small incision in the proximal colon below the caecum where a canula was inserted and secured with a simple ligature. Warm HBSS was carefully flushed until the wash out was clear. A proximal colon segment of 2 cm length was isolated (pcLoop) by making one small incision in the mesocolon right below the caecum and ligated, the second incision and ligation was made 2 cm distally from the first incision. To induce the recruitment of neutrophils to the intestinal lumen, 200 µl of sterile HBSS+ containing 1 µM fMLP was injected intraluminally using a 30-gauge needle; control mice received an intraluminal injection of saline solution. The colon was reinserted into the abdominal cavity and the abdomen was closed with a

double suture. After 60 min the abdomen was reopened, the pcLoop was excised and placed on a clean petri dish on ice. Luminal content was recovered by flushing the pcLoop with 500 µl cold HBSS containing 2mM EDTA followed by 500 µl cold HBSS containing 5 mM 1,4-dithiothreitol (DTT). Luminal exudates were filtered through a 50 µm nylon mesh cell strainer, collected in 5 ml round-bottom tubes and then centrifuged at 1500 rpm for 5 min at 4°C. Supernatants were discarded, and cells were resuspended in 100 µl of PBS 1X for flow cytometry staining.

7.3.11 Isolation of epithelial and immune cells from the lamina propria by enzymatic digestion

Colons from healthy and colitic mice were washed in Ca/Mg-free HBSS at RT [89]. Each colon was cut into approximately 1.5 cm pieces and placed into separate 50mL tubes (1 colon per tube) containing 30 mL pre-warmed Ca/Mg-free HBSS with 5% FBS and 2mM EDTA. Tubes were placed in an orbital shaker at 250 rpm for 20 min at 37°C. Then, the content was filtered using a mesh wire strainer to recover the 1.5 cm colon pieces and epithelial enriched fraction. Colon pieces were placed again inside a 50 ml tube with 30 ml fresh pre-warmed Ca/Mg-free HBSS + 5% FBS and 2mM EDTA in an orbital shaker (this step was repeated 2 times).

For the epithelial enriched fraction: 2mM EDTA was added and cells were centrifuged at 1500 rpm for 5 minutes at 4°C, washed with Ca/Mg-free HBSS and snap frozen in liquid nitrogen and lysed for Western Blot analysis as described in chapter 7.3.7.

For the immune cells enriched fraction: colon pieces were recovered and minced into smaller pieces, then placed in 20 ml of collagenase solution in an orbital shaker at 200 rpm for 15 min at 37°C. Colon pieces were filtered through a 100 µm cell strainer into a 50 ml tube containing 50 ml of cold Ca/Mg-free HBSS and 5% FBS and the cell suspension was centrifuged at 1500 rpm for 5 min at 4°C. The supernatant was removed, and the pellet resuspended in 100 µl ice-cold Ca/Mg-free HBSS containing 5% FBS for flow cytometry staining.

7.3.12 Flow cytometry staining

An equal number of cells isolated either from the intestinal lumen were blocked using anti-CD16/CD32 mAb for 15 min. Then, cells were co-stained using the antibodies listed in Table 4 for 15 min and fixed with 200 µl PFA 4% for 20 min. Data were acquired on a BD FACSCanto II flow cytometer and data analysis was performed using FlowJo V10 software.

Table 4. Antibodies for flow cytometry analysis

Marker	Fluorochrome
Anti-CD45	Pacific Blue
Anti-CD11b	PE
Anti-Gr1	APC/Cy7
Anti-Ly6G	APC/Cy7
Anti-CD170	APC
Anti-F4/80	PE/Cy5

7.3.13 Human tissue samples

Human tissue samples were kindly provided by Dr. Nicolas Schlegel, Department of Surgery I, University Hospital, Würzburg, Germany. Samples were obtained from UC patients or colon carcinoma patients (resection margins used as control) (Table 5). The study was approved by the ethical committee of the University of Würzburg (proposal numbers 113/13, 46/11, 42/16) [90].

For Western Blot, tissues were snap frozen in liquid nitrogen and lysed in RIPA buffer as described above.

For Immunofluorescence, tissues were fixed in 4% PFA, embedded in paraffin, sectioned (1µm) and stained for immunofluorescence as described above after removal of paraffin [91].

Table 5. Human tissue samples characteristics.

IDENTIFICATION	SEX	AGE	MEDICATION	HISTOLOGY	LOCATION
CONTROL 1	F	72	none	Colon ascendens carcinoma	Colon ascendens (resection margin)
CONTROL 2	F	60	none	Colon ascendens carcinoma	Colon ascendens (resection margin)
CONTROL 3	M	82	none	Colon ascendens carcinoma	Colon ascendens (resection margin)
CONTROL 4	M	49	none	High grade intra-epithelial neoplasia coecum	Colon ascendens (resection margin)
CONTROL 5	M	76	none	Colon ascendens carcinoma	Colon ascendens (resection margin)
CONTROL 6	M	72	none	Rectal cancer	Colon descendens (resection margin)
UC 1	M	53	Infliximab, prednisolone	Active ulcerative colitis	Colon descendens
UC 2	M	62	none	Colitis associated carcinoma colon descendens, active ulcerative colitis	Colon ascendens
UC 3	M	54	Budenoside, mesalazine	Active ulcerative colitis, neuroendocrine tumor rectum	Rectum (resection margin)
UC 4	M	41	Vedolizumab	Active ulcerative colitis	Colon sigmoideum
UC 5	M	53	Adalimumab, prednisolone, mesalazine	Active ulcerative colitis	Colon descendens
UC 6	M	59	Adalimumab, prednisolone	Chronic ulcerative colitis	Colon descendens

8. RESULTS

8.1 HS1-KO mice are less susceptible to experimental colitis

To determine experimental colitis susceptibility in HS1-KO mice, WT and HS1-KO mice were exposed to DSS for 7 days. To determine the clinical progress of colitis, the disease activity index (DAI) consisting of weight loss percentage, intestinal bleeding and stool consistency was monitored daily (Figure 9A). During DSS-colitis development, WT mice gradually increased weight loss (Figure 9B), intestinal bleeding (Figure 9C) and presence of occult blood in feces until gross bleeding was observed (Figure 9D). Importantly, all DAI parameters, i.e. weight loss, intestinal bleeding and changes in stool consistency were significantly ameliorated in HS1-KO mice with strongest protective effects after day 5. Moreover, these mice showed a slight improvement in stool consistency and intestinal bleeding at the end of the experiment.

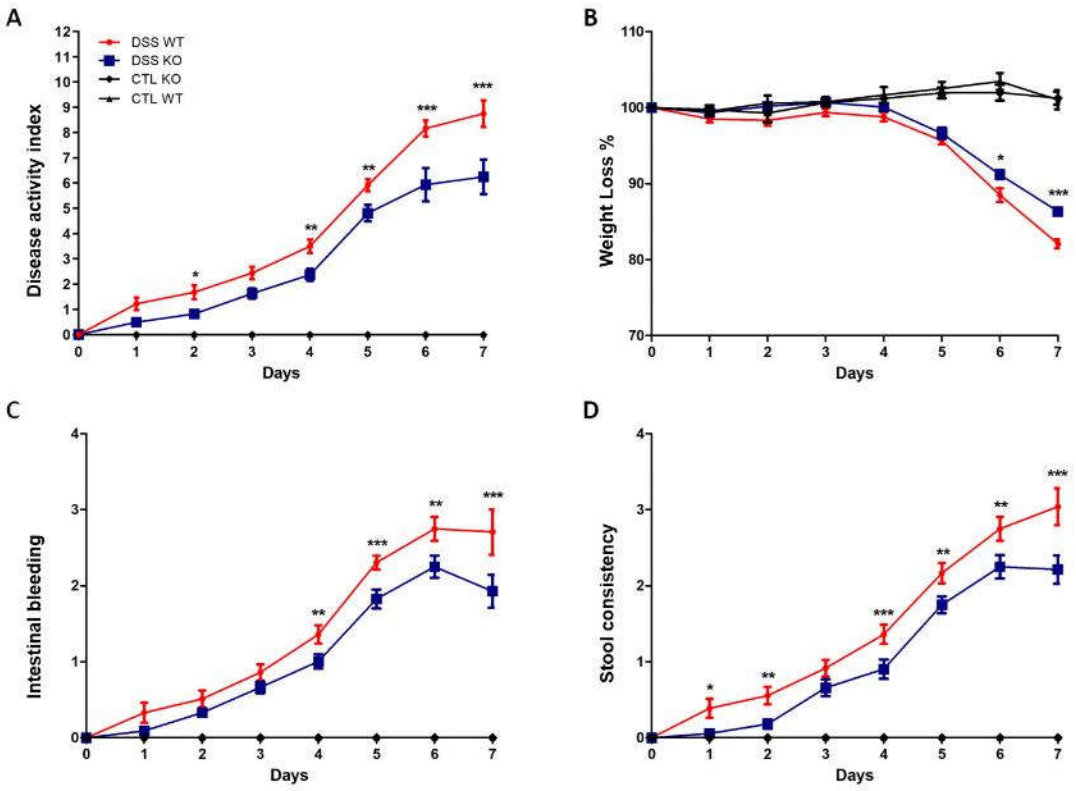

Figure 9. DSS-colitis is ameliorated in HS1-KO mice. (A) Disease activity index consisting of the daily determination of 3 parameters: **(B)** Weight loss percentage, **(C)** Intestinal bleeding and **(D)** stool consistency. Control WT (n=8) and KO (n=7) groups received water and maintained a score of 0.

DAI of colitic WT (n=18) and KO (n=26) increased gradually over time. However, KO-mice developed significantly less colitis signs compared to the WT. Values are displayed as mean±standard deviation of the mean (SDM). ***p < 0.001, **p < 0.01 and *p< 0.05. DSS WT vs DSS KO.

We also analyzed whether HS1 is involved in the healing process using a DSS recovery phase model. After DSS treatment for 5 days, mice received normal drinking water and were allowed to recover for 5 days (Figure 10A). WT mice slowly recovered, however 50% of the group died after day 7 (Figure 10B), whereas HS1-KO mice recovered faster and the complete group survived until day 10. Stool consistency and intestinal bleeding in HS1-KO mice completely recovered to control levels; and weight gain improved but did not yet reach control levels after day 10 (data not shown).

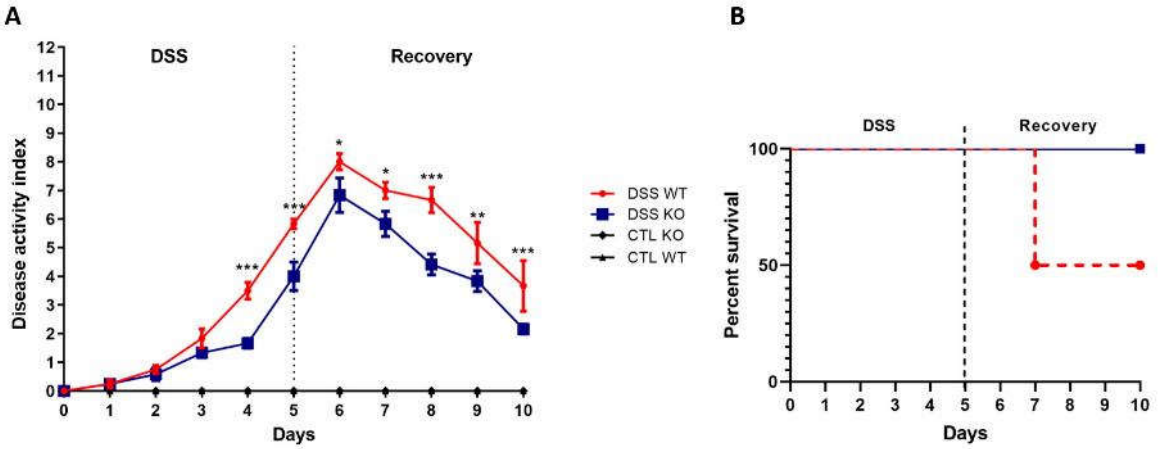

Figure 10. HS1-KO mice recovered faster after DSS-colitis. DSS was administered for 5 days; then mice received normal water to allow recovery. **A)** Control WT (n=3) and KO (n=3) groups received water and maintained a score of 0. DAI of colitic WT (n=4) and KO (n=4) increased gradually over time and decreased once the administration of DSS was stopped. **B)** 50% of the WT colitic group died after day 7, whereas all the HS1-KO mice survived. Values are displayed as mean±standard deviation of the mean (SDM). ***p < 0.001, **p < 0.01 and *p< 0.05. DSS WT vs DSS KO.

8.2 DSS-induced tissue damage and oxidative stress is ameliorated in HS1-KO mice

To analyze in more detail the underlying mechanism of how HS1 deficiency is conferring this protective effect, we first analyzed colon histology by hematoxylin and eosin stainings of swiss rolls. Normal morphology was observed in control WT and HS1-KO mice that both presented intact surface epithelium, colon crypts, lamina propria and muscularis mucosa; and showed similar numbers of goblet cells (Figure 11). DSS-colitis in WT mice induced surface epithelium erosion, loss of intestinal crypts, absence of goblet cells, infiltration of leukocytes into the lamina propria and edema formation as expected (Figure 11). However, in HS1-KO colitic mice surface epithelium was still observed, intestinal crypt depletion was significantly reduced and goblet cells were still present. Leukocyte infiltration into the lamina propria was visibly reduced in HS1-KO mice and in most cases edema formation was not observed (Figure 11). A histological inflammation score was determined by a pathologist in a blinded fashion considering extent of inflammatory, immune cell infiltrates and changes in intestinal architecture [85]. In the absence of HS1, the extent of inflammatory cell infiltration and damage to the intestinal architecture was significantly reduced by 36.54% during DSS colitis (Table 6).

Table 6. Histological inflammation score. Hematoxylin and eosin stainings of 3 independent tissue preparations were analyzed for the indicated parameters. Scores of control WT, control KO, DSS WT and DSS KO groups are shown. Values are means ± SE. **p<0.01.

	CTL WT	CTL KO	DSS WT	DSS KO	p VALUE DSS WT vs DSS KO
EXTENT OF INFLAMMATORY CELL INFILTRATE	0.50 ± 0.35	0.75 ± 0.32	2.91 ± 0.08	1.83 ± 0.27	**
INTESTINAL ARCHITECTURE	0.43 ± 0.25	0.37 ± 0.23	3 ± 0	1.97 ± 0.23	**
TOTAL SCORE	0.93 ± 0.48	1.12 ± 0.55	5.91 ± 0.08	3.75 ± 0.42	**

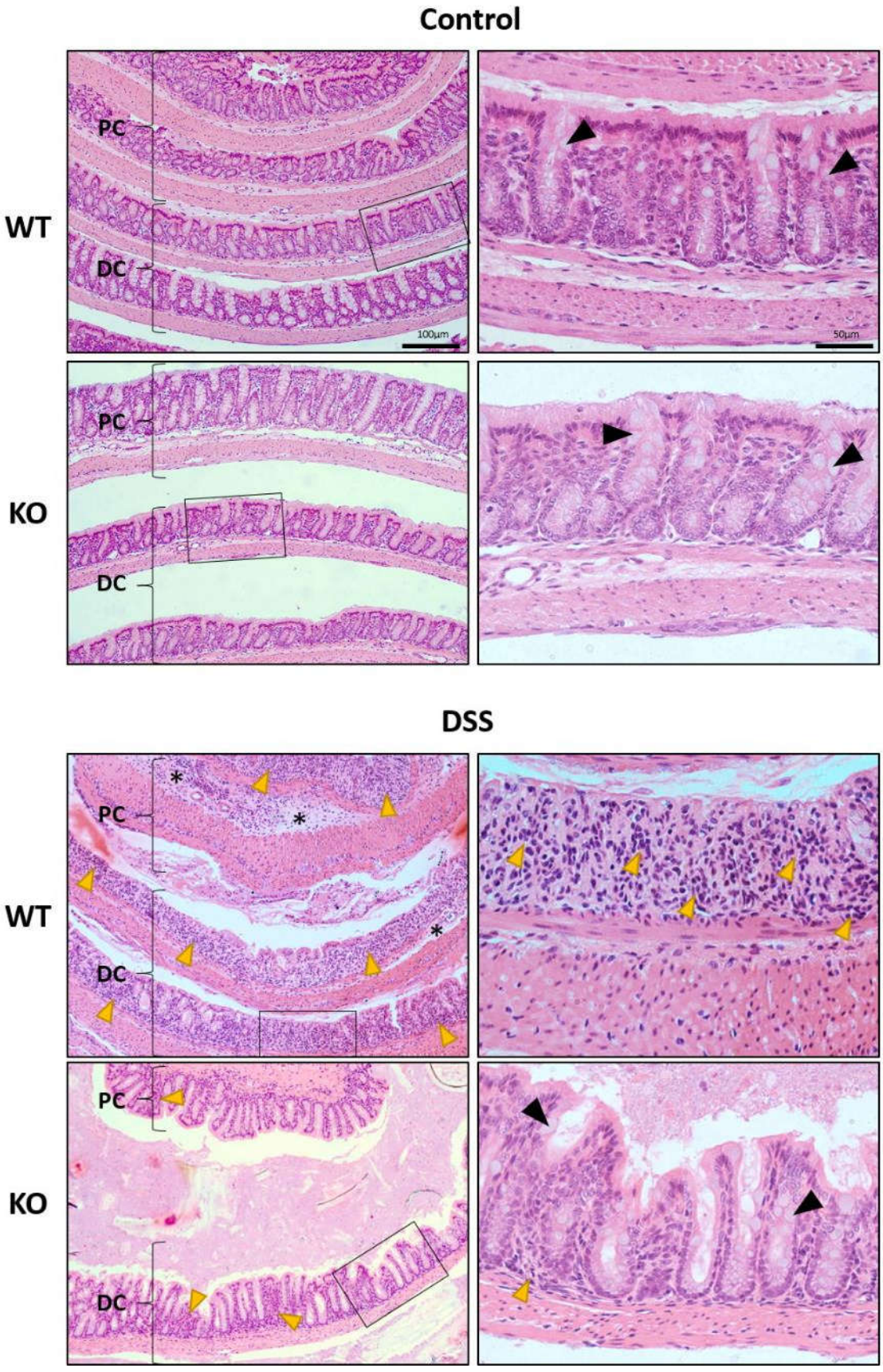
Control
WT
KO
PC
DC
100μm
50μm
DSS
WT
KO
PC
DC

Figure 11. Tissue damage induced by DSS-colitis is attenuated in the absence of HS1. DSS-colitis was induced in WT and HS1-KO mice for 7 days. Left panel: Swiss rolls showing proximal (PC) and distal colon (DC). Right panel: Boxed magnifications. Black arrowheads indicate goblet cells, leukocyte infiltrations are shown with yellow arrowheads and edema formation marked with asterisks. Representative images of 3 independent experimental groups of 3 mice each. (H&E; magnification left panel: 40x; bar=100µm; right panel 10x; bar=50 µm).

During DSS-colitis the formation of submucosal edema and hypertrophy of the muscularis and mucosa layers lead to colon shortening. Thus, colon lengths were determined in the different experimental groups (Figure 12). In control WT (75.1mm ±1.89) and HS1-KO (78.9mm ±1.98) mice colon lengths were similar, whereas WT colitic (55.9mm ±1.12) mice presented the expected significant colon shortening, which was ameliorated in HS1-KO mice (67.3mm ±2.02).

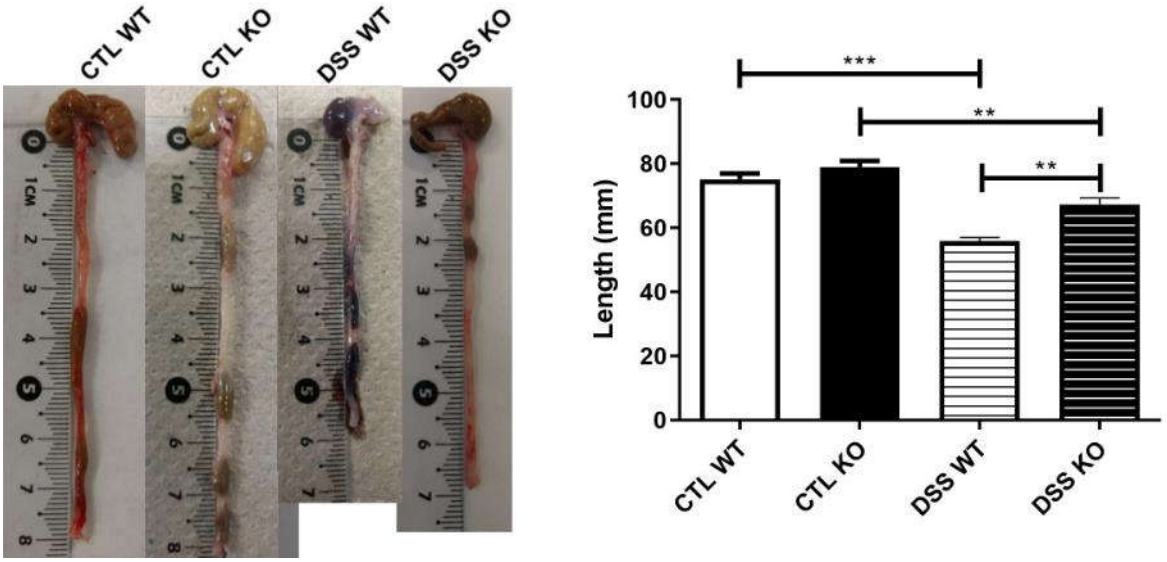

Figure 12. Colon shortening is ameliorated in HS1-KO mice during experimental colitis. Morphology and gross appearance of colons of control WT (75.1mm ±1.89) and HS1-KO (78.9mm ±1.98) mice were compared to colons of WT (55.9mm ±1.12) and HS1-KO (67.3mm ±2.02) mice that received DSS. n=10 per group. Values given as means±SDM. **p<0.01, ***p<0.001.

DSS-colitis induces a considerable influx of leukocytes into the lamina propria that produce and release ROS for bacterial clearance [92]. However, excessive release of ROS leads to oxidative stress harming surrounding tissue by inducing protein dysfunction and DNA damage, which eventually lead to cell death [93]. Given that less leukocyte influx was observed in colitic HS1-KO mice, it was tempting to speculate that oxidative stress in the colon was reduced without HS1. To evaluate the impact of HS1 deficiency on oxidative stress *in vivo*, colon cryosections from control and colitic WT and HS1-KO mice were incubated with dihydroethidium (DHE). During DSS-colitis, WT mice showed a strong increase in oxidative stress, whereas in the absence of HS1 oxidative stress was significantly reduced (Figure 13).

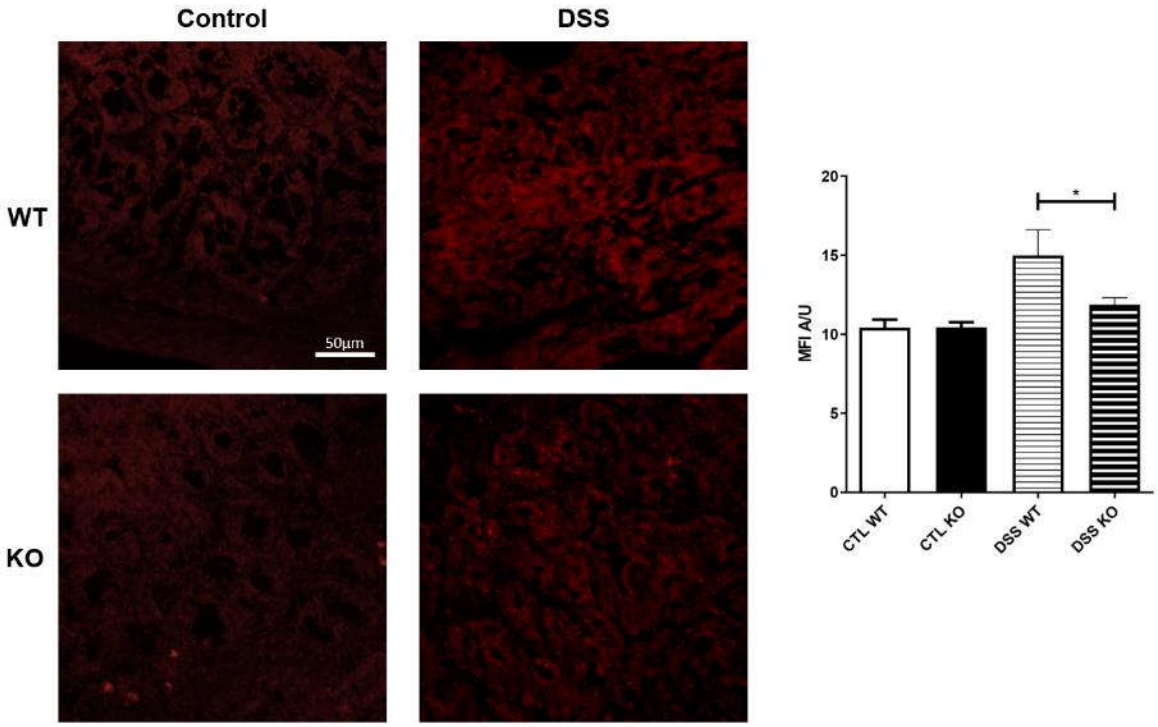

Figure 13. Oxidative stress induced by leukocyte influx to the lamina propria is ameliorated in the absence of HS1. Fresh colon cross-sections were incubated with 5µM dihydroethidium for 1 h. Representative fluorescent images of oxidized ethidium are shown. The bar graph shows mean fluorescence intensity (MFI) in arbitrary units (AU). n=3 in each group. Bar= 50 µm. *p< 0.05.

8.3 TJ modifications and epithelial permeability induced by DSS are prevented in HS1-KO mice

Given that epithelial barrier functions depend on correct composition of intercellular junctions, we investigated TJ architecture during DSS-colitis in the absence of HS1. Protein levels of ZO-1, claudin-1 and claudin-2 were similar in control WT and HS1-KO mice (Figure 14). However, WT mice that received DSS for 7 days showed a significant downregulation of ZO-1 and claudin-1; and upregulation of the pore-forming claudin-2 as expected. Of note, these effects were partially reverted in HS1-KO colitic mice.

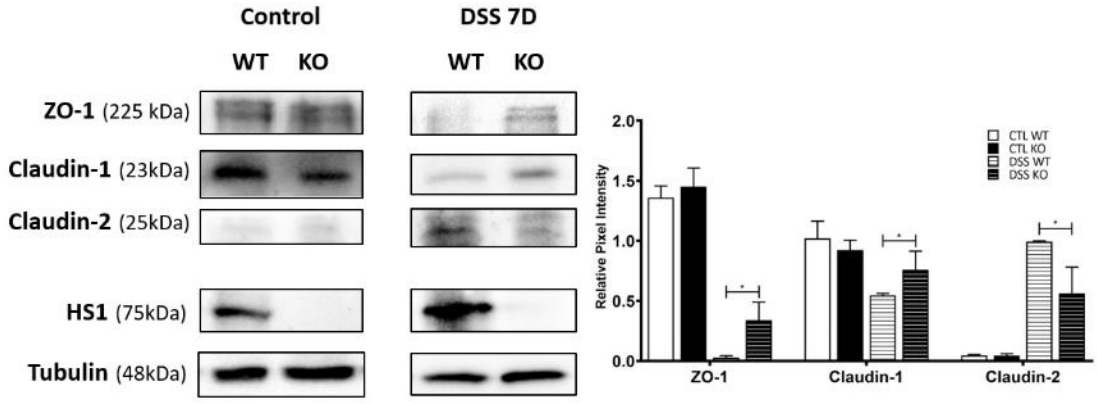

Figure 14. Expression of tight junction molecules is maintained in HS1-KO mice during DSS-colitis. Blots of ZO-1, claudin-1 and claudin-2 from colons of control and colitic WT and HS1-KO mice. Blots for HS1 proved HS1 deficiency in the KO mice. Tubulin was used as loading control. The graph shows quantification of at least 5 animals per group. Values given as mean±standard deviation of the mean. *$p<0.05$.

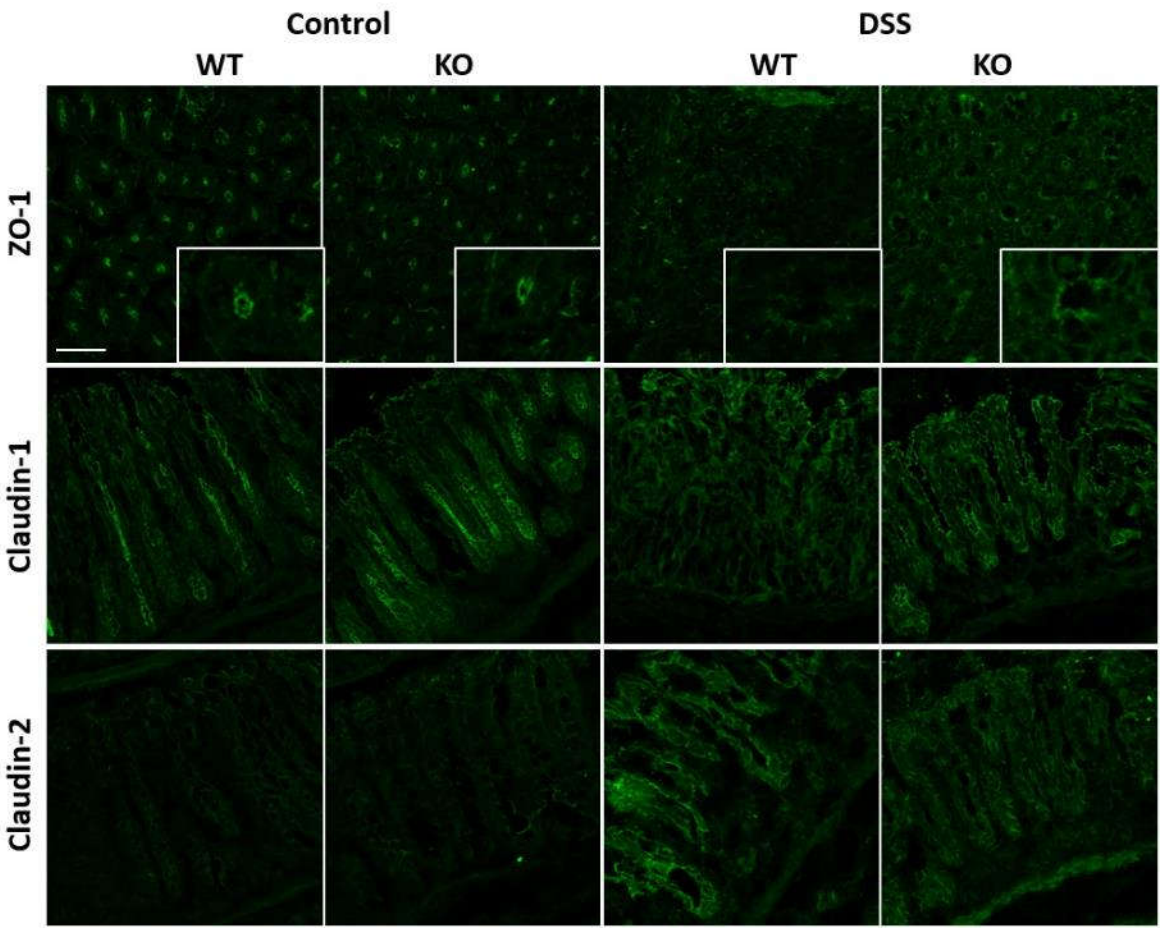

Figure 15. Representative images of TJ molecules by immunofluorescence of colon tissues derived from WT and HS1-KO mice. After DSS exposure, colitic WT mice lose expression of ZO-1 and claudin-1, whereas claudin-2 is upregulated. However, TJ architecture is better preserved in colitic HS1-KO mice where expression and localization of ZO-1 and claudin-1 is maintained and severe upregulation of claudin-2 is prevented. n=5 in each group. Bar= 50 μm.

Immunofluorescence stainings of colonic TJ proteins in WT and HS1-KO mice indicated an unaltered localization of ZO-1, claudin-1 and claudin-2 before the administration of DSS in both WT and HS1-KO mice (Figure 15). After DSS-colitis, WT mice lost expression of ZO-1 and claudin-1. By contrast, HS1-KO mice maintained a similar localization pattern than the one observed in healthy mice (Figure 15). Claudin-2 upregulation was clearly observed in WT colitic mice, whereas in HS1-KO claudin-2 was barely expressed (Figure 15).

Tight junctions regulate epithelial permeability for ions and macromolecules, which increases during inflammation. Considering that DSS-induced TJ architecture disruption was prevented in HS1-KO mice, we wondered whether epithelial hyperpermeability was also prevented (Figure 16). Preliminary results showed that healthy WT and HS1-KO mice presented similar barrier permeability to 4kDa FITC-Dextran. After DSS exposure permeability in WT colons significantly increased, but this was not observed in HS1-KO mice.

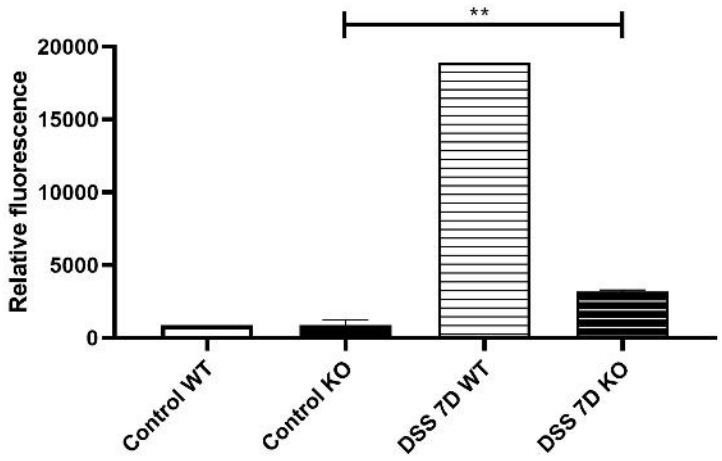

Figure 16. FITC-Dextran in vivo permeability assay. Intestinal permeability was assessed by measuring FITC-dextran fluorescence in peripheral blood 4 hours after oral gavage. WT mice exposed to DSS showed excessive permeability, whereas HS1-KO mice presented a slight increase. n=1-3 in each group. Values given as means±SDM. **p<0.01.

8.4 Neutrophil recruitment and transepithelial migration are reduced in the absence of HS1

Given that exacerbated neutrophil influx to the lamina propria leads to tissue injury (compare chapter 8.2), we analyzed neutrophil recruitment to the lamina propria during DSS-colitis in WT and HS1-KO mice by immunofluorescence stainings using an antibody against the neutrophil surface receptor GR1 (Figure 17). The number of $GR1^+$ cells in colitic WT mice was significantly higher than in HS1-KO mice, likely explaining the observed reduction in oxidative stress, lower barrier permeability and overall less susceptibility to the damage induced by DSS.

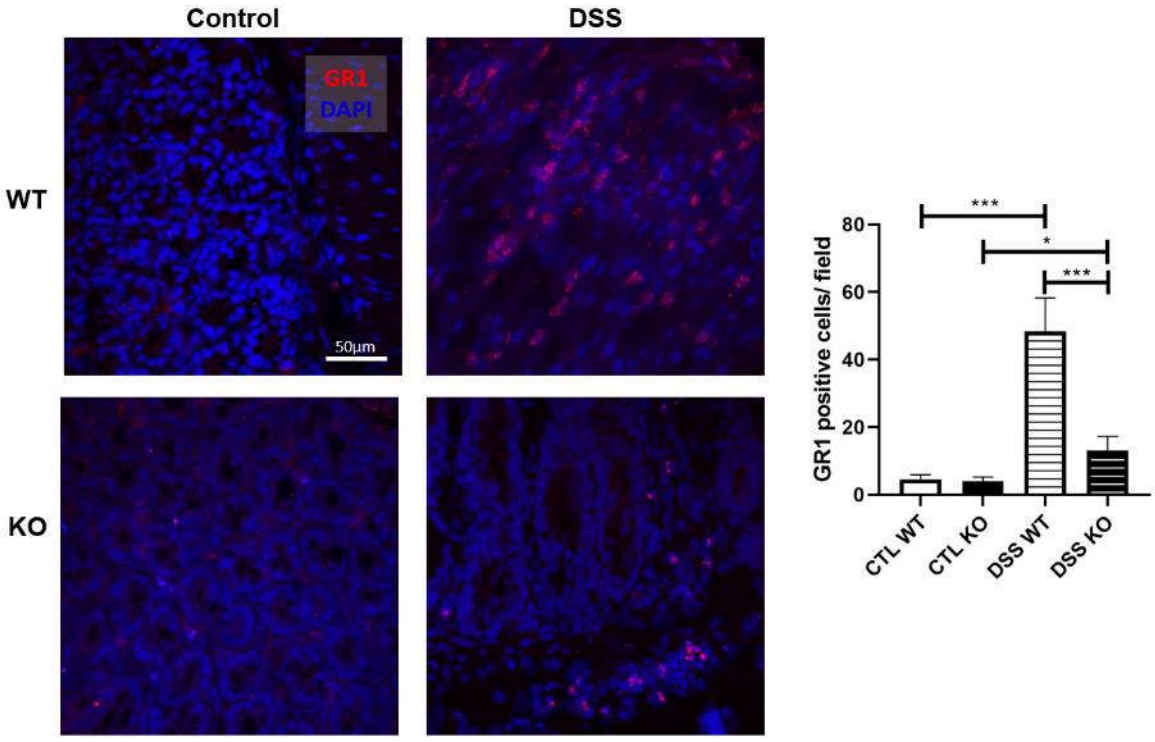

Figure 17. GR1+ cells were abundant in the lamina propria of colitic WT, but not HS1-KO mice. Gr1 positive cells (red) were increased in the WT colitic mice. Nuclei were stained with DAPI (blue) Quantification shows the number of GR1+ cells from 5 different fields per group of at least 5 mice. Bar= 50 µm.

After reaching the lamina propria, neutrophils migrate through the intestinal epithelium to eliminate luminal bacteria. Excessive TEpM is associated with mucosal damage and an increase in barrier permeability [51, 52]. Therefore, we evaluated TEpM *in vivo* in WT and HS1-KO mice during basal or inflammatory conditions using the pcLoop model as previously published [88]. Under basal conditions where mice received saline solution intraperitoneally and intraluminally, the number of neutrophils in the lumen is low and comparable between WT and HS1-KO mice. However, during inflammation induced by the administration of TNF-α and IFN-γ intraperitoneally and fMLP intrluminally, fMLP-dependent recruitment of neutrophils to the intestinal lumen significantly increased in WT mice, but not in HS1-KO mice.

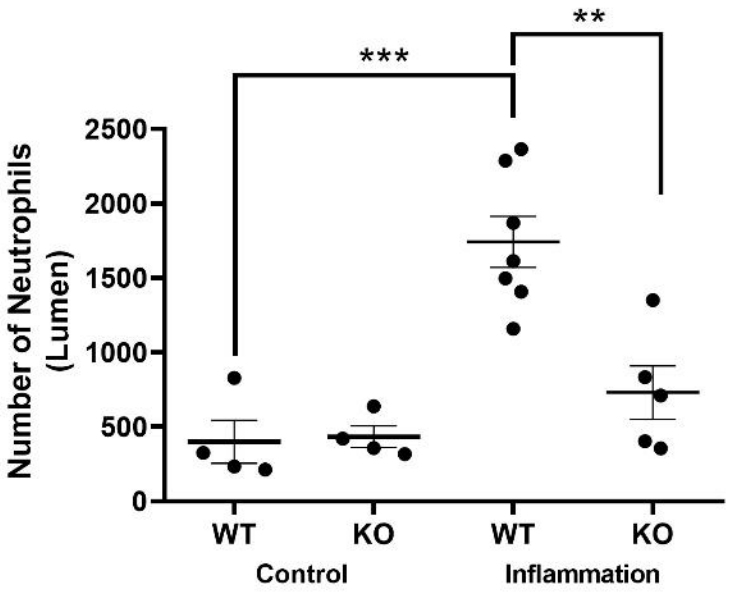

Figure 18. The number of neutrophils recruited to the intestinal lumen of the pcLoop is increased during inflammation in WT, but not in HS1-KO mice. Depicted are neutrophil numbers in the colon loop of control WT (n=4) and HS1-KO (n=4) mice that received saline solution intraperitoneally and intraluminally, compared to inflamed WT (n=7) and HS1-KO (n=5) mice that received 100ng TNF-α/IFN-γ intraperitoneally and intraluminal fMLP. Values are given as mean±standard deviation of the mean. **p<0.01, ***p<0.001.

8.5 More HS1 is detected in the mucosa of ulcerative colitis patients

To assess the severity of immune cell infiltration during human UC, and confirm that HS1 is not expressed in human intestinal epithelial cells, we analyzed the expression of HS1 in tissue samples from UC and uninflamed control patients suffering from other pathologies that required colon surgery. E-cadherin was used as an epithelial cell marker to show that HS1 is not present in epithelial cells. As previously reported [94], E-cadherin is downregulated in UC patients. Western blot analysis showed increased levels of HS1 in UC tissue samples (Figure 19A). This finding is in agreement with what we observed in mice during DSS colitis, where HS1 levels in colon lysates were significantly increased compared to WT control mice (Figure 19B). HS1 was not detected in the colon carcinoma cell line Caco-2 and isolated mouse epithelial cells. Presence of HS1 was detected by HS1

immunofluorescence stainings in immune cell infiltrates in the lamina propria of colon sections of UC and control patients. No HS1 signals were observed in epithelial cells. However, UC tissue showed much stronger HS1 signals compared to control tissue (Figure 20).

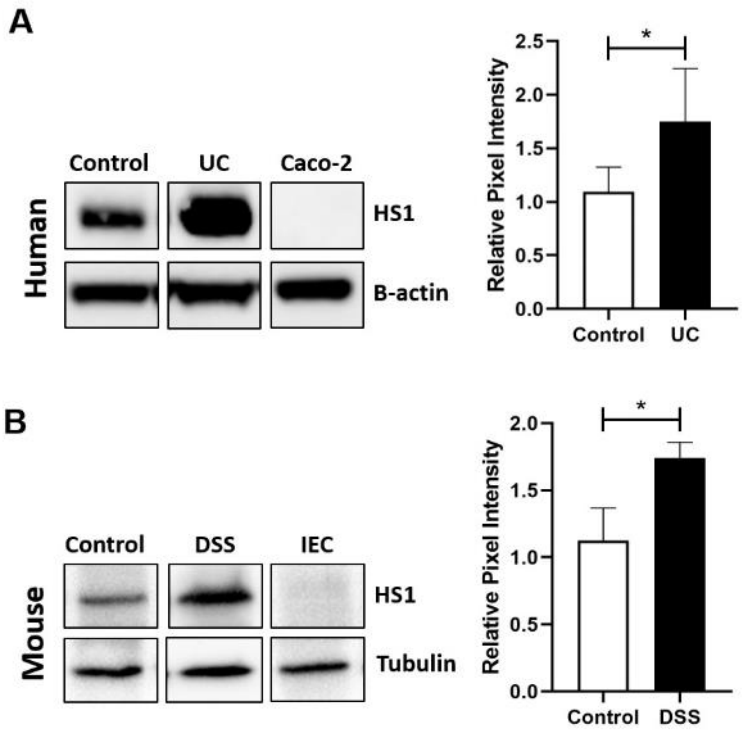

Figure 19. HS1 protein expression in human and mouse colon lysates during inflammation. HS1 levels in (A) colon tissue samples from control patients, UC patients and Caco-2 cells; and (B) in mouse colon lysates and isolated intestinal epithelial cells after DSS-colitis. Quantification of pixel intensity values are given as mean±standard deviation of the mean. *p<0.05.

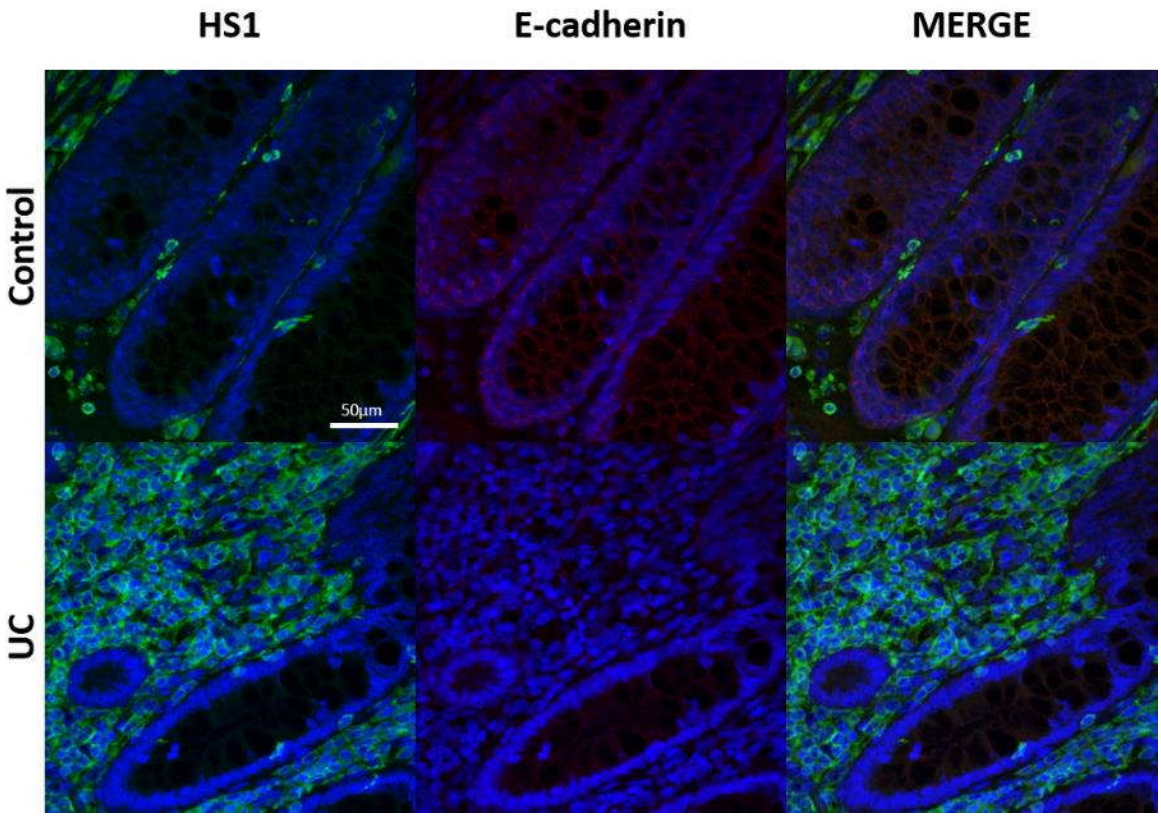

Figure 20. HS1 is detected in colon mucosa, but not in epithelial cells. Longitudinal sections of intestinal crypts from control and UC patients visualized using confocal fluorescence microscopy (green shows HS1, red shows E-cadherin and blue shows nuclei stained with DAPI). Bar=50 µm.

9. DISCUSSION

Inflammatory bowel diseases are chronic inflammatory disorders that affect millions of people worldwide. These diseases can be debilitating and lead to other life-threatening complications such as colitis-associated colorectal cancer [95]. Unfortunately, the pathogenesis of IBD is still not understood, but it is known that an excessive, dysregulated inflammation triggers tissue destruction mainly due to excessive neutrophil influx to the intestinal mucosa and accumulation of activated neutrophils in the lamina propria, epithelial layer and the colon lumen [6, 45]. Neutrophils are important effector cells during acute inflammations that aid in controlling bacterial infection and, at the same time, promote mucosal healing and resolution of inflammation. By contrast, exacerbated neutrophil presence contributes to tissue damage due to excessive release of ROS and matrix metalloproteases that further triggers the inflammatory response and may eventually lead to chronic inflammatory disorders [35, 96]. Thus, it is intuitive to think that depletion of neutrophils may have beneficial effects during chronic inflammation. However, many studies have shown that this is not the case. For example, when DSS-treated mice were injected intravenously with an anti-Gr-1 antibody to deplete $Gr1^{+}CD11b^{+}$ myeloid-lineage cells and $Gr\text{-}1^{+}$ granulocytes, which differentiate into macrophages or neutrophils, respectively, exacerbated DSS-colitis and slow recovery were observed compared to mice that did not received anti-Gr-1 [97]. Of note, another study showed that inhibition of neutrophil infiltration by blocking the chemokine receptor CXCR2 using the pharmacological compound SB225002 during DSS-colitis resulted in milder colitis and higher survival rate compared to non-treated mice. Whole blood neutrophil counts were comparable between groups, therefore, the observed effects were clearly due to reduced neutrophil influx and not reduced neutrophil numbers [98]. These data clearly show that a correct balance of neutrophil recruitment is of utmost pathophysiological importance during innate immune responses. Thus, further studies elucidating the molecular mechanisms involved in neutrophil recruitment are warranted to open

new therapeutic avenues to modulate the number of recruited neutrophils to ensure an effective, but contained inflammatory response.

In this thesis, we analyzed the role of the actin-binding protein HS1 in the development of experimental colitis and its implications on neutrophil recruitment to the inflamed colon. We found that during experimental colitis induced by DSS, mice deficient in HS1 developed less severe colitis compared to WT mice thus confirming previous data from our group showing that in a mouse model of lethal sepsis induced by cecal-ligation and puncture, HS1-KO mice showed increased survival, reduced leukocyte infiltration into the lung and reduced tissue damage (unpublished data). We also observed that HS1-KO colitic mice not only developed milder colitis but also recovered faster with complete normalization of stool consistency and absence of intestinal bleeding 5 days after removal of DSS. Moreover, the characteristic histomorphological changes observed in the colon of WT mice exposed to DSS such as extensive inflammatory cell infiltrates, epithelial changes including apical erosions, ulcerations, crypt dysplasia and depletion of goblet cells [85] were ameliorated in the absence of HS1. Therefore, we propose that HS1 is modulating severity of DSS-induced colitis by regulating neutrophil influx to the lamina propria.

During colitis, neutrophils are recruited to the colon from the blood stream following a chemokine gradient released by epithelial cells and resident immune cells [99]. In order to reach the site of inflammation, neutrophils require to exit the blood vessels by a complex multistep mechanism called transendothelial migration which consists of dynamic adhesive interactions between endothelial cells and neutrophils[36]. Recent studies have shown that neutrophil extravasation is reduced by approximately 50% in the absence of HS1 due to impaired activation of the β2-integrin LFA-1, which is critical for adhesion of neutrophils to the endothelium [75]. However, we showed here that still a significant amount of HS1-KO neutrophils can reach the inflamed colon, although to a much lesser extent than in WT mice. Consequently, intracellular ROS accumulation was also reduced in HS1-KO colitic mice. These data suggest that while HS1-KO neutrophils are less efficient to migrate

to the lamina propria, their ROS production functions normally *in vivo* to aid in bacterial clearance. This is in agreement with *in vitro* data from our group (unpublished) and data in dendritic cells [61] showing that HS1 is not a critical protein for the regulation of effector functions such as phagocytosis and respiratory burst; and likely explains why tissue injury and colitis severity is ameliorated in HS1-KO mice as high ROS levels destroy surrounding tissue by affecting the function of lipids, proteins and nucleic acids leading to apoptosis of epithelial cells and epithelial barrier dysfunction [100]. However, it will be important to elucidate whether this is a secondary effect due to reduced extravasation or additionally caused by defective extravascular interstitial migration of HS1-KO neutrophils.

Several studies have shown that epithelial ABPs such as cortactin, a homologue of HS1, are involved in regulating intestinal barrier function by directly orchestrating actin dynamics that control intercellular junctions architecture [101]. However, HS1 is not expressed in intestinal epithelial cells, therefore its role in barrier function regulation is indirect since it regulates leukocyte migration and chemotaxis [60, 75]. Excessive intestinal epithelial barrier dysfunction is a key characteristic of IBD [33]. And while increased permeability by itself is not an onset factor, as it has been reported in several studies that direct relatives of IBD patients presented increased intestinal permeability without developing the disease [102], it greatly contributes to intestinal barrier dysfunction. Epithelial permeability is mainly regulated by TJ proteins including ZO-1, claudins and occludin. Alterations in TJ structure induced by proinflammatory cytokines are detrimental for epithelial barrier functions. For instance, IFN-γ induces internalization of TJ proteins by a micropinocytosis-like mechanism leading to a leaky epithelial barrier [103]. TNF-α induces caveolin1-dependent endocytosis of occludin also causing increased permeability [104]. TJ proteins are also cleaved by enteric microbial proteases [105]. During TEpM neutrophils release elastase which cleaves E-cadherin, thus further disrupting the epithelial barrier [51].

When TJ architecture is altered, passage of ions and macromolecules is increased allowing the translocation of luminal antigens through the epithelial barrier, thus

exacerbating the inflammatory response. During DSS-colitis, ZO-1 and occludin expression is almost completely ablated, whereas the expression of claudins is switched from a sealing claudin-1 to a pore forming claudin-2 [106, 107]. Our results show that in absence of HS1 during DSS-colitis, complete depletion of ZO-1 is prevented and the claudin switch is ameliorated likely providing an explanation for our finding that absence of HS1 protects against excessive intestinal epithelial permeability in vivo during DSS-colitis. Therefore, we conclude that the observed protective effect on TJ architecture is due to a reduction in neutrophil presence in the mucosa and neutrophil TEpM.

While neutrophil TEpM is critical for intestinal mucosal defense, excessive neutrophil presence at the luminal side of the epithelium is detrimental due to the release of effector molecules such as proteases and ROS that in high quantities induce epithelial cell apoptosis and disruption of intercellular junctions [35]. After reaching the lamina propria, neutrophils interact with the basolateral side of the epithelium through interactions between the integrin Mac1 and epithelial fucosylated ligands [108], then navigate through the intercellular space guided by the binding of Sirpα to the epithelial glycoprotein CD47 [109, 110]. Then crossing AJ and TJ involves interactions between neutrophil JAML and epithelial CAR to reach the luminal side of the epithelium [111]. Considering that several studies show that HS1 is involved in signaling via the integrin LFA-1 [59, 75], and other receptors such as BCR, TCR, FcγRIIIA and CXCR4 [57, 112] in different hematopoietic cells, it will be important to elucidate whether HS1 is required for Mac1, Sirpα and JAML activation and/or signaling downstream of their engagement; and in this way further controlling neutrophil TEpM.

Apical bound neutrophils produce abundant 5'-adenosine monophosphate (5'-AMP), which binds to the epithelial receptor A2B inducing a passive water flux towards the lumen leading to the development of diarrhea [113]. Additionally, accumulation of neutrophils in the lumen of intestinal crypts, also known as crypt abscesses, and presence of neutrophils in stool samples are correlated with UC

severity [114]. Therefore, we analyzed the number of neutrophils recruited to the intestinal lumen during inflammation. In line with reduced neutrophil presence in the lamina propria, also the number of neutrophils in the lumen of inflamed HS1-KO mice was significantly lower than in inflamed WT mice. Further research is needed to elucidate if the TEpM process itself is affected in the absence of HS1 or whether this effect is a consequence of lower neutrophil numbers in the lamina propria.

While our findings using a mouse model of UC indicate that HS1 is relevant for the development and progression of intestinal inflammation, it is important to analyze whether HS1 is also of significance in human pathology. Moreover, since its discovery, HS1 has only been found in cells of the hematopoietic lineage [63]. However, a recent study identified HS1 in the cytoplasm of ovarian epithelial carcinoma cells, but not in normal ovarian tissue [115]. Thus, we decided to analyze the expression and localization of HS1 in colon tissue samples from control and UC patients. Our findings demonstrate that HS1 is not detected in human or mouse epithelial cells. Of note, HS1 is clearly increased in UC patients and in colitic mouse colons as shown by WB and IF. This is in agreement with findings in platelets, where platelets were incubated with LPS resulting in significantly increased HS1 protein levels [116]. However, more detailed PCR and WB analyses are needed to prove HS1 upregulation in different hematopoietic cells under inflammatory conditions.

Taken together, our findings clearly show that HS1 regulates neutrophil recruitment to the lamina propria and the colon lumen during experimental colitis. Absence of HS1 reduces colon tissue damage caused by excessive production of ROS, thus preventing intestinal epithelial barrier dysfunction and ameliorating overall colitis severity.

CONCLUSION

We conclude that inhibition, but not depletion of neutrophil recruitment as induced by HS1 deficiency ameliorates signs of severe colitis development. Thus, targeting HS1 in chronic inflammatory diseases could be a promising therapeutic approach to ameliorate neutrophil-inflicted tissue damage without preventing neutrophil-induced resolution of inflammation and wound healing. However, it remains to be proven whether the observed effects are a consequence of reduced neutrophil extravasation, or whether HS1 also regulates TEpM directly.

10. PERSPECTIVES

a) To corroborate whether HS1-deficient neutrophils show defective transepithelial migration *in vitro*.

b) To evaluate leukocyte extravasation in the mesenteric microvasculature *in vivo* during inflammation

c) To analyze levels of inflammatory mediators in HS1-KO mice during the development of experimental colitis.

d) To determine the role of HS1 in colitis-associated colorectal cancer development.

11. PUBLICATIONS

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