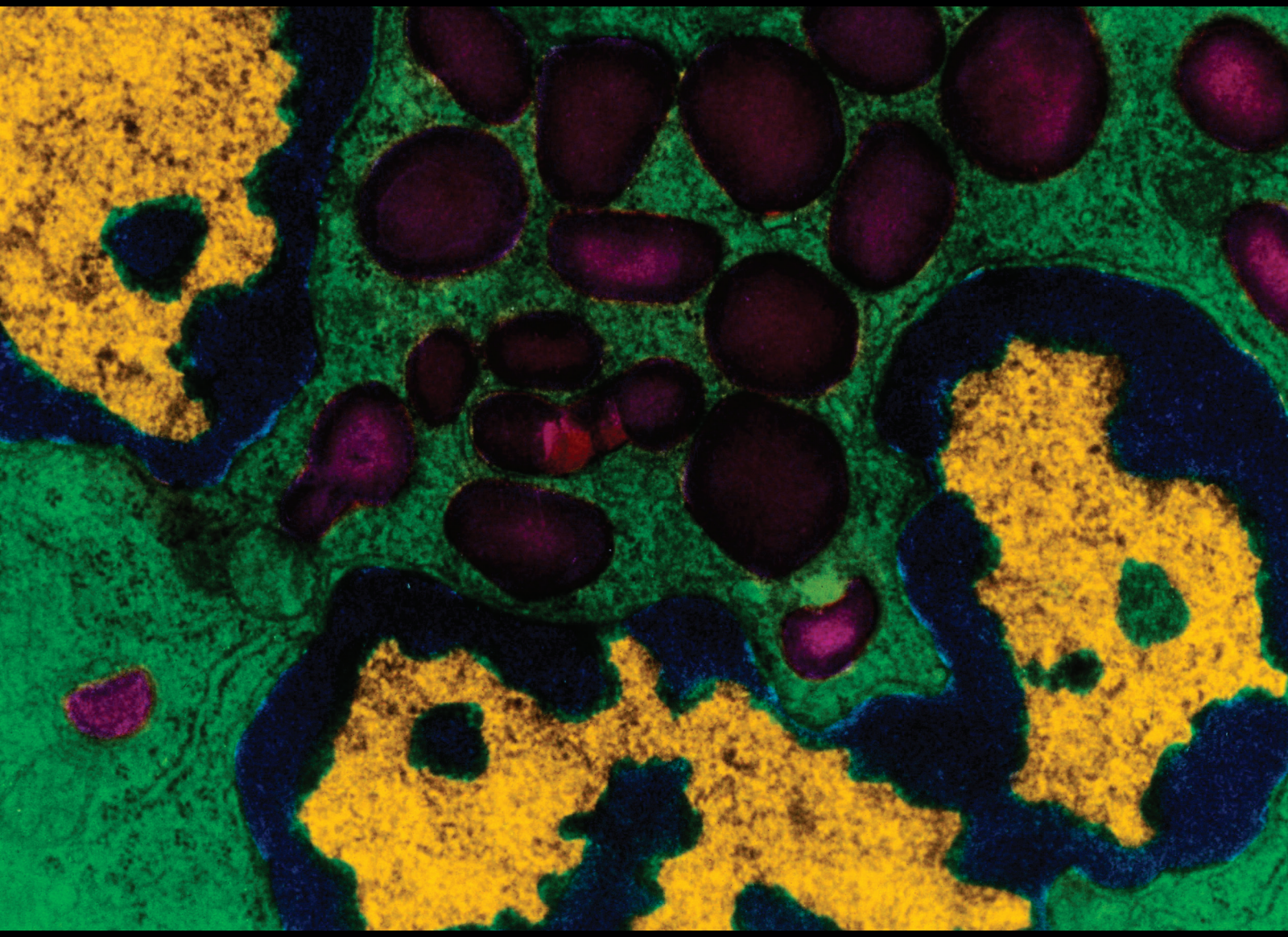
Mediators of Inflammation

Recruitment of Immune Cells into Inflamed Tissues: Consequences for Endothelial Barrier Integrity and Tissue Functionality

Guest Editors: Michael Schnoor, Pilar Alcaide, Mathieu-Benoit Voisin, and Jaap D. van Buul



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Editorial Board

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Editorial

Recruitment of Immune Cells into Inflamed Tissues: Consequences for Endothelial Barrier Integrity and Tissue Functionality

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Immune cell trafficking is critical for survival. In order to fulfil their functions in surveillance and immune responses, leukocytes need to exit the blood stream to reach sites of infection or injury in peripheral tissues [1]. Once transmigrated, leukocytes trigger pathogen clearance, resolution of inflammation, and wound healing. On the other hand, excessive extravasation of leukocytes, as seen in pathological inflammatory conditions including sepsis, autoimmune, and cardiovascular diseases, can provoke tissue damage due to excessive release of proinflammatory cytokines, chemokines, and reactive oxygen species. These substances cause endothelial overactivation triggering leukocyte transmigration and endothelial dysfunction [2]. The complex nature of physiological and pathological leukocyte extravasation makes it tricky to define a threshold where beneficial transmigration ends and where detrimental excessive extravasation starts. Thus, it is of utmost importance to better understand the mechanisms that control leukocyte-endothelial interactions during the extravasation cascade and leukocyte behaviour within peripheral tissues that ensure appropriate immune responses. The purpose of this special issue was to publish original research and review articles addressing recent conceptual advances in the field of inflammatory immune cell recruitment, endothelial dysfunction, and resolution of inflammation.

The editors contributed a review article in which they highlight certain mechanisms that different subsets exploit to achieve the goal of transmigration. This is actually very important when it comes to the treatment of immune diseases caused by a certain leukocyte subset. In this case, it is desired to only interfere with the transmigration of this certain subset without affecting others to avoid complete immune suppression that would cause severe side effects. Such mechanisms include tyrosine phosphorylation of endothelial adhesion receptors that are summarized in the review by Dr. A. P. Adam. He highlights signalling pathways downstream of proinflammatory cytokines and adhering leukocytes that induce tyrosine phosphorylation of junctional proteins such as VE-cadherin. He discusses the consequences of these phosphorylations that may or may not lead to junction disassembly and excessive leukocyte transmigration. J. Kryczka and J. Boncela nicely review the multidimensional impact of leukocytes in skin fibrosis emphasizing current knowledge about recruitment of different leukocyte types in wounded tissues and the mechanisms triggering wound enclosure. A fine balance between scar formation and massive fibrosis is required to avoid excessive fibrosis while promoting wound healing. M. C. Souza and colleagues present a comprehensive review on endothelial dysfunction in cerebral malaria. The authors summarize known leukocyte interactions with the

brain and lung endothelium and highlight the challenges of using experimental malaria data in animals to interpret and understand human severe malaria. Moreover, the J. Millán group contributed a comprehensive review on how leukocytes, once extravasated, interact with polarized epithelial cells in inflamed tissues. They emphasize that polarized epithelial cells not only secrete chemokines asymmetrically, but also polarize adhesion receptors that may guide leukocytes across epithelial monolayers. In the review of J. Cedervall and colleagues the latest findings on the alterations of vascular functions by tumor cells and tumor-associated leukocytes are summarized. They also discuss how circulating tumor-derived factors drive systemic inflammation in the blood vasculature of distant organs.

In a clinical study, A. Lukasz and colleagues elegantly showed that plasma angiopoietin-2 levels may have predictive value for a complicated clinical course of hemolytic uremic syndrome. Using endothelial cells in vitro, the authors showed that serum from these patients reduced barrier stability. S. Denk and coworkers showed the diagnostic implications of the presence of JAM-1 in the circulation for the detection of experimental and clinical polytrauma. C. Kirchhoff and colleagues developed a method that may potentially help identifying traumatic brain infarction by showing that monocytes accumulate in the circulation shortly after onset of the infarct. In another research article, E. Gallego-Colon and colleagues demonstrate that the number of infiltrating proinflammatory monocytes decreased in the heart after myocardial infarction whereas the number of anti-inflammatory macrophages increased in mice overexpressing insulin-like growth factor-1Ea (IGF-1Ea) leading to reduced scar formation and increased cardiac functional recovery. These data highlight the importance of the balance between pro- and anti-inflammatory leukocytes for tissue repair after myocardial infarction, which is in part controlled by IGF-1Ea. S. A. Dorosz and colleagues provide new evidence that IL-27 promotes proinflammatory signals in endothelial cells that are downregulated by the damage-associated molecular pattern molecule calprotectin through mechanisms involving STAT-1. These findings highlight calprotectin as a modulator of inflammation and early vascular dysfunction.

We hope that this special issue will be beneficial for clinicians and researchers not only in the field of leukocyte transmigration but also for those working on acute and chronic inflammatory diseases. The studies published in this special issue provide new insights into physiologically relevant mechanisms of inflammation and inflammatory diseases that will hopefully stimulate the development of novel research ideas and therapeutic strategies.

Acknowledgments

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Michael Schnoor
Pilar Alcaide
Mathieu-Benoit Voisin
Jaap D. van Buul

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Review Article

Cellular Barriers after Extravasation: Leukocyte Interactions with Polarized Epithelia in the Inflamed Tissue

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During the inflammatory response, immune cells egress from the circulation and follow a chemotactic and haptotactic gradient within the tissue, interacting with matrix components in the stroma and with parenchymal cells, which guide them towards the sites of inflammation. Polarized epithelial cells compartmentalize tissue cavities and are often exposed to inflammatory challenges such as toxics or infections in non-lymphoid tissues. Apicobasal polarity is critical to the specialized functions of these epithelia. Indeed, a common feature of epithelial dysfunction is the loss of polarity. Here we review evidence showing that apicobasal polarity regulates the inflammatory response: various polarized epithelia asymmetrically secrete chemotactic mediators and polarize adhesion receptors that dictate the route of leukocyte migration within the parenchyma. We also discuss recent findings showing that the loss of apicobasal polarity increases leukocyte adhesion to epithelial cells and the consequences that this could have for the inflammatory response towards damaged, infected or transformed epithelial cells.

1. Introduction

Leukocyte recruitment into the inflamed parenchyma requires successive interactions with cellular and stromal barriers that establish mechanical, chemotactic and haptotactic gradients to guide immune cells towards the inflammatory focus. The first stage of this immune steeplechase, the leukocyte transendothelial migration, is a multi-step cascade of interactions that have been extensively studied in recent years in different vascular beds and experimental models, and some comprehensive reviews on this topic can be found in this special issue [1–5]. The events that follow leukocyte extravasation are perhaps less well characterized, although significant advances have been made with the advent of high-resolution intravital microscopy and the development of more sophisticated culture systems to investigate leukocyte migration and interactions in three dimensions. Particular attention has been paid to elucidating how leukocytes can migrate through the stroma, the way these cells remodel their morphology and sense cues that guide them towards dysfunctional tissue areas. These areas are often made up of polarized parenchymal epithelial cells that form barriers to compartmentalize functions in cavities of the liver, intestine or lungs (Figure 1). Compared to the endothelium, the molecular mechanisms involved in the interaction of infiltrated or tissue-resident immune cells with parenchymal barriers have not been so extensively studied. Polarized epithelial barriers establish two types of interactions. On the one hand, similar to endothelial cells, parenchymal epithelia must guide leukocytes to traverse them in order to reach a localized inflammatory focus, for example, in the lung or intestinal mucosa. These interactions are thus transient and often occur in two directions, from the parenchyma to the lumen and viceversa [6]. On the other hand, these barriers contain damaged or infected cells that are part of the inflammatory focus and the endpoint of the leukocyte

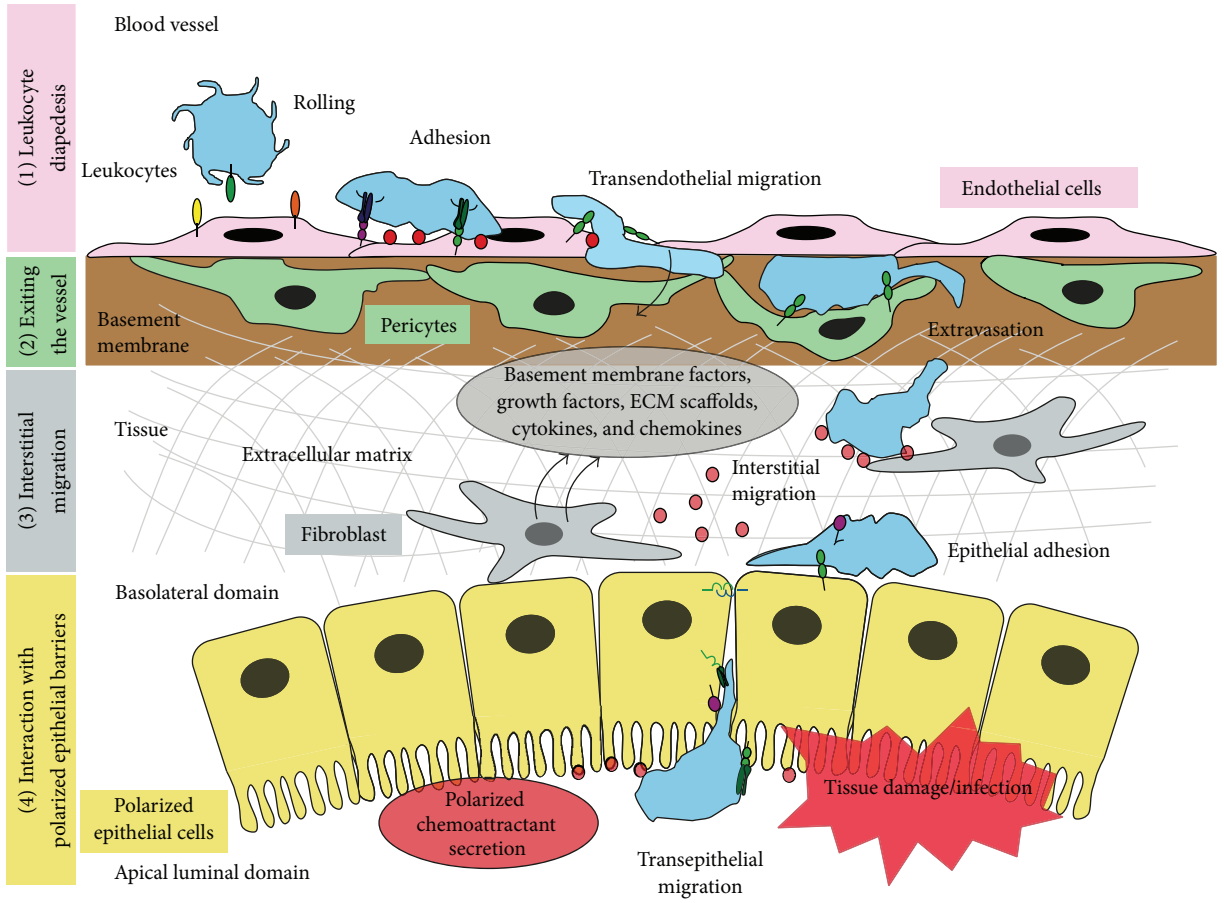

Figure 1: Endothelial and epithelial barriers determine the different stages of leukocyte migration in its journey towards the inflammatory focus in complex tissues. The parenchymal three-dimensional organization contributes to establish an haptotactic and chemotactic gradient (1) Leukocyte adhesion and transendothelial migration or diapedesis. (2) Exiting the vessel. Once they reach the subendothelial space, leukocytes traverse the basement membrane and interact with pericytes, which promote the complete extravasation of immune cells via adhesion receptors. (3) Interstitial migration. Leukocytes switch from a two-dimensional to a less adhesive three-dimensional migration to circumvent topological constraints in the stromal barrier. Fibroblasts help leukocyte navigation by maintaining protein scaffolds and secreting mediators such as cytokines and growth factors, which act as chemotactic cues. (4) Interaction with polarized epithelial barriers. Following haptotactic and chemotactic gradients, leukocytes encounter polarized epithelial cells and often undergo transepithelial migration. The polarized distribution of the adhesive and chemotactic machineries mediates leukocyte guidance through the parenchymal epithelia for immunosurveillance or the clearance of pathogens and dysfunctional cells.

migratory journey, so some sort of footprint, which is not completely understood, must exist in these cells to promote a preferential adhesion with infiltrated leukocytes. So far, most of the *in vivo* and *in vitro* approaches to study leukocyte migration across the tissue parenchyma have addressed the role of each single tissue barrier that immune cells encounter in their journey to the inflammatory focus. We believe that successfully combining our current knowledge about leukocyte extravasation, three-dimensional migration through the stroma and the sequential interactions with parenchymal cell barriers, which include adopting unified experimental models, will help shed light on the entire migratory route of each immune cell type and on the specificity of the innate inflammatory responses in each type of tissue.

2. The Long Journey towards the Parenchymal Inflammatory Focus after Leukocyte Transendothelial Migration: Leaving the Vessel

Most of the leukocyte efflux from the bloodstream occurs in postcapillary venules, small vessels covered by pericytes and other mural cells and the basement membrane, which are a secondary barrier that extravasating leukocytes have to traverse [9]. Endothelial cells initiate leukocyte extravasation, but subendothelial, leukocyte-pericyte interactions are required for the final egression of leukocytes into the interstitium (Figure 1). Similar to endothelial cells, pericytes express adhesion receptors in response to inflammatory cytokines

and establish adhesive tracks at least in the case of neutrophils [10]. Few studies have addressed the contribution of abluminal endothelial surfaces, the basement membrane and the pericyte barriers to leukocyte trafficking into the tissue, but the most recent reports suggest a pro-active role for pericytes in controlling leukocyte navigation into the parenchyma through the intercellular adhesion molecule-1 (ICAM-1), the counter-receptor of β2-integrins, which is expressed in pericytes at levels comparable to those in inflamed endothelial cells [10–12]. This interaction occurs mainly in postcapillary venules, where myeloid leukocytes egress through regions between pericytes that have low density of matrix proteins [13]. A subset of these extravasated leukocytes interacts with pericytes surrounding capillaries and arterioles. Pericytes in these microvessels express macrophage inhibitory factor (MIF), which attracts fully extravasated neutrophils and macrophages and, as a consequence, these leukocytes are guided to sites of (sterile) inflammation [12]. Pericytes are not the only perivascular cell type that regulates leukocyte trafficking. Two other cell types have been shown to be critical for neutrophil extravasation: first, resident perivascular macrophages from dermal venules are the main source of neutrophil chemoattractants and secrete the chemokines CXCL1, CXCL2, CCL2, CCL3, and CCL4 in an experimental model of bacterial infection of the skin [14]; second, vessel-associated mast cells secrete the chemoattractants CXCL1 and CXCL2 to induce neutrophil extravasation in a model of intraperitoneal lipopolysaccharide (LPS) stimulation [4].

Leukocyte extravasation constitutes a transition between a two-dimensional migration mode, in the presence of shear stress, to a three-dimensional migration mode upon infiltration into the connective tissue (Figure 1). Immune cells in the interstitium interact with the extracellular matrix and the stromal cells adopting a migratory mode called ameboid, in which cells with a rounded morphology can easily change shape and squeeze between matrix fibers with little stromal remodeling [15]. This ameboid movement requires less adhesiveness than two-dimensional motility, and is mainly based on the ability of the leukocytes to reshape their actomyosin cytoskeleton, emitting pseudopods and inducing contractility at the rear of the cell. Within the heterogeneity of the tissue, leukocytes usually combine adhesion-independent and -dependent motility modes and rapidly adapt their migratory requirements to the microenvironment of the parenchyma [16].

Various fibroblast subtypes reside in the stroma and are adjacent to parenchymal epithelial barriers. Pericryptal fibroblasts, hepatic Ito cells and glomerular mesangial cells support epithelial cell function [17, 18]. These fibroblasts regulate tissue homeostasis and repair by secreting basement membrane factors that contribute to the architecture of the internal epithelial barriers and regulate epithelial cell proliferation and differentiation [19, 20]. Stromal fibroblasts have been attributed a role of maintaining a healthy environment in the lymphoid and non-lymphoid tissue by having an immunomodulatory effect on neighboring endothelial and immune cells [17, 20, 21]. Stromal fibroblasts mediate leukocyte parenchymal navigation through damaged areas by secreting extracellular matrix components as well as cytokines, chemokines and growth factors as soluble chemotactic cues. Direct fibroblast-leukocyte interactions have been investigated in the context of allergic, inflammatory and cancer pathologies [22, 23]. Fibroblasts from patients with rheumatoid arthritis can present autoantigens to infiltrated T-cells and, reciprocally, these cells can induce a proinflammatory status in the fibroblast-like synoviocytes [24, 25]. A probably aberrant leukocyte-fibroblast interaction also occurs in systemic sclerosis, a disease of unknown etiology, in which immune cells cause fibrosis by inducing collagen synthesis in skin fibroblasts [26]. However, the main role of tissue fibroblasts in leukocyte migration seems to be the maintenance of the stromal protein scaffolds and the secretion of mediators that attract or activate migrating immune cells. In conclusion, leukocytes not only follow chemotactic and haptotactic cues within the vessel. The parenchyma establishes a full program of immune cell guidance towards the inflammatory focus. Many cell types orchestrate this program either directly, by interacting with leukocytes, or indirectly, by secreting mediators and extracellular matrix components.

3. Reaching the Polarized Epithelia in the Parenchyma

In several tissues, parenchymal cells need to establish lumens to perform specialized functions, including filtration, absorption, secretion and protection. Polarized epithelia and the underlying basement membranes form different mucosal, blood-brain, bile duct or renal barriers, which are exposed to internal cavities and are therefore prone to infections and intense mechanical, toxic and inflammatory stresses. The compartmentalization properties of epithelial barriers arise from the ability to polarize and form intercellular junctions that separate apical from basolateral membrane domains. Columnar epithelial cells place their apical domains facing the lumens that form relatively large tube-shaped cavities [27]. Other polarized epithelia, such as hepatocytes, form smaller apical lumens between adjacent cells, giving rise to smaller tubules that form the bile canaliculus, an intricate network of channels that drains bile into the bile ducts and eventually into the intestine [28]. Importantly, as we shall see below, the compartmentalization properties of the parenchymal epithelia are also fundamental for controlling immune cell trafficking.

Resident leukocytes traverse parenchymal epithelial barriers to survey luminal surfaces exposed to extra-tissular material [29, 30]. Epithelial cell dysfunction can also produce an inflammatory response and additional leukocyte infiltration. Such infiltration may have the final aim of reaching internal cavities, such as the intestinal lumen, to fight luminal pathogens, but leukocytes may also interact with dysfunctional epithelia that themselves constitute the inflammatory focus. Hence, immune cells preferentially contact with impaired epithelial cells to eliminate or receive information from them, so mechanisms must exist to help leukocytes discriminate in the same inflammatory microenvironment between sick cells and inflamed, but still-operative, adjacent cells. On the other hand, leukocytes, particularly neutrophils

in the intestine, play an important role in the resolution of epithelial inflammation. Neutrophils transmigrate across inflamed epithelial monolayers and release factors that contribute to tissue repair. It is therefore critical to understand the signals that mediate the leukocyte-epithelium interaction in diverse physiological and pathological scenarios (Figure 1).

4. Polarized Secretion of Signals Involved in Leukocyte Attraction to Epithelia

The remarkable compartmentalization occurring between apical and basolateral environments in polarized epithelia led researchers to hypothesize that leukocyte chemoattractants are released in a polarized way. IL-8 is one of the main chemokines driving leukocyte infiltration into the intestine. In an *in vitro* model of human intestinal epithelial cells, Sonnier and colleagues, elegantly showed the different effects on IL-8 secretion caused by polarized TNFα stimulation. Whereas basolateral TNFα stimulation resulted in secretion of IL-8 from both apical and basolateral membrane regions, apical stimulation induced the secretion of IL-8 exclusively from these surface domains. By combining blocking antibodies, the authors proposed that the receptor 2 for TNFα (TNFR2) is apically confined and responsible for polarized IL-8 secretion [31]. Interestingly, the IL-8 receptor CXCR1 is also apically distributed in these cells, suggesting the existence of an autocrine pathway for this chemokine on the luminal side of the intestinal epithelium [32]. Other polarized epithelia, such as endometrial epithelial cells, also vectorially secrete IL-8 depending on the membrane domain receiving the inflammatory stimulus [33]. These cells also release other inflammatory mediators from the apical surfaces such as IL-6 or prostaglandins [33, 34].

The polarized secretion of inflammatory mediators is probably defined by the cell type and the nature and location of the stimulus that induces secretion; indeed, the secretion of IL-8 and other CXC chemokines has been found to be preferentially basolateral in other polarized epithelial beds and with other pathogen-derived stimuli [35–38]. Chemokine secretion polarity has been investigated in more detail with IL-8, but other chemokines involved in lymphocyte attraction to intestinal epithelia, such as interferon γ–inducible protein (IP)-10, monokine induced by interferon γ (MIG) and MDC/CCL22, are basolaterally secreted and have a differential effect in the basolateral and apical milieu, attracting lymphocytes preferentially from epithelial basolateral membranes, at least for CCL22 [39, 40].

Chemokines are not the only polarized parenchymal chemoattractants. The eicosanoid Hepoxilin A3 (HXA3) is a proinflammatory lipid that is also selectively released from the apical membrane domains of polarized epithelia in intestine and lung. In these two organs, HXA3 is a potent neutrophil chemoattractant that follows the effect of IL-8 to promote neutrophil transepithelial migration [41, 42]. The mechanisms regulating the polarized secretion of this lipid mediator are not well understood, but the apical epithelial marker, multidrug-resistance associated transporter 2 (MRP2) is clearly involved in the apical secretion of HXA3 in the intestine. HXA3 and MRP2 are both induced in experimental models of chronic intestinal inflammation and so constitute a potential therapeutic target [43]. In conclusion, the apicobasal polarity of these chemoattractants is important for efficient leukocyte guidance towards different physiological or pathological inflammatory foci within the complex three-dimensional organization of tissues.

5. Apicobasal Polarity of Adhesion Receptors Involved in Leukocyte-Epithelial Cell Interactions

The leukocytes chemoattracted by parenchymal epithelial cells finally make contact. Leukocytes first encounter basolateral epithelial membranes containing fucosylated proteoglycans that can interact with β2 integrins [44]. This binding is often the initial step in the process of leukocyte transepithelial migration. It has been reported that macrophages traverse retinal-pigmented epithelial cells from diabetic rats through caveolin-1-positive transcellular pores [45], similar to those found in the transcellular diapedesis of vascular endothelial cells [46, 47]. However, this route of transmigration has not been observed in other polarized epithelial beds. Since transcellular transmigration occurs in the areas of lower membrane resistance [48] the columnar shape that many of the polarized epithelial barriers acquire probably makes it very difficult for immune cells to break through single epithelial cells. Thus, immune cells preferentially negotiate epithelial transmigration following a paracellular route between two cells. The receptor from the immunoglobulin superfamily CD47 is located at lateral cell surfaces and interacts with the signal regulatory protein-(SIRP)-α from at least neutrophils, macrophages and dendritic cells [49–51] thereby mediating basolateral-to-apical transepithelial migration. The interaction of these pair of molecules inhibits phagocytosis between immune and cancer cells [51], so these molecules may well regulate the stability of leukocyte-epithelial cell interactions to promote the leukocyte movement across the barrier from the basolateral side. CD47 is also expressed in leukocytes and associates with and regulates their integrin machinery [52]. However, a possible function of CD47 in modulating leukocyte integrin activation from the epithelium has not been reported.

Similar to endothelial cells, some surface receptors forming epithelial cell-cell junctions play a dual role and mediate the process of paracellular transepithelial migration by guiding immune cells across the boundaries between two epithelial cells. Of these, junctional adhesion molecules (JAMs) are paradigmatic receptors from the immunoglobulin superfamily that switch between epithelial cell-cell junctions to heterotypic interactions between endothelial or epithelial cells and leukocytes. The intestinal epithelium mainly expresses JAM-A, JAM-C, JAM4, Coxsackie virus and adenovirus receptor (CAR) and the more distantly related CAR-like membrane protein (CLMP) [53]. It has been reported that the interaction of desmosomal JAM-C with β2-integrins mediates PMN transepithelial migration [54]. Desmosomes are more basolaterally located than tight junctions, so

this interaction probably precedes that established between epithelial CAR and JAM-L expressed in neutrophils, which also participates in the leukocyte transepithelial migration [53, 55]. In contrast, JAM-A helps maintain the integrity of the epithelial monolayer but does not seem to mediate leukocyte crossing of polarized epithelia [56, 57].

PMNs also proactively contribute to open paracellular spaces during transmigration by secreting serine-proteases such as elastase and cathepsin G. These serine-proteases activate basolateral epithelial protease-activated receptor (PAR)-1 and -2 [58]. PARs are G-protein coupled receptors, which are activated by the proteolytic cleavage of their extracellular domain [59]. Active PARs induce actomyosin-mediated contraction and transiently reduce barrier function to facilitate the transepithelial passage of the immune cell [58].

In summary, the first polarized molecular complexes that leukocytes find during their abluminal interactions with the polarized epithelium are those localized mostly in the lateral cell-to-cell junctions, which play a double function maintaining the epithelial barrier function and facilitating the transmigration of immune cells.

6. The Apical Adhesion Machinery

Once leukocytes interact with the epithelial protein machinery exposed in the basolateral domains and traverse the epithelial monolayer, they encounter adhesion proteins confined in the epithelial apical membrane domains. The intercellular adhesion molecule (ICAM)-1 belongs to the immunoglobulin superfamily of transmembrane receptors and interacts with $\beta 2$ integrins from immune cells. Unlike other adhesion receptors that are selectively expressed in endothelial cells, such as E-selectin or VCAM-1, ICAM-1 is broadly expressed in different cell types upon proinflammatory stimulation, including pericytes and parenchymal epithelia. In polarized epithelia, the stimulation of human intestinal epithelial cells with IFNγ or by exposure to enteropathogenic bacteria increases apical ICAM-1, suggesting that luminal adhesion of immune cells is important for the inflammatory response to gastrointestinal pathogens [8, 60, 61]. Apical ICAM-1 promotes neutrophil adhesion and crawling on the apical surface of intestinal epithelial cells [61] as well as luminal-to-abluminal neutrophil transepithelial migration [8]. The engagement of apical ICAM-1 signals to the actomyosin cytoskeleton in these cells, inducing contraction and compromising cell barrier function *in vivo* and *in vitro* [61], in a manner comparable to that previously found in endothelial cells [62, 63]. This suggests that a pathological accumulation of PMN cells in the lumen of the intestine may contribute to intestinal barrier dysfunction in response to infections or during chronic proinflammatory diseases. It is of note that ICAM-1-blocking antibodies only have an effect in apical-to-basolateral transepithelial migration and do not affect basolateral-to-apical migration [8]. This suggests that interactions with ICAM-1 probably follow the leukocyte crossing through JAMs and CD47 basolateral surfaces and mediate the return of immune cells from the epithelial lumen towards the parenchyma.

The hyaluronic acid receptor CD44 also belongs to the immunoglobulin superfamily and participates in a wide range of pathological diseases [64]. CD44 displays a notable heterogeneity due to the alternative splicing from the transcription of a single gene, and by the different glycosylation that each isoform undergoes [64–66]. A blocking antibody against the CD44 isoform CD44v6 inhibits the detachment of PMN from the apical membrane domains of polarized intestinal epithelial cells [67, 68]. Although the molecular mechanisms mediating the role of CD44v6 in the apical membrane have not been described in great detail, C44v6 shedding upon PMN interaction seems to be involved. Specific O-glycosylation of this isoform with sialyl Lewis A is required for PMN interaction with CD44v6, but this is not dependent on integrin interactions (Brazil et al., 2013). An increase in expression of the CD44v6 variant in the apical membrane domains has been detected in inflamed colonic mucosa of patients with ulcerative colitis, suggesting a role for CD44 in mobilizing neutrophils to chronic inflammatory lesions in the intestinal tract. In contrast, other epithelial CD44 isoforms, such as CD44v13, which interact with leukocyte $\beta 2$ integrins, are basolaterally expressed in polarized intestinal epithelial cells and so probably mediate other steps in the haptotactic gradient of the intestinal tissue [69].

Two anti-adhesive molecules are also polarized in the apical membrane domains of the parenchymal epithelium. The decay-accelerating factor, CD55, and the ecto-5′-nucleotidase, CD73, are glycosylphosphatidylinositol (GPI) anchored proteins that are apically expressed in intestinal epithelial cells [70, 71]. Similar to CD44v6, CD55 has anti-adhesive properties, although its ligand for infiltrated leukocytes has not been identified yet. CD73 converts AMP to adenosine, which is anti-inflammatory and, in endothelial cells at least, reduces leukocyte adhesion and transmigration [71]. The effect of apical CD73 on leukocyte passage across epithelial barriers has not been investigated in great detail. In epithelial cells, CD73 participates in the generation of adenosine, required for Cl-secretion. Pathological Cl-release causes secretory diarrea, so a role has been proposed for CD73 on PMN-mediated Cl-secretion and diarrea in pathological conditions [70]. CD73 also helps metabolize the ATP secreted by platelets associated with PMN leukocytes that cross the intestinal barriers in some mucosal diseases, which promote bacterial clearance in inflammatory conditions [6, 72]. In conclusion, apical CD73 is central to regulate not only adhesion, but also the effects of immune cells on epithelial barrier function in a pathological context.

Taken together, the data accumulated so far with respect to ICAM-1, CD44v6 and CD55 suggest that they orchestrate the mobilization of immune cells to the luminal side of the intestine, which determines the intensity, duration and resolution of the inflammatory response. However, as mentioned, the specific ligands of CD55 and CD44v6 on luminally-infiltrated leukocytes have not been identified yet and hence, the molecular machinery in leukocytes involved in their detachment from epithelial apical membrane domains remains to be fully elucidated.

7. Loss of Apicobasal Polarity and Leukocyte Adhesion to Epithelial Cells

A common feature of most epithelial pathologies is the loss of apicobasal polarity. Cell death and cancer transformation are the most evident dysfunctions that cause depolarization, but the loss of polarity also underlies inflammatory disorders, such as the inflammatory bowel disease (IBD), which disrupts cell barrier function in the gut mucosal epithelium [73, 74]. In hepatocytes, hepatitis C virus infection disrupts tight junctions and diminishes the canalicular density in infected livers [75]. Some important diseases in kidney, such as polycystic kidney disease directly affect the formation of apical membranes in renal epithelia [76]. Tubular necrosis or autoimmune renal diseases lead to the malfunction of glomerular filtration by altering epithelial tight junctions [77]. No studies have been performed so far on how apicobasal polarity affects the role in the innate immune response of CD44v6, CD55 or CD73. However, CD44v isoforms and CD73 are mesenchymal markers and appear upregulated not only in inflammatory diseases, but also in malignant transformation of epithelial cells such as in hepatocellular carcinoma [78, 79], and in cancer stem cells [80–82]. Thus, changes in the expression and/or the polarized distribution of these plasma membrane proteins could be involved in the interaction of immune cells with epithelial cells undergoing de-differentiation events during cancer or tissue repair (Figure 2).

However, ICAM-1 polarity has received more attention in polarized hepatic cells. Similar to intestinal epithelial layers, ICAM-1 appears apically localized in cholangiocytes and hepatocytes in human tissues from inflammatory diseases that preserve their parenchymal architecture. In contrast, ICAM-1 is depolarized in regions with inflammatory damage and T-cell infiltration, in which parenchymal organization is clearly affected [7]. Interestingly, *in vitro*, upon hepatic cell depolarization, ICAM-1 is dispersed from apical membrane domains, but remains localized at microvilli at the cell surface and becomes accessible to immune cells (Figure 2(a)). Adhesion experiments with human memory T-cells have shown an inverse relation between the apicobasal polarity and the ability to interact with lymphocytes of these hepatic cells. Moreover, loss-of-function experiments by gene silencing or by using blocking antibodies demonstrated that the increase of T-cell adhesion to depolarized hepatic cells is mediated by ICAM-1 exposed upon loss of polarity [7]. Further characterization of the molecular bases of ICAM-1 apical localization indicates that ICAM-1 interaction with the underlying F-actin scaffold is required for the complete confinement of the receptor in the apical plasma membrane domain. In addition, the analysis of the dynamics of photoactivatable ICAM-1-GFP protein has shown that ICAM-1 follows an indirect route of transport toward the apical membrane domain: ICAM-1 can reach the basolateral membrane of polarized hepatic cells, but is rapidly redirected to the apical domains, probably by transcytosis [7]. Thus, polarized, functional hepatic cells have mechanisms, beyond the regulation of protein expression, for depleting ICAM-1 from basolateral membrane domains potentially exposed to hepatic vessels and immune cells. It is of note that, since columnar epithelial cells also confine ICAM-1 in apical membrane domains, loss of apicobasal polarity may have a similar effect on leukocyte-epithelial adhesion, although this remains to be investigated (Figure 2(b)).

The intracellular vesicular trafficking in these polarized cells seems to be important for limiting the accesibility of these receptors to immune cells and thereby their adhesion, at least *in vitro*. Presumably, other apically polarized receptors involved in leukocyte adhesion, which also interact with the submembranal actin cytoskeleton, such as CD44 isoforms, should be more exposed to parenchymal immune cells upon loss of apicobasal polarity caused by cell transformation, damage or infection, and thus, may modulate leukocyte adhesion to those cells that specifically lose their shape within the epithelial barriers. CD44 and ICAM-1 both interact in their cytoplasmic segments with the ezrin-radixin-moesin (ERM) protein subfamily that connects them to filamentous actin [83, 84]. Long-term stimulation with the inflammatory cytokine TNFα activates ERMs in different cell types [85, 86]. Interestingly, in polarized hepatic cells, TNFα preferentially activates ERMs at the basolateral membrane domains, causing an increase in ICAM-1 exposure, probably by retaining the receptor at the basolateral surface [7] (Figure 2(a)). The molecular requirements for CD44 and ICAM-1 interaction with ERM proteins are quite similar, so it is plausible that inflammatory cytokines may also alter CD44 isoform polarity in different epithelial beds through ERM activation (Figure 2(b)). Therefore, inflammatory cytokines may regulate not only the expression of epithelial adhesion receptors, but also their localization in polarized epithelial cells. Further research on this issue is required, but these findings could have important consequences in the tissue inflammatory response in which infiltrated immune cells need to discriminate operative from dysfunctional, depolarized epithelial cells (Figure 2).

8. Concluding Remarks

In this review we have presented evidence of an essential role for epithelial apicobasal polarity in generating a chemotactic and haptotactic gradient for leukocytes in specific tissues containing parenchymal epithelial layers. This evidence comes from studies of the secretion of several chemokines and the polarization of a few receptors involved in the epithelial–leukocyte interaction. Therefore, a major challenge in the near future will be the systematic analysis of the apical and basolateral secretome and "surfaceome" of the main parenchymal cell types. A systems biology approach integrating all this information is likely to reveal the whole set of molecular cues that leukocytes encounter in the inflamed parenchyma during their journey after extravasation. On the other hand, polarized epithelial cells have sophisticated intracellular machinery for sorting surface proteins to each plasma membrane domain. Immunologists have begun to pay closer attention to the endocytic machinery in leukocytes, which appears to be an important player for the immune response. It is time to investigate how vesicular trafficking of receptors and soluble molecules in polarized epithelial cells

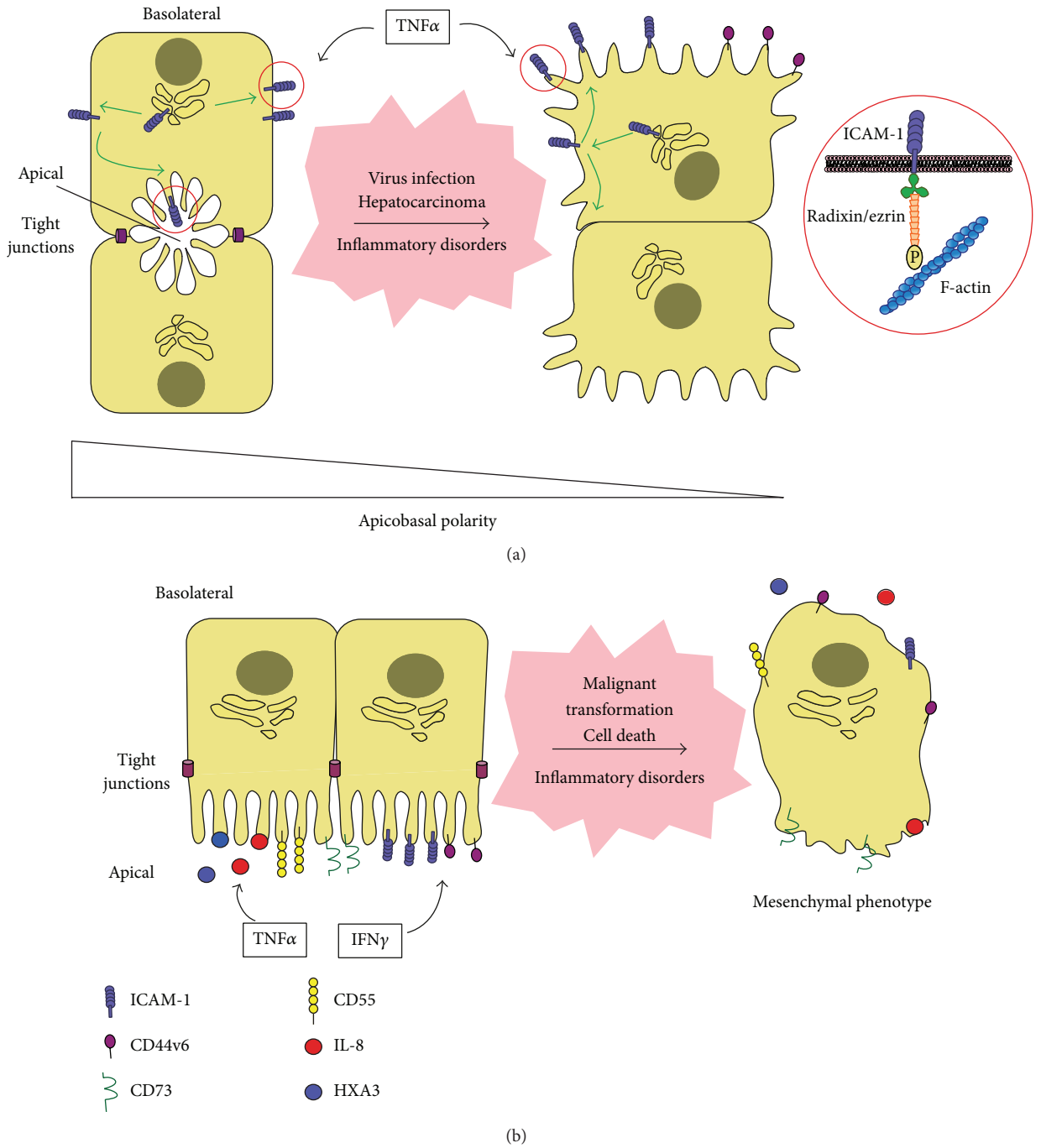

Figure 2: Redistribution of surface receptors and soluble chemoattractants upon loss of apicobasal polarity in epithelial cells. (a) In polarized hepatocytes, ICAM-1 is able to reach the basolateral membrane but is rapidly redirected to the apical membrane domains. Upon hepatic cell depolarization or in response to persistent stimulation with the inflammatory cytokine TNFα, ICAM-1 and ezrin-radixin-moesin (ERM) proteins, which connect the receptor to the underlying actin cytoskeleton, are oriented towards the stromal milieu and become accessible to immune cells and small hepatic vessels [7]. Other apically polarized receptors involved in leukocyte adhesion, which also interact with the submembranal actin cytoskeleton, such as CD44 isoforms, should be more exposed to parenchymal immune cells upon loss of apicobasal polarity. (b) Columnar epithelial cells, such as intestinal cells, express on their apical membrane key chemokines and lipid mediators, adhesion receptors and other membrane proteins involved in the mobilization of immune cells to the luminal side of the epithelial barrier during inflammation. The proinflammatory cytokine IFNγ is central to increase the expression of some of these receptors, namely ICAM-1 [8]. TNFα may also contribute, for example, by stimulating IL-8 secretion from the basolateral or the apical membrane domains. Following epithelial pathologies or cell death, epithelial cells acquire a "mesenchymal phenotype" and therefore, it is plausible to speculate about the loss of polarized distribution of immune cues also in these epithelial cells. However, the relationship between apicobasal polarity and leukocyte adhesion remains to be investigated in columnar epithelial cells.

affect the parenchymal inflammatory response. The effect of inflammatory mediators on this trafficking and, in general, on epithelial apicobasal polarity may provide new therapeutic opportunities for modulating physiological and pathological inflammation in complex tissues.

Conflict of Interests

The authors declare that no conflict of interests exists.

Authors' Contribution

Natalia Reglero-Real and Diego García-Weber equally contributed to this work.

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Clinical Study

Involvement of Angiopoietin-2 and Tie2 Receptor Phosphorylation in STEC-HUS Mediated by *Escherichia coli* O104:H4

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Escherichia coli O104:H4-associated hemolytic uremic syndrome (HUS) is characterized by Shiga toxin-induced vascular damage. As indicated by recent studies, dysregulation of the angiopoietin (Angpt)/Tie2 ligand receptor system may be crucial for endothelial dysfunction in HUS. Early Angpt-2 levels quantified in 48 adult HUS patients were predictive for a complicated clinical course, in particular for need of hemodialysis and mechanical ventilation as well as occurrence of seizures. *In vitro* challenge of human umbilical vein endothelial cells with patients' sera indicated an injurious mediator role of Angpt-2 opening future perspectives for mitigating endothelial activation in HUS.

1. Introduction

Hemolytic uremic syndrome (HUS) is characterized by acute hemolytic anemia, thrombotic microangiopathy, and kidney injury often caused by intestinal infections with Shiga-like toxin-producing *E. coli* (STEC-HUS). STEC-HUS is a medical emergency with a mortality rate of 4% [1]. A significant proportion of patients suffer from complications such as bloody diarrhea, neurological symptoms up to generalized seizures, and acute kidney injury (AKI) or need for renal replacement therapy (RRT) [1]. Many patients with STEC-HUS show spontaneous remission and complete recovery within a few weeks. However, in some patients the disease progresses rapidly requiring best supportive treatment, intensive care, and interventional therapy. Definition of relevant prognostic parameters for early identification of high-risk patients remains a challenge for treating physicians. Instruments of risk evaluation such as prognostic biomarkers remain highly desirable.

Global endothelial damage of the microvasculature and subsequent organ failure is pathophysiological hallmark of STEC-HUS [2]. Some pathological details remain unidentified, while Shiga toxins are known to alter expression of chemokines, chemokine receptors, and molecules of cell adhesion, thereby increasing leucocyte recruitment [3]. In addition, platelets [4] and neutrophil granulocytes [5] are activated. Endothelial reactions include an increased release of tissue-factor [6] and activation of several pathways [7]. Concerning endothelial activation, we also focused on a potential involvement of angiopoietins.

Angiopoietin-1 (Angpt-1) and Angpt-2 are antagonistic ligands of the endothelial tyrosine kinase Tie2. The Angpt/Tie2 ligand receptor system is a nonredundant gatekeeper of endothelial activation and controls the endothelial

phenotype during angiogenesis and inflammation. Angpt-1 is continuously released by pericytes and maintains endothelial quiescence. In contrast, Angpt-2 competitively inhibits binding of Angpt-1 to Tie2 and thereby disrupts protective Angpt-1 signalling, causing loss of vessel integrity, vascular leakage, and expression of inflammatory genes [8]. In a recent study published, the dysregulation of the Angpt/Tie2 system was associated with a diagnosis of STEC-HUS in children [9]. However, clinical and prognostic impact of this phenomenon still remains elusive, and no data is available assessing the role of circulating angiopoietins in adult STEC-HUS patients.

On a single centre basis, we analyzed Angpt-2 plasma levels in adult STEC-HUS patients during the largest reported outbreak of *Escherichia coli* O104:H4 in northern Germany from May till July 2011 [10, 11]. We aimed to determine early predictive factors for complications and outcome.

2. Materials and Methods

2.1. Patients and Study Design. Forty-eight patients with STEC-HUS treated in Hannover Medical School during the outbreak in 2011 were included in this study after obtaining written informed consents from the patients or their legal representatives. The study was performed in accordance with the declaration of Helsinki and approved by the Institutional Review Board at Hannover Medical School (number 1123-2011).

STEC-HUS was diagnosed when patients had EHEC and/or Shiga toxin-2 positive stools or history of bloody diarrhoea between May and July 2011 and fulfilled all of the following three criteria: (1) platelet count below 150/nL, (2) haemolytic anaemia with haemoglobin below the lower limit of normal, and (3) kidney injury as indicated by an increase of urea or creatinine [12]. All of the above patients fulfilled these criteria.

2.2. Blood Sampling and Quantification of Circulating Angpt-1 and Angpt-2. EDTA plasma samples for quantification of Angpt-1 and Angpt-2 were obtained for each patient on different time points (admission and day three after admission or referral to our tertiary care hospital), immediately centrifuged, divided into aliquots, and stored at −80°C. Plasma Angpt-1 and Angpt-2 were measured as described previously [13]. Plasma samples of controls were also anticoagulated with EDTA.

2.3. Endothelial Cell Culture. Passage 5 human umbilical vein endothelial cells (HUVECs) (Lonza, Basel, Switzerland) were cultured, treated, and blotted as described previously in detail [14].

In order to perform fluorescent immunocytochemistry HUVECs were grown to confluence on glass coverslips, fixed, permeabilized, and stained with an anti-VE-cadherin antibody and with phalloidin to visualize F-actin as described previously [15]. These experiments were each time done in duplication and the results were reproducible (data not shown).

Endothelial cells were incubated with EBM-2 containing 5% fetal bovine serum (FBS) to grow to a confluent monolayer. When the experiment was started the tissue culture medium was changed to EBM-2 containing 5% of patients' plasma.

2.4. Measurements of Tie2. Tie2 and phosphoTie2 (pTie2) were measured as described previously [16, 17]. Shortly, antibodies against pTie2 (Y992) (R&D systems, Minneapolis, MN) and Tie2 (C-20) (Santa Cruz Biotechnology, Heidelberg, Germany) were used. For immunoblotting, equal amounts of lysed protein were resolved on 0% polyacrylamide gel and transferred to polyvinylidene fluoride (PVDF) membranes (Merck Millipore, Darmstadt, Germany). Membranes were blocked for 1 hour at room temperature in 3% bovine serum albumin (BSA). All washing steps were carried out in TBST (20 mM Tris, 150 mM NaCl, and 0.1% Tween 20 (Merck)). Afterwards, membranes were incubated with anti-phosphotyrosine antibody (R&D systems) or anti-Tie2 antibody (Santa Cruz). Proteins were visualized by secondary antibodies conjugated to horseradish peroxidase (HRP) (Santa Cruz) and by enhanced chemiluminescence. An exemplary blot from HUVEC lysates stimulated plasma from our patients is shown in Supplemental Figure 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2015/670248.

2.5. Statistical Analysis. Continuous variables are expressed as medians with corresponding 25th and 75th percentiles (Interquartile Range (IQR)) and compared by using the Mann-Whitney rank sum test. Correlations between variables were assessed by Spearman's correlation test or linear regression. Data analysis and figure preparation were performed using GraphPad Prism (GraphPad Prism Software Inc., San Diego, California, USA).

3. Results

3.1. Patients' Characteristics. Our STEC-HUS cohort contained 48 patients (73% female) with a median age of 45 (27–57) years. None had a history of chronic kidney disease. All patients developed haemolysis, thrombocytopenia, and moderate to severe inflammation (Table 1). Median admission creatinine was 259.5 (163–342) μmol/L and 43 patients (89.6%) suffered acute kidney injury (AKI) AKIN I or higher.

3.2. Angiopoietin Levels. Compared to healthy control patients, we found significantly lower plasma levels of Angpt-1 in STEC-HUS patients (1.36 [0.95–2.29] versus 2.5 [1.5–3.4] ng/mL, $p < 0.05$) as well as significantly elevated Angpt-2 (2.6 [1.8–3.28] ng/mL versus 1.39 [0.91–1.64], $p < 0.0001$, resp.) on day 3 after admission.

3.3. Association of Angiopoietins with a Complicated Clinical Course of STEC-HUS. Focusing on STEC-HUS patients, we compared Angpt-1 and Angpt-2 levels within subcollectives following different outcome parameters. In particular, early plasma levels of angiopoietins were then compared between patients being either more severely or less afflicted by clinical symptoms and complications during the course. 32 patients required RRT (66.7%) a median of 5 (3–7.3) days

Table 1: Characteristics, chemistry values, and clinical symptoms in 48 patients with STEC-HUS mediated by *Escherichia coli* O104:H4.

Variable	Admission	Day 3
Demographics		
Number of patients (*n*, %)	48 (100)	
Age (years, median (IQR))	45 (27–57)	
Female sex (*n*, %)	35 (72.9)	
Positive test for Shiga toxin or EHEC (*n*, %)	45 (93.8)	
Onset of diarrhea (median (IQR))	2 (1–4)	
Medical history (*n*, %)		
Arterial hypertension	11 (22.9)	
Diabetes mellitus	2 (4.2)	
Coronary heart disease	2 (4.2)	
Chronic kidney disease	0 (0)	
Laboratory data (median (IQR))		
Creatinine (μmol/L)	202 (120–335)	264.5 (184.8–344)
eGFR (mL/min/1.73 m^2)	26 (15–51.5)	20 (13–33)
LDH (U/L)	1018 (749–1429)	886 (651–1142.3)
Platelets (/nL)	46 (32–64)	52 (23–72)
Hemoglobin (g/dL)	11.2 (9.8–12.4)	9.2 (8.3–10.4)
Angiopoietin-1 [ng/mL]	1.5 (0.7–2.1)	1.36 (0.95–2.29)
Angiopoietin-2 [ng/mL]	2.4 (2–3.2)	2.6 (1.8–3.28)
Clinical data (*n*, %)		
Complicated course	32 (66.7)	
Need of RRT	32 (66.7)	
Seizures	10 (20.8)	
Need of mechanical ventilation	9 (18.8)	

after admission. Angpt-2 levels were significantly higher in patients requiring RRT compared to those without (2.9 [2.1–3.8] ng/mL versus 1.8 [1.2–2.8] ng/mL, $p < 0.05$) (Figure 1(a)). Angpt-2 levels on day three after admission discriminated between patients requiring RRT and those who did not with an area under the receiver operating characteristic curve (ROC) of 0.73 (95% confidence interval, 0.567–0.888; $p = 0.01$) (data not shown). Even on day of admission, Angpt-2 levels were associated with patients requiring RRT compared to those without (2.7 [2.2–3.6] ng/mL versus 1.9 [1.3–2.2] ng/mL, $p < 0.001$) (data not shown).

A median of 10.5 days after admission, 10 patients (20.8%) developed neurological complications ranging from mild cognitive disorder to generalized seizures. Again, early Angpt-2 on day three was significantly increased in those patients with seizures during the course compared to those without (3.1 [2.5–5.7] ng/mL versus 2.4 [1.7–3.1] ng/mL, $p < 0.05$) (Figure 1(b)). The corresponding ROC curve showed an area under the curve (AUC) of 0.71 (95% CI, 0.547–0.882, $p = 0.04$) (data not shown).

Mechanical ventilation was needed in 9 patients (18.8%). Angpt-2 on day three after admission was higher in patients requiring mechanical ventilation compared to those without (3.8 [2.7–5.9] ng/mL versus 2.3 [1.7–3.1] ng/mL, $p < 0.001$) (Figure 1(c)). The AUC of the corresponding ROC curves was 0.80 (95% CI, 0.652–0.952, $p = 0.005$) (data not shown).

Angpt-2 levels on admission did not correlate with occurrence of seizures and need of mechanical ventilation (data not shown). Angpt-2 levels on admission did not correlate with occurrence of seizures and need of mechanical ventilation (data not shown). On admission and day three, neither Angpt-1 nor the Angpt-2/Angpt-1 ratio was associated with need of RRT, mechanical ventilation, or occurrence of seizures (data not shown).

3.4. Disruption of Endothelial Integrity In Vitro Might Be Induced by Angpt-2. Parikh et al. have previously shown that, in sepsis, excess Angpt-2 diminishes Tie2 activation, which leads to paracellular gap formation driven by actin-myosin-based contraction of the cytoskeleton. Addition of Angpt-1 reverses this process [18]. To explore a potential mediator role of Angpt-1 and Angpt-2 we challenged endothelial cells with patients' plasma. We chose plasma from patients with high Angpt-2 levels (Angpt-2: 13.6 and 15.6 ng/mL) and challenged ECs to test the effect of higher Angpt-2 levels on the endothelial phenotype. Fluorescent immunocytochemistry for F-actin, a structural protein of the cytoskeleton, and VE-cadherin, a main constituent of endothelial adherens junctions, were performed. ECs treated with controls' plasma did not affect the confluent, adjacent cell monolayer with cortical actin architecture and the typical cell border localization of VE-cadherin. The same quiescent phenotype was observed using plasma from those HUS patients with a particularly high Angpt-1 concentration (Angpt-1: 15.5 and 17.2 ng/mL) (Figure 2). However, patients' plasma with a high Angpt-2 (Angpt-2: 13.6 and 15.6 ng/mL) disrupted the endothelial

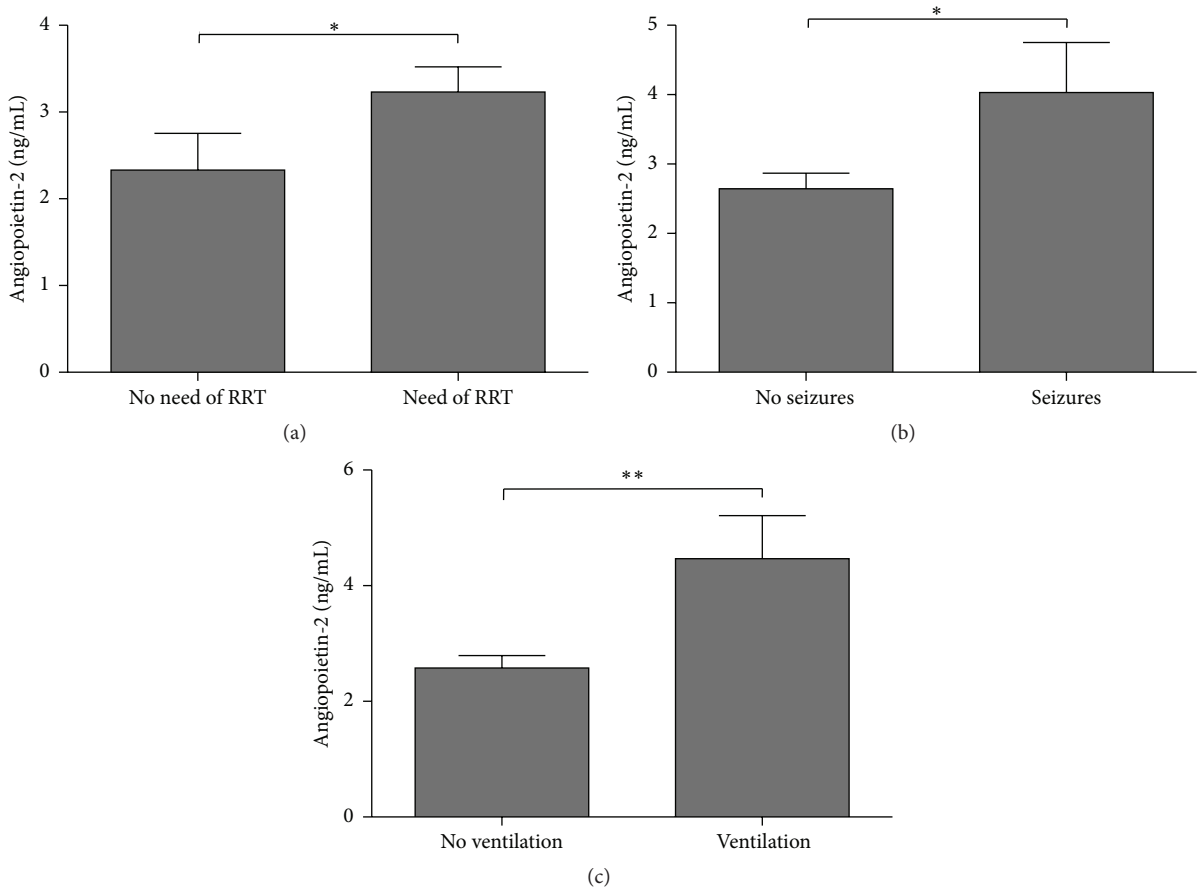

Figure 1: Association of Angpt-2 levels on day three after admission with clinical complications. Boxplots showing associations between Angpt-2 levels and clinical outcomes ((a) need of RRT, (b) occurrence of seizures, and (c) need of mechanical ventilation); $^{*}p < 0.05$ and $^{**}p < 0.01$.

architecture, as depicted by increased actin stress fibers (ASF) and distinct endothelial gap formation. We then performed an experiment to test the effect of plasma with high Angpt-1 and Angpt-2 levels on the endothelial integrity. Exposure of patient's plasma with both high Angpt-1 and Angpt-2 levels (Angpt-1: 12.1 and 10.1 ng/mL, Angpt-2: 9.3 and 12.9 ng/mL, resp.) completely abolished the formation of ASF and interendothelial gaps.

3.5. Phosphorylation of the Tie2 Receptor In Vitro. Ligation of Angpt-1 to Tie2 leads to its tyrosine phosphorylation, whereas Angpt-2 can antagonize Tie2 activation. To test whether or not gap formation is consistent with degree of Tie2 phosphorylation, we immunoblotted EC lysates for pTie2 after having been challenged with the Angpt-1 and Angpt-2 plasma constellations mentioned. The Angpt-1/Angpt-2 ratio was significantly associated with the phosphorylated Tie2/total Tie2 ratio (pTie2/tTie2) ($r^2 = 0.27$, $p = 0.02$) (Figure 3) indicating that these circulating molecules were indeed biologically active and that they target their canonical common receptor as a pathophysiological feature of HUS-associated endothelial dysfunction.

4. Discussion

Although STEC-HUS is categorized as a thrombotic microangiopathy, the incidences of thrombotic events or embolism were rare in the German outbreak 2011 [19]. Renal impairment seems to be also provoked by direct acute tubular damage even when thrombotic events are foreclosed by choice of mouse models [20]. In addition, the contribution of microvascular damage to clinical symptoms in STEC-HUS was indicated by prolonged T2 relaxation times in quantitative MRI scans of patients with neurological complications [21]. For the purpose of investigating mechanisms of endothelial damage and dysfunction in our patients, we correlated angiopoietin levels with outcome parameters.

From our data, we conclude that angiopoietins and Tie2 receptor alterations contribute to the early stage of STEC-HUS. It appears capable of indicating the severity of potential initial endothelial damage and thereby also a prognostic value for the onset of further complications. Affliction of endothelial integrity might be more important than thrombotic events, lightening a new aspect on this disease.

The endothelial integrity is supported by high Angpt-1 levels and impaired by elevated Angpt-2. Endothelial cells

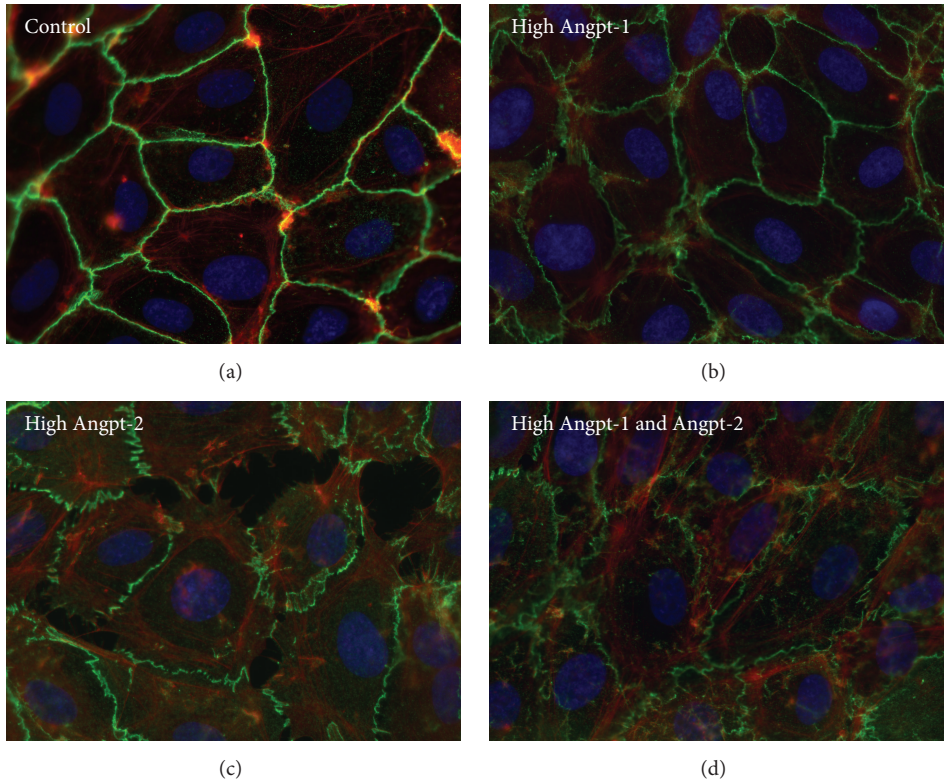

Figure 2: Fluorescent immunocytochemistry microscopy images showing immunofluorescence staining for F-actin (red) and VE-cadherin (green) performed on 100% confluent P5 HMVECs. Cells were treated with 5% prefiltered human EDTA plasma. EDTA plasma samples were collected from healthy controls (a), patients with high Angpt-1 (Angpt-1: 15.5 and 17.2 ng/mL) (b), high Angpt-2 (Angpt-2: 13.6 and 15.6 ng/mL) (c), and both high Angpt-1 and Angpt-2 (Angpt-1: 12.1 and 10.1 ng/mL, Angpt-2: 9.3 and 12.9 ng/mL, resp.) (d).

exposed to plasma with low Angpt-2 and high Angpt-1 levels showed intact cell-cell contacts and a cortical configuration of the cytoskeleton whereas exposure to high Angpt-2 without elevated Angpt-1 levels induced severe gap formation. In particular, the contribution of Angpt-2 is emphasized in our results, predicting the requirement of RRT and mechanical ventilation as well as occurrence of seizures at an early stage of the disease. As a Weibel-Palade body-stored protein, Angpt-2 can be rapidly released upon various stimuli including cytokines, thrombin, hypoxia, activated leucocytes, and platelets [22]. In children with *E. coli* O157:H7 mediated STEC-HUS early dysregulation of angiopoietins has been described recently [9]. During the pre-HUS phase, children exhibit prothrombotic coagulation abnormalities with evidence of early vascular injury preceding HUS with renal injury [23]. However, triggering factors inducing Angpt-2 release are still largely unknown, and clinical and prognostic impact of Angpt-2 plasma levels in STEC-HUS patients have been missing so far. A possible confounder of endothelial barrier disruption could be the presence of EDTA. By analogous confrontation of samples from patients and controls with EDTA, EDTA specific effects should be minimized from the observed differences.

Plasma levels of Angpt-1 showed no significant correlation with severity of affliction in our patients. Due to the sudden initiation of our study during the German outbreak 2011, Angpt-1 measurements could not be performed widely enough and thereby present a limitation to our study. As being expressed more constitutively by pericytes and vascular smooth muscle cells [22, 24], the adaption to a rapid increase of Angpt-2 is likely following delayed to clinical complications. Whether or not the administration of Angpt-1 to individuals afflicted by STEC-HUS could protect from endothelial, renal, or neurological impairment could be a valuable target of future studies. Other studies underlining diagnostic and prognostic potential of circulating Angpt-1 and Angpt-2 in various infectious diseases like malaria and/or sepsis with and without multiorgan dysfunction syndrome (MODS) [13, 18, 25, 26] indicate that detailed investigations of prognostic and therapeutic options for angiopoietins and the Tie2 receptor are worth considering intensely.

Our conclusions are that *E. coli* O104:H4 induced STEC-HUS is likely to be associated with early elevations of Angpt-2 and disruption of endothelial integrity. Early elevated plasma levels supply a valuable approach for prognostic parameters in patients. As STEC-HUS remains a rare disease predominantly affecting children, we encourage monitoring Angpt-2 levels for risk assessment.

In addition, we want to promote the concept of larger, prospective multicenter studies needed to confirm the

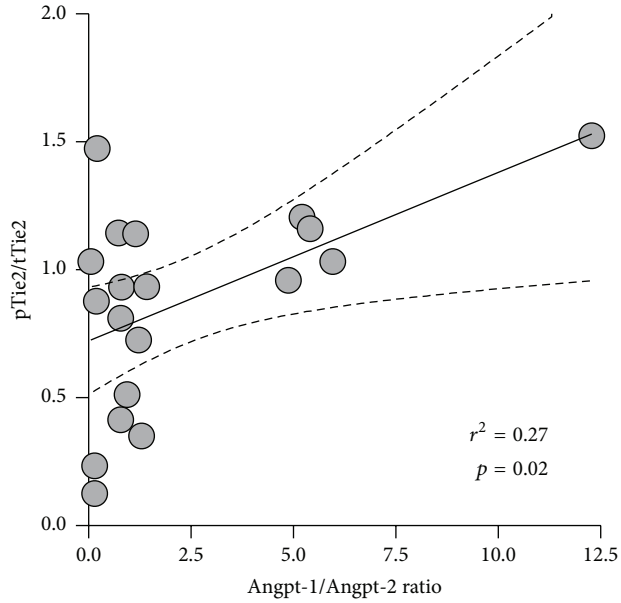

Figure 3: Linear regression shows the correlation between the Angpt-1/Angpt-2 ratio and the phosphorylated Tie2/total Tie2 ratio (pTie2/tTie2).

reliability of prognostic values and possibilities of therapeutic intervention in the Angpt/Tie2 pathway.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Alexander Lukasz and Jan Beneke contributed equally to the paper and are both considered first authors.

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Review Article

Tumor-Induced Local and Systemic Impact on Blood Vessel Function

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Endothelial dysfunction plays a role in several processes that contribute to cancer-associated mortality. The vessel wall serves as a barrier for metastatic tumor cells, and the integrity and activation status of the endothelium serves as an important defense mechanism against metastasis. In addition, leukocytes, such as cytotoxic T-cells, have to travel across the vessel wall to enter the tumor tissue where they contribute to killing of cancer cells. Tumor cells can alter the characteristics of the endothelium by recruitment of leukocytes such as neutrophils and macrophages, which further stimulate inflammation and promote tumorigenesis. Recent findings also suggest that leukocyte-mediated effects on vascular function are not limited to the primary tumor or tissues that represent metastatic sites. Peripheral organs, such as kidney and heart, also display impaired vascular function in tumor-bearing individuals, potentially contributing to organ failure. Here, we discuss how vascular function is altered in malignant tissue and distant organs in individuals with cancer and how leukocytes function as potent mediators of these tumor-induced effects.

1. Introduction

During the last decades, it has become increasingly clear that cancer is a complex disease with systemic effects, which contribute significantly to the mortality. Indeed, the absolute majority of cancer-related deaths is caused by tumor-induced systemic events, such as metastasis and thrombosis. The vasculature is central in these processes, since it is a transport system that spans all organs of the individual. Via this route, tumor-derived factors, as well as disseminating tumor cells, can spread to distant organs, where they contribute to the disease state directly by promoting formation of metastases or indirectly, for example, by induction of thrombosis. In this review, we discuss how endothelial function is affected in individuals with cancer and how the primary tumor dictates these alterations by activation and recruitment of leukocytes. Furthermore, the consequences for tumor progression as well as distant organ function and systemic inflammation in the afflicted individual will be addressed. A summary of the effects discussed in the text can be found in Figure 1.

Tumors stimulate and recruit leukocytes not only to the local tumor microenvironment, but also to other sites in an individual with cancer. For example, tumors express cytokines and growth factors, such as G-CSF and VEGF, which modulate leukocyte stimulation and trafficking over the endothelium. The effects of these tumor-produced factors are however not limited to the site of the primary tumor. Tumor-derived cytokines and growth factors can spread systemically by free transport in the blood or be distributed by carriers such as platelets or microvesicles [1, 2]. Several of these tumor-derived factors affect the integrity and function of the endothelium, either directly or secondary to changes in endothelial-leukocyte interactions.

2. Local Effects in the Tumor Microenvironment

Compared to healthy vessels under physiological conditions, the tumor vasculature is frequently poorly functional with

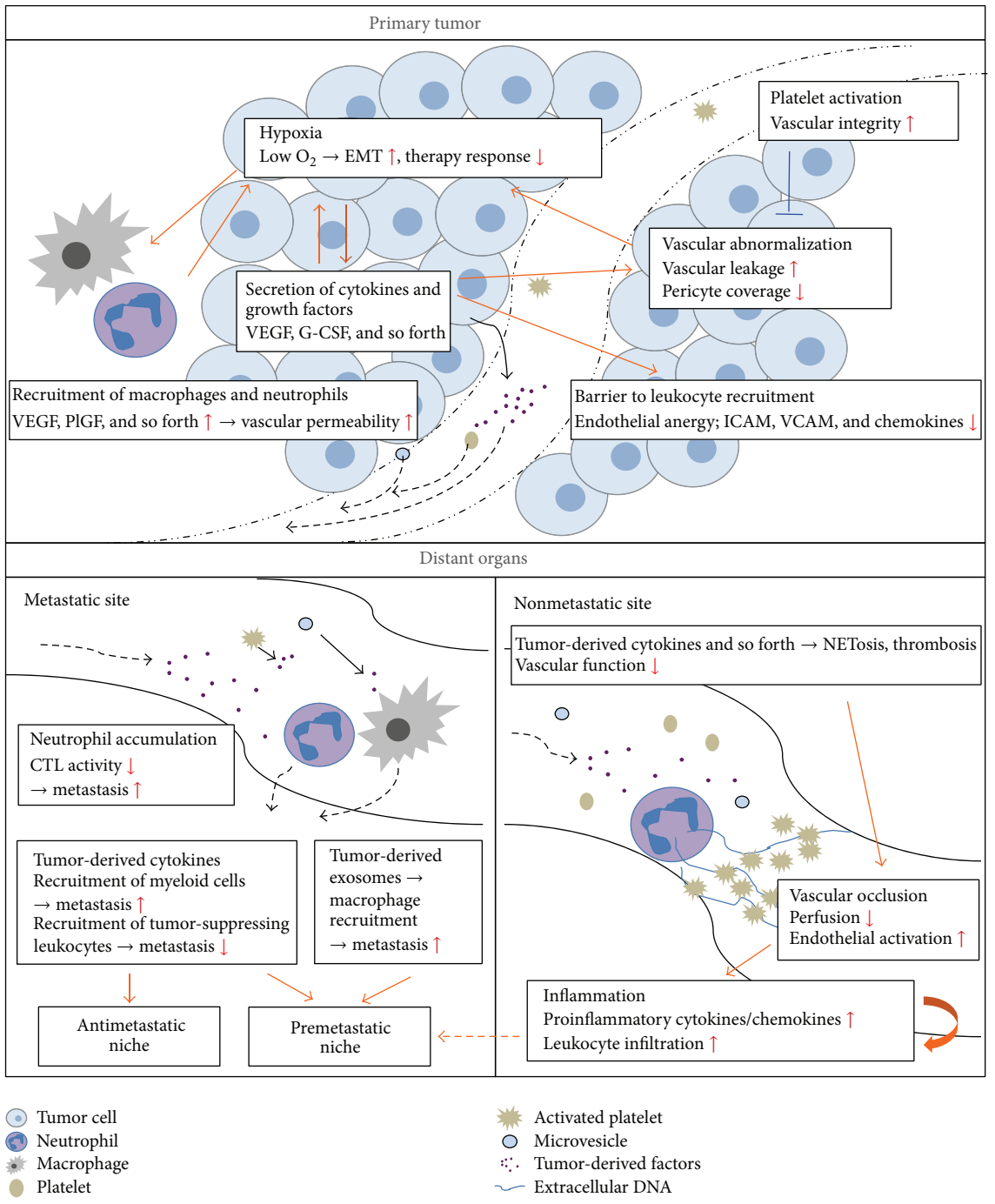

Figure 1: Altered function of blood vessels in tumor tissue and distant organs in individuals with cancer. Vascular function is impaired both at local tumor level and systemic level in an individual with cancer. The primary tumor secretes proangiogenic growth factors that contribute to vascular abnormalization with enhanced permeability and anergic endothelial cells within the tumor. The poor vascular function leads to hypoxia and subsequent recruitment of macrophages and neutrophils that further contribute to vascular permeability by secretion of additional proangiogenic factors. Hypoxia stimulates tumor invasiveness by induction of EMT and contributes to impaired therapy response. Effects on the vasculature are not limited to the actual tumor, but altered vascular function is also found in distant organs of tumor-bearing individuals. Tumor cell-derived cytokines are spread throughout the body in plasma or as cargo in platelets or microvesicles and can contribute to formation of pre- or antimetastatic niches in organs that exert sites for metastasis. These effects are mainly mediated by recruitment of leukocytes to the metastatic sites, which prepare the microenvironment to facilitate metastatic colonization. Furthermore, tumor-derived factors stimulate NETosis and thrombosis in distant organs leading to vascular occlusion and systemic inflammation also in organs that are not sites for metastasis.

permeable and leaky vessels, and the hierarchical organization is often lost and replaced by a chaotic vascular system with disturbed blood flow [3]. This typical characteristic of the tumor vasculature has extensive impact on tumor progression. Poor vascular function leads to intermittent or chronic hypoxia, which affects the tumor phenotype directly and contributes to increased tumor invasiveness and metastasis by induction of Epithelial-Mesenchymal Transition (EMT) [4]. The vascular function also affects the response to therapy, since good vascular perfusion is crucial for delivery of therapeutic substances to the tumor, and maintained oxygen tension and physiological pH are required for efficient killing of tumor cells by radiation and chemotherapy. Importantly, the vasculature regulates recruitment of leukocytes to the tumor, and the recruited leukocytes in turn affect vascular function.

2.1. The Tumor Endothelial Barrier. During inflammation and wound healing, proinflammatory cytokines stimulate endothelial cells to upregulate adhesion molecules and chemokines that together mediate the capture and extravasation of leukocytes from the blood to the tissue. Tumor endothelial cells are anergic in the sense that they respond poorly to proinflammatory stimulation. This is at least in part due to constant stimulation by proangiogenic factors, including FGF and VEGF, which inhibit TNF-α-induced upregulation of ICAM, VCAM, and chemokines through interference with NF-kappaB-signaling pathways [5–9]. Consequently, antiangiogenic therapy can restore adhesion molecule expression in tumor endothelial cells and induce leukocyte recruitment [8, 9]. The tumor vessels may also block the activation of T-cells that are recruited to the tumor tissue by expressing inhibitory molecules such as PDL1 and IDO1 or directly induce T-cell apoptosis by expression of death-receptor family members including TRAIL or FASL [10, 11]. Thus, tumor endothelial gene expression may significantly affect the quantity and activation of leukocytes recruited to the tissue. Indeed, endothelial expression of the Endothelin B receptor has been shown to inhibit T-cell recruitment in ovarian cancer and decrease efficacy of cancer immunotherapy [12]. The location and quantity of tumor-promoting macrophages and tumor-inhibiting cytotoxic T-cells are predictive of survival in many types of solid tumors [13], and the success of cancer immunotherapy strictly depends on efficient recruitment of tumor-targeting immune cells [14]. Therefore, the endothelial barrier represents an attractive target for treatment of cancer [15]. Importantly, the recruited immune cells also affect tumor vessel quality and gene expression, as delineated below.

2.2. Tumor-Promoting Effects. Cells of the innate immune system, such as macrophages and neutrophils, are crucial regulators of angiogenesis and vascular properties in the tumor microenvironment. Macrophages are often classified into two subpopulations: the proinflammatory M1 macrophages with tumor-suppressing properties and the immunosuppressive M2 macrophages considered as tumor promoters. However, it is now emerging that the division into two distinct macrophage subpopulations is too simplified and that macrophages likely display a spectrum of phenotypic variation [16]. Macrophage recruitment is stimulated by hypoxia and infiltration into hypoxic tumor areas is guided by tumor-derived factors such as VEGF or CCL2 [17–19]. Upon arrival, the hypoxic tumor microenvironment stimulates macrophages to produce VEGF and MMPs, which promotes angiogenesis and contributes to permeability of the tumor vasculature. In addition, macrophages produce numerous other growth factors (PlGF, FGF, PDGF, M-CSF, and TGF-β) and cytokines (IL-1, IL-8, and TNF-α) that stimulates angiogenesis and activates the endothelium [20, 21].

Similar to macrophages, neutrophils are potent regulators of tumor angiogenesis. Recruitment and transendothelial migration of neutrophils are mediated via chemokine signaling, and tumor-derived CXCL8 has been suggested to play an important role in these processes [22, 23]. At the tumor site, TNF-α can induce direct release of VEGF from the neutrophils [24]. Furthermore, neutrophils secrete MMP-9, which contribute to increased release of VEGF bound to the extracellular matrix and further promote angiogenesis and vessel permeability [25, 26]. Innate immune cells such as macrophages and neutrophils hence contribute significantly to the permeable and leaky vascular phenotype observed in tumors, mainly by increasing the concentration of bioavailable VEGF in the microenvironment.

Another cell type that has been shown to maintain the endothelial barrier and increase tumor growth is the platelet. In tumor vessels, platelets play an important role in protecting tumor vessels from hemorrhage [27–29]. Depleting mice with established tumors from platelets results in bleeding specifically in the tumor tissues [27]. Furthermore, it has been demonstrated that inflammation and associated leukocyte infiltration are causing the tumor hemorrhage during thrombocytopenia [27, 30]. If neutrophil infiltration into the tumor tissue is reduced by genetic deletion of beta2-integrin (CD18−/−), tumor hemorrhage is suppressed after platelet depletion [28, 30]. A role for macrophages in tumor-induced bleeding during thrombocytopenia was also described [28]. Recently, the importance of leukocytes was further supported by a study showing that diapedesis of neutrophils through the endothelium is crucial for hemorrhage during thrombocytopenia in several mouse models of inflammatory disease [31]. It was further demonstrated that the vessel-protective effect of platelets is mediated by secretion of platelet granules rather than platelet adhesion to the endothelium [27]. Platelet granule secretion was suggested to provide factors that suppress permeability, such as serotonin and angiopoetin-1, and hence balance the permeability promoting effect of VEGF. It has also been demonstrated that platelets contribute to integrity and function of the tumor vasculature by affecting pericyte coverage [32]. Platelet depletion of transgenic RIP1-Tag2 mice with insulinoma resulted in significantly decreased pericyte coverage of the tumor vasculature and severely impaired perfusion. How platelets support pericyte coverage of the vasculature in a tumor remains to be explored.

2.3. Tumor-Suppressing Effects. Infiltration of innate immune cells may not only play a tumor-promoting role but can also under certain conditions and in some types of cancers exert tumor-suppressing effects. While a high number of tumor infiltrating neutrophils correlate with poor survival in a variety of different tumors types [33–37], the opposite has been demonstrated, for example, in patients with gastric cancer [38]. Furthermore, tumor suppressive effects of infiltrating neutrophils have also been demonstrated in various experimental models of breast cancer. Using an *in vitro* approach, it was shown that neutrophil-derived elastase (NE) was taken up by breast cancer cells and contributed to T lymphocyte-mediated tumor cell lysis [39]. In an orthotopic mouse model of breast cancer, neutrophils were further found to suppress metastasis by preventing metastatic seeding in the lungs [40]. In addition to the more prominent proangiogenic role of neutrophils described earlier, they also contain antiangiogenic mediators such as NE that can suppress VEGF-mediated angiogenesis and leakage and hence support integrity of the tumor vasculature [41–43]. The high number of tumor infiltrating macrophages correlates in the majority of tumor types with poor prognosis, reflecting the fact that macrophages mainly exert tumor-promoting effects. Some reports however suggest a correlation between high level of macrophage infiltration and positive prognosis in patients with osteosarcoma and gastric cancer [44, 45]. The tumor-suppressing effects are mediated by proinflammatory macrophages, often referred to as M1 macrophages. Macrophages of the proinflammatory phenotype, induced, for example, by IFN-γ, produce Reactive Oxygen Species (ROS) and proinflammatory cytokines such as IL-1β and IL-6 that contributes to activation of the endothelium [46]. This further promotes recruitment of cytotoxic T-lymphocytes to the tumor microenvironment, which can suppress growth of the tumor.

The adaptive immune system has mainly been attributed a tumor-suppressive role. However, B-lymphocytes may support inflammation-associated epithelial carcinogenesis [47] and regulatory T-lymphocytes are frequently induced in the tumor microenvironment and suppress the antitumorigenic activity of cytotoxic T-lymphocytes [48]. Classifying tumors according to the "immunoscore," which takes into account the location and prevalence of different leukocyte subsets in the tumor microenvironment, can be used to predict patient survival for several solid tumor types [49]. Immune checkpoint therapy, involving reactivation of cytotoxic T-cells with antibodies targeting CTLA4 or PDL1/PD1, has recently gained success in the clinical treatment of cancer [50].

3. Systemic Effects on Peripheral Vasculature and Organ Function in Individuals with Cancer

Leukocyte-mediated effects on vascular function are not limited to the local tumor microenvironment but appear to reach far beyond the actual tumor. Altered endothelial integrity and recruited immune cells can affect malignant progression directly by altering the milieu in organs that represent sites for metastasis—even before the tumor cells arrive. Furthermore, recent data show that vascular function is impaired in distant organs not directly affected by either the primary tumor or metastases in mice with cancer.

3.1. Tumor-Induced Effects on Organs that Represent Metastatic Sites. Metastasis, responsible for the absolute majority of cancer-related deaths, is a complex and challenging process for the tumor cells. Indeed, only a small fraction of the disseminating tumor cells will eventually succeed in establishing a secondary tumor in a distant organ.

It has however been demonstrated that the primary tumor can facilitate metastatic colonization by orchestrating systemic processes that prepare the distant organ before the metastatic tumor cells arrive, that is, creating a premetastatic niche. This was first suggested more than a decade ago, when several studies showed that tumor-derived VEGF-A, PlGF, TGF-β, and TNF-α contribute to recruitment of CD11b+ myeloid cells to the lungs in tumor-bearing mice before tumor dissemination and that this results in enhanced recruitment of metastatic cells to the lung [51–53]. Since then, additional tumor-derived factors (LOX, CCL2, and VCAN) have been shown to stimulate recruitment of bone-marrow-derived cells (BMDCs) and hence contribute to formation of the premetastatic niche in a similar manner [54–56]. Besides a few exceptions [56–58], these studies focus on lung tissue, and whether the described effects occur also in other organs with metastatic growth, or even throughout the body, has not been firmly established. Some lines of evidence do support that this is a general phenomenon. A few years ago, a study revealed that systemic inflammation, induced by arthritis, enhanced metastasis in a transgenic mouse model of mammary carcinoma [59]. This effect was observed not only in lung but also in bone marrow, indicating that systemic inflammation may be a general promoter of metastasis. This hypothesis was recently confirmed by data from Coffelt and colleagues demonstrating that systemic neutrophil expansion and accumulation in multiple organs occurs in a mouse mammary tumor model with spontaneous lung metastases [60]. These tumor-induced neutrophils suppressed the ability of CD8+ cytotoxic T-cells to kill tumor cells, thus resulting in an increased metastatic burden. Another recent paper also reports on systemic accumulation of neutrophils in peripheral organs in mice with distinct tumor types such as mammary carcinoma and insulinoma [61]. Furthermore, upregulation of leukocyte adhesion markers as well as proinflammatory cytokines such as IL-1β, IL-6, and CXCL1 was detected in the kidney tissue, indeed supporting an ongoing systemic inflammation in individuals with cancer [61].

While the factors responsible for formation of the premetastatic niche may be distributed freely in the circulation, they were recently also reported to spread as cargo in tumor-derived exosomes. This mechanism was first described in mouse models of melanoma [62, 63] but was recently demonstrated also in mice with pancreatic ductal adenocarcinoma (PDAC) [64]. Costa-Silva and colleagues showed that primary tumor-derived exosomes promote enhanced metastatic burden in the liver. This effect was mediated by increased

macrophage recruitment from the bone marrow, induced by macrophage migration inhibitory factor (MIF) expressed in the exosomes [64].

In contrast to the situations discussed above, some reports suggest that tumor-derived factors can stimulate leukocytes to function as metastatic suppressors and as such contribute to formation of antimetastatic niches. It was, for example, demonstrated a few years ago that tumor-entrained neutrophils (TENs), upon stimulation by tumor-derived G-CSF and CCL2, prevent lung metastasis [40].

3.2. Tumor-Induced Effects on Organs that Do not Represent Sites for Metastases. While a vast amount of research has focused on organs that represent sites for metastasis, less is known about cancer-induced effects in distant organs that are not affected by either primary or secondary tumor growth. However, one recently published paper demonstrate that mice with cancer display significantly impaired function of the vasculature in heart and kidney, organs that are not targets for metastasis in the tumor models used [61]. Furthermore, it was shown that the reduced peripheral vascular function was caused by formation of Neutrophil Extracellular Traps (NETs), which occlude peripheral vessels in tumor-bearing mice [61]. NET formation (NETosis) was first described in 2004 as a novel mechanism used by neutrophils to fight bacterial infections [65]. During NETosis, neutrophils secrete their chromatin together with proteases such as Myeloperoxidase (MPO) and Neutrophil Elastase (NE). However, NETs are also highly prothrombotic, mainly due to the negatively charged chromatin and associated histones. In this way, neutrophils undergoing NETosis may also stimulate thrombosis, leading to further vascular occlusions [66, 67]. Removal of the intravascular NETs by DNase treatment restored functionality of the peripheral vessels in tumor-bearing mice [61]. In addition to occluding the vessels, NETs may damage the vasculature in other ways. It was previously shown that NETs have cytotoxic effects on the endothelium and that they directly induce endothelial damage in other pathological conditions [68–70].

Organ failure in general, and acute renal failure (ARF) in particular, is a cause of substantial morbidity in cancer patients and is characterized by hypoperfusion of the kidney vasculature [71]. The mechanisms behind tumor-induced organ failure are however poorly studied. Systemic intravascular NET formation offers a potential explanation for how these fatal effects occur.

A link between cancer and NETosis was first demonstrated in 2012, when Demers and colleagues showed that cancer is a predisposing factor for NETosis in mice and that this subsequently contributes to thrombosis [72]. Formation of NETs can also directly contribute to malignant progression. In mice with liver tumors exposed to sepsis, NETs that formed due to the infection were reported to sequester circulating tumor cells and promote metastasis [73]. These data imply that an infection is a potential risk factor for metastasis. It is also possible that NETs facilitate metastasis by inducing inflammation and upregulation of adhesion molecules in peripheral vessels [61], thereby offering a route for extravasation in a secondary organ. It has, for example, been shown that VCAM-1 can be used by tumor cells to adhere to the endothelium and hence facilitates transendothelial migration [74]. Furthermore, tumor cell expression of E-selectin binding ligands such as Sialyl Lewis (a) has been correlated to malignancy and prognosis in the clinic [75, 76]. Whether NETs really promote extravasation remains to be explored.

4. Conclusion and Perspective

Endothelial activation and vascular integrity are crucial regulators of tumor progression and related systemic effects (see summary in Figure 1). Serving as a barrier for infiltrating leukocytes and metastasizing tumor cells, the endothelium plays an important role in protecting us from the fatal processes responsible for cancer-related deaths. When designing new cancer therapies, it is therefore of utmost importance to consider potential effects on the vasculature in the local tumor microenvironment, as well as in peripheral organs. For immunotherapeutic approaches it would be beneficial to enhance endothelial transmigration of cytotoxic T-lymphocytes into the tumor, to improve the killing of tumor cells. On the systemic level, inflammation and endothelial activation should probably be kept as low as possible, to avoid tumor extravasation into secondary tissues.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Role of Calprotectin as a Modulator of the IL27-Mediated Proinflammatory Effect on Endothelial Cells

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An underlying endothelial dysfunction plays a fundamental role in the pathogenesis of cardiovascular events and is the central feature of atherosclerosis. The protein-based communication between leukocytes and inflamed endothelial cells leading to diapedesis has been largely investigated and several key players such as IL6, TNFα, or the damage associated molecular pattern molecule (DAMP) calprotectin are now well identified. However, regarding cytokine IL27, the controversial current knowledge about its inflammatory role and the involved regulatory elements requires clarification. Therefore, we examined the inflammatory impact of IL27 on primary endothelial cells and the potentially modulatory effect of calprotectin on both transcriptome and proteome levels. A qPCR-based screening demonstrated high IL27-mediated gene expression of *IL7*, *IL15*, *CXCL10*, and *CXCL11*. Calprotectin time-dependent downregulatory effects were observed on IL27-induced *IL15* and *CXCL10* gene expression. A mass spectrometry-based approach of IL27 ± calprotectin cell stimulation enlightened a calprotectin modulatory role in the expression of 28 proteins, mostly involved in the mechanism of leukocyte transmigration. Furthermore, we showed evidence for STAT1 involvement in this process. Our findings provide new evidence about the IL27-dependent proinflammatory signaling which may be under the control of calprotectin and highlight the need for further investigations on molecules which might have antiatherosclerotic functions.

1. Introduction

Atherosclerosis is characterized by the narrowing of arteries caused by plaque formation and is a prominent representative of cardiovascular diseases [1, 2]. Interestingly, development of atherosclerosis occurs often prior to other cardiovascular events such as strokes or heart attacks. Under healthy conditions, the vascular system contains an endothelium composed of a monolayer of cells and forms a barrier between the circulating blood and the vessel wall [3]. The autocrine, paracrine, and endocrine mechanisms of the vascular endothelium can exert modulatory effects like the capacity to regulate cell activation as well as proliferation influencing the growth and metabolism of the surrounding tissue [4]. Furthermore, it has a key role being a gatekeeper by regulating leukocyte trafficking between the blood and the underlying tissue [5]. Alterations of endothelial functions can be caused by several risk factors including smoking, hypercholesterolemia, hyperglycemia, genetic factors, hypertension, ageing, or inflammation [1, 2, 6–8]. While a normal quiescent endothelium induces almost no expression of proinflammatory molecules [6], the recognition of endo- and exogenous danger signals by endothelial cells (ECs) can lead to inflammatory responses through the expression of adhesion molecules, secretion of inflammatory proteins, and morphological changes of ECs [9, 10].

A key event in the early vascular inflammation process is the recruitment and adhesion of leukocytes prior to

transendothelial migration into inflammatory sites, a mechanism which involves cytokines, hormones, pathogen associated molecular pattern molecules (PAMPs), and damage associated molecular pattern molecules (DAMPs) [11, 12]. The role of cytokines and DAMPs, especially their atherogenic activities and their involvement in the acceleration of vascular diseases, is already well documented [13–18]. Regarding the IL6-family member IL27 cytokine, composed of the subunits p28 and Epstein-Barr-virus-induced gene 3 (EBI3), its participation in immune responses and diseases is more and more accepted [19]. Contradictory observations regarding its pro- or anti-inflammatory role are however reported [20, 21]. It has been shown *in vivo* that IL27 reduced inflammation by suppressing excessive Th1 immune responses during infection, and *in vitro* in several T cell subtypes it has been shown that IL27 induced the production of the anti-inflammatory IL10 [22, 23]. More recently, the anti-inflammatory role of IL27 as an upstream activator of the STAT3 pathway was also established [24]. In the context of atherosclerosis, Hirase and coworkers demonstrated that mice with IL27 receptor deficiencies develop atherosclerotic lesions [25].

On the other side, other data rather strive for proinflammatory activity of IL27, as described by Guzzo and collaborators in primary monocytes [26] or by Nam and coworkers who demonstrated that IL27 is secreted from pre- and normal adipocytes under inflammatory conditions [27]. Moreover, in terms of atherosclerosis development, IL27 is known to induce in HUVECs the upregulation of the chemokines CXCL9 and CXCL10, implicated in the transendothelial cell migration [19, 28]. In a human study, higher serum levels of IL27 were detected in patients suffering from coronary artery diseases (CAD) such as myocardial infarction and stable and unstable angina pectoris [29]. A pathway analysis of primary tissue from different coronary atherosclerotic lesion demonstrated an upregulation of IL27 in the early developed lesions of atherosclerotic material, which emphasizes an important role of IL27 in the development of atherosclerosis [30]. Altogether these contradictory data about the exact role of IL27 in inflammation suggest the existence of cytokine-specific regulation processes occurring during cell to cell communication.

Calprotectin, a S100A8/S100A9 heterodimer member of the S100 protein family, is also known to be involved in acute and chronic inflammation [31, 32]. Most of the publications propose a proinflammatory function for calprotectin, and regarding the development and progression of atherosclerosis, both *in vitro* and *in vivo* studies suggest a proatherogenic role for calprotectin [33–36]. On the contrary, it has been reported that administration of calprotectin induces immunosuppressive functions in rat animal models [37, 38], indicating that, similar to IL27, calprotectin may have opposite regulatory functions.

In this study, we enlightened the involvement of IL27 and calprotectin in the regulation of the inflammatory state of the endothelium in terms of pro- and antiatherogenic functions. Moreover, with the aim of identifying potential synergistic, additive, or antagonistic effects from other mediators, we analysed the role of calprotectin in IL27-mediated transcriptome and proteome regulation.

2. Materials and Methods

2.1. HUVEC Isolation, Purification, and Activation. After informed consent of parturients (Comité National D'Ethique de Recherche Luxembourg 2013/01v1.0), primary Human Umbilical Vein Endothelial cells (HUVECs) were isolated with 1 mg/mL collagenase NB4 (SERVA) from fresh umbilical cord veins from planned C-sections (protocol adapted from [39]). The HUVEC cell cultures were grown on 0.2% gelatine-coated tissue flasks and with complete M199 (SIGMA) supplemented with EGM2 SingleQuots (LONZA, Verviers, Belgium) and 2 mM L-glutamine (SIGMA) at 37°C in humid atmosphere with 5% CO_2. Purity of HUVEC cell cultures was assessed by flow cytometry; the following antibodies were used: mouse anti-human CD31-PE, mouse anti-human CD144-A647, and mouse anti-human CD146-PerCP-Cy5.5 antibodies (all from BD Sciences, Erembodegem, Belgium). Passage numbers 2–4 were used for the activation of HUVECs. Stimulation experiments with HUVECs were prepared with a cell density of 2.5×10^5 cells/mL in 6-well plates (Greiner, Vilvoorde, Belgium) with 1 mL/well complete M199 media for 24 h, and with a subsequent depletion phase of 12 h with EGM2 SingleQuots-depleted M199 media supplemented with 2% FBS (LONZA), gentamycin (LONZA), and 2 mM L-glutamine (SIGMA, MA). To determine the optimal stimulation concentrations, HUVECs were incubated for 12 h with ranges of 10, 30, and 100 ng/mL IL27 (R&D Systems, Abingdon, UK) and 1, 5, and 10 μg/mL calprotectin (Hycult, Uden, Netherlands) (3 biological replicates). In the costimulation assays, for transcriptome analysis, HUVECs were stimulated with IL27 (30 ng/mL) ± calprotectin (1 μg/mL) for 3, 6, 12, and 24 h (6 biological replicates) and for intracellular proteome analysis a time course of 6, 12, and 24 h was performed (9 biological replicates). Furthermore, HUVECs were stimulated with TNFα (2 ng/mL) (Peprotech, NJ) ± calprotectin (1 μg/mL) for 3, 6, 12, and 24 h (3 biological replicates). Stimulations were stopped by washing the adherent HUVECs with 1 mL of ice cold sterile PBS and the 6-well plates were directly frozen at −80°C. Cell viability was assessed by LDH release using cytox96 nonradioactivity cytotoxicity assay (Promega, Leiden, Netherlands).

2.2. RNA Extraction and RT-qPCR. RNA extraction from HUVECs was performed according to the instructions and solutions of the ReliaPrep RNA Cell Miniprep System (Promega). 250 μL of the lysis buffer was added to each well and cells were scratched off. Afterwards, purified RNA was eluted with 30 μL nuclease-free water. RNA yield and purity were determined by spectrophotometer NANOdrop 2000 (Thermo Scientific, CA). For reverse transcription, 0.2 μg random primer (Promega) was added to 1 μg RNA and incubated for 5 min at 70°C, followed by a subsequent addition of 5x reaction buffer, 20 U (final) RNAsin Ribonuclease inhibitor (Promega), 0.5 mM (final) dNTP (Promega), and 160 U (final) GoScript reverse transcriptase (Promega) with

incubations of 5 min at 25°C, 60 min at 42°C, and 10 min at 70°C.

For determination of optimal stimulation concentration, a 96-multigene array TaqMan Human Immune panel (cat. 4370573) was used. The cDNA (437.5 ng) was mixed with TaqMan Universal Master mix II (Applied Biosystems, CA) and added per well. Parameters for qPCR were set as follows: 2 min at 50°C, 10 min at 95°C, and 40 repetitions of 15 s at 95°C and 60 s at 60°C (QuantStudio 12K Flex v1.1, Applied Biosystems). Data analysis was performed by Expression Suite v1.1 software. For time course analysis, the following TaqMan primers were used: IL7 Hs00174202_m1, IL15 Hs00174106_m1, CXCL10 Hs00171042_m1, CXCL11 Hs00171138_m1, and the HKGs ACTB Hs99999903_m1, GAPDH Hs99999905, and GUSB Hs99999908_m1. In total, 25 ng of cDNA was mixed with TaqMan Universal Master mix II (Applied Biosystems) and added per well of a MicroAmpFast 96-well plate (Applied Biosystems). Parameters for qPCR were set as follows: 10 min at 93°C and 40 repetitions of 15 s at 95°C and 60 s at 60°C (QuantStudio 12K Flex v1.1, Applied Biosystems).

Quantitative analysis was performed by the method of Willems and collaborators [40]. Internal normalization was performed to ACTB, GAPDH, and GUSB housekeeping genes (HKGs).

2.3. Proteomics

2.3.1. Sample Preparation. For cells lysis, 50 μL 8 M urea was added directly to each well and cells were scratched off, transferred, and subsequently sonicated for 1 h at 4°C. Afterwards 200 μL of 0.8% RapiGest (Waters) within 50 mM NH_4HCO_3 and 2 mM DTT were added and incubated for 1 h at room temperature (RT), followed by addition of 10 mM MMTS (Pierce, Erembodegem, Belgium) and incubation of 1 h at RT in the dark. Digestion of proteins in peptides was achieved by addition of 1 μg Trypsin Gold (Promega) for 24 h at RT and stopped by addition of 1% TFA for 1 h at RT. Afterwards samples were salted out by using 3 M Empore cartridges (3 M Bioanalytical, MS). Samples were loaded on cartridges, eluted with 200 μL of 70% acetonitrile (ACN) within 0.1% TFA, and further dried in vacuum centrifuge (Speedvac, Thermo Scientific). Dried samples were resuspended with 20 μL of 0.1% TFA. Samples were loaded on Zip Tips C18 (ZTC18S960, Millipore, Molsheim, France) and eluted with 20 μL 70% ACN within 0.1% TFA and dried in vacuum centrifuge (Speedvac, Thermo Scientific). Before measurement, samples were resuspended in 10 μL of 2% ACN within 0.1% TFA.

2.3.2. LC-MS/MS. Peptide analysis was performed by LC-MS/MS on an EASY-nLC Ultra HPLC (Thermo Scientific) coupled to a hybrid dual-pressure linear ion trap/orbitrap mass spectrometer (LTQ Orbitrap Velos Pro, Thermo Scientific). Dissolved peptide samples were separated on a 75 μm (inner diameter), 25 cm, PepMap C18-column (Dionex-Thermo Sciences). A solution gradient ranging from 2% to 40% ACN within 0.1% formic acid at a constant flow rate of 300 nL/min for 200 min enabled the preseparation of peptides. Eluting peptides were ionized in a nanospray interface. Regarding the MS/MS settings, collision-induced dissociation (CID) was applied for the 15 most abundant ions detected in the full MS scan. Essential MS settings were as follows: full MS (FTMS; resolution 60000; m/z range 400–2000) and MS/MS (linear trap; minimum signal threshold 500; isolation width 2 Da; dynamic exclusion time setting 30 s; singly charged ions were excluded from selection; normalized collision energy was set to 35% and activation time to 10 ms).

2.3.3. Label-Free Quantification. Progenesis QI for proteomics (Waters, Nonlinear Dynamics, MA) was used for label-free quantification of LC-MS derived data. First, alignment of the two-dimensional ion intensity map representing the retention time and mass to charge ratio of peptides was performed, followed by quantification of signals and finally peptide and protein identification by database search. In order to rely on proteins that were confidently identified, a cut-off for at least two unique peptides per protein was applied.

2.3.4. Data Normalization. The progenesis QI for proteomics quantified proteins was still skewed toward high abundances. Thus, abundances were further processed by using *R* (v3.2.1, [41]) for data normalization using the *R* bioconductor package variance stabilization and normalization (VSN [v3.36.0], [42]) and the function *vsn2*.

In order to assess the variability between the biological replicates, calculation of the peak sum per replicate was performed; that is, a ratio between each protein abundance to the sum of all protein abundances of the considered replicate was calculated. Afterwards for each time point, the peak sum medians per replicates and a peak sum median of all replicates were calculated. Application of a cut-off standard deviation (sd) of 1.25 allowed an elimination of outlier replicates. In detail, 2 replicates for the 6 h time point, 6 replicates for the 12 h time point, and 7 replicates for the 24 h time point were removed. Additionally, two biological replicates, control replicates 1 and 2 for $t = 24$ h, despite fulfilling the cut-off of sd of 1.25 showed a shifted distribution and were subsequently removed (Figures S1–S3 in Supplementary Material available online at http://dx.doi.org/10.1155/2015/737310).

2.3.5. Differential Protein Expression Calculation. Retained replicates were implemented to the Linear Models for Microarray Data (*limma*) bioconductor *R* package [v3.24.10], for determination of differential expression [43]. This package was already successfully applied to proteomics [42, 44, 45]. *limma* uses an empirical Bayes *t*-test that takes into account the global variance to avoid the noise of local variance for small samples.

Correction for multiple testing was performed by computing the false discovery rate (termed as *q*-values) using the *p* values provided by limma and calculated using the *R* bioconductor package *q*-value [v2.0.0] [46]. Lastly, logarithmic base 2 FC (LogFC) values were converted back to original log2FC using the function sinh. Plots were performed using

the *ggplot2 R* package [v1.0.1] [47]. Significance threshold of the q-values is determined by plotting q-values over p values and identification of intersection (Figure S4) [45]. Statistically significant q-values were indicated with $^{*}q < 0.15$, $^{**}q < 0.10$, and $^{***}q < 0.01$. Of note, all statistical analyses were performed on a mac OSX architecture (x86_64-apple-darwin14.3.0 (64-bit)).

2.4. Western Blot. HUVEC samples were scratched off with a Triton-x lysis buffer. A 10% tris-glycine polyacrylamide gel was prepared and approximately 20 μg protein was loaded (mixed with 5x Laemmli buffer). 3 μL of PageRuler Plus Prestained protein Ladder (Thermo Scientific) was transferred at least to one well, and samples of interest were transferred to free wells of the gel. Furthermore, a 0.2 μm PVDF membrane was used. Antibodies were from BD Biosciences: pSTAT1 pY701 (612233), STAT1 (610116), and STAT3 (610190); cell signaling: pSTAT3 pY705 (9145S); Pierce Thermo: tubulin (PA1-38814). Secondary antibodies were anti-goat IRdye800CW (ODYSSEY, 926-32214) and anti-mouse Alexa Fluor 680 (Invitrogen, A10038). Membranes were detected by fluorescent labelled target proteins, a photo sensor of the LI-COR Odyssey Infra-Red Imaging System (LI-COR, NE). For densitometry analysis, pSTAT1 ($n = 3$) and pSTAT3 ($n = 3$) were normalized to tubulin.

2.5. Pathway Analysis. Data were analysed through the use of QIAGEN's Ingenuity Pathway Analysis (IPA, QIAGEN Redwood City; http://www.ingenuity.com/). Normalized transcriptome data of different concentrations per stimulus and proteomic data of 6, 12, and 24 h were merged, respectively. Core analyses were performed with the following settings: p value < 0.05 (transcriptome data) or q-value < 0.15 (proteome data); reference set was the Ingenuity Knowledge Base (Genes + Endogenous Chemicals), where only experimental observations, direct and indirect relationships, and the Fisher exact t-test were chosen; the knowledge bases from all species and all cell types were included. Regarding transcriptome data, a comparative analysis of IL27- and calprotectin-regulated genes was performed and the top 20 biological functions were chosen based on scoring the z-scores. Afterwards, a hierarchical clustering was performed on the top 20 biological functions based on Euclidean distance using the function heatmap.2 from the *R* package gplots.

2.6. Statistical Analysis. The statistical analysis was performed using the software GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). For more than 5 biological replicates and unequal variances, an unpaired t-test with Welch's correction and, for less than 5 biological replicates, an unpaired t-test were applied. Statistically significant p values were indicated with $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

3. Results

3.1. IL27 and Calprotectin-Dependent Regulation of the Endothelial Cell Gene Expression. To examine the effect of IL27 and calprotectin on endothelial cell gene expression, a multiplex gene array analysis of 96 genes including cytokines, growth factors, and other immune response genes was performed. In order to optimize the stimulation concentrations for IL27 and calprotectin, 3 different concentrations for IL27 (10, 30, and 100 ng/mL) and for calprotectin (1, 5, and 10 μg/mL) were used, according to published data [19, 33, 48]. The relationship of significant differentially expressed genes following stimulation with IL27 is shown in Figure 1(a). Nineteen unique significantly expressed genes were found among which 15 were upregulated and 4 were downregulated. Interestingly, the highest upregulations were observed for *IL7*, *IL15*, *CXCL10*, and *CXCL11* (Table S1). Regarding calprotectin cell stimulation, 15 unique significantly expressed genes were found, among which 11 genes were upregulated and 4 genes were downregulated, with the gene *PTGS2* being upregulated after 1 and 10 μg/mL calprotectin treatment and downregulated after 5 μg/mL calprotectin stimulation. The highest differential gene expression was observed for IL7 and CCL5 (Table S1).

We next performed functional gene enrichment analyses using the Ingenuity Pathway Analysis (IPA) tool. The significantly regulated unique genes were merged for each stimulus. A core analysis was carried out and biological annotation enrichment was generated based on genes. The top 20 biological functions derived from calprotectin and IL27-regulated genes are shown in Figure 2. IL27-mediated gene expression showed that IL27 activated all of the presented top 20 biological functions, including, for example, inflammatory response and activation, stimulation, and migration of leukocytes (z-score ≥ 2). However, calprotectin only mediated the activation of 1 out of the 20 presented biological functions. This pathway analysis reveals that while IL27 appears to be involved in typical inflammatory functions, the activity of calprotectin is less obvious.

3.2. Calprotectin-Dependent Modulatory Effects on IL27-Mediated Gene Expression of IL7, IL15, CXCL10, and CXCL11. To evaluate the possible role of calprotectin in the regulation of the expression of the IL27-dependent upregulated genes *IL7*, *IL15*, *CXCL10*, and *CXCL11*, HUVECs were treated with IL27 (30 ng/mL) ± calprotectin (1 μg/mL) and the relative gene expression was determined by RT-qPCR for the different time points 3, 6, 12, and 24 h as shown in Figure 3. Calprotectin induced downregulation of IL27-mediated gene expression of *IL15* and *CXCL10* while no significant effect was observed neither on *IL7* nor on *CXCL11*. Interestingly, calprotectin decreased IL27-induced gene expression of *IL15* by half at all time points, whereas its downregulating effect on *CXCL10* was weaker and limited to the early time points 3 h and 6 h. These results point to a specific downregulatory role of calprotectin in the endothelial IL27-dependent signaling leading to gene expression of *IL15* and *CXCL10*.

In order to confirm the proinflammatory activity for IL27 and the calprotectin modulatory effects, the HUVECs were stimulated with TNFα (2 ng/mL) ± calprotectin (1 μg/mL) for 3, 6, 12, and 24 h, and the gene expression of *IL7*, *IL15*, *CXCL10*, and *CXCL11* was analysed by RT-qPCR (Figure 4). We observed similar TNFα-mediated gene upregulation of

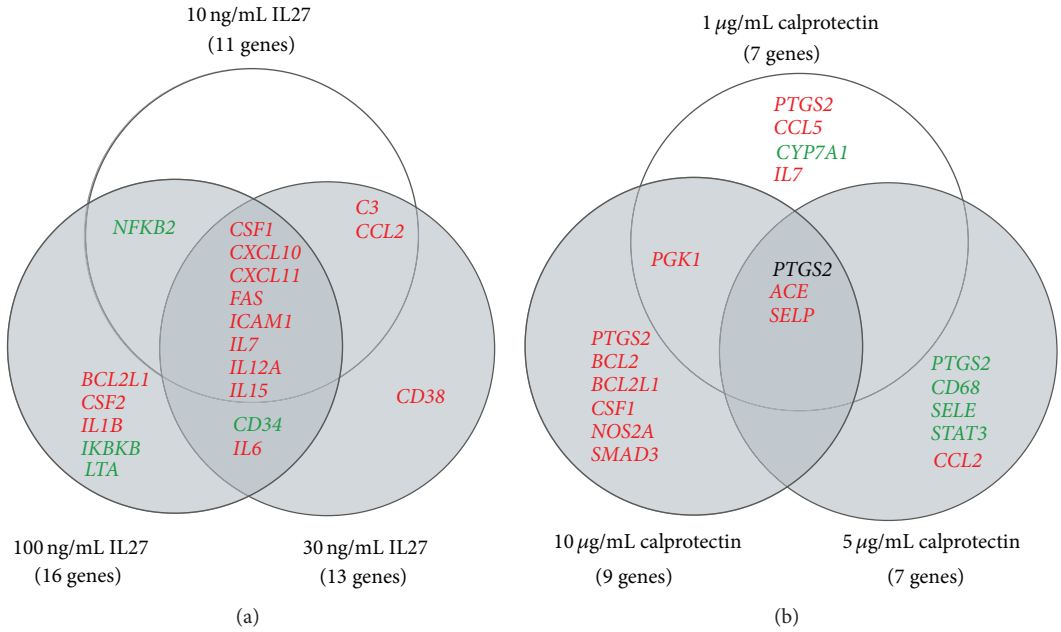

Figure 1: Venn diagrams on IL27- and calprotectin-stimulated HUVECs including significant regulated genes with p value $p < 0.05$: red: upregulated, green: downregulated, and black: up- and downregulation different in compared concentrations. (a) Venn diagram: HUVECs stimulated with IL27 (10, 30, and 100 ng/mL). (b) Venn diagram: HUVECs stimulated with calprotectin (1, 5, and 10 μg/mL). ACE, angiotensin-converting enzyme; BCL2, B-cell lymphoma 2; BCL2L1, BCL2-like 1; C3, complement component 3; CD, cluster of differentiation; CSF, colony stimulating factor; CCL2/MCP-1, monocyte chemotactic protein-1; CCL5/RANTES, regulated on activation, normal T cell expressed and secreted; CXCL10, interferon gamma-induced protein 10; CXCL11, interferon-inducible T cell alpha chemoattractant; CYP7A1, cholesterol 7 alpha-hydroxylase; FAS, Fas cell surface death receptor; ICAM1, intercellular adhesion molecule 1; IKBKB, inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta; IL, interleukin; LTA, lymphotoxin-α; NFKB2, nuclear factor-kappa-B p100 subunit; NOS2A, inducible nitric oxide synthase; PGK1, phosphoglycerate kinase 1; PTGS2, prostaglandin G/H synthase and cyclooxygenase; SELE, selectin E; SELP, selectin P; SMAD3, mothers against decapentaplegic homolog 3; STAT, signal transducers and activators of transcription.

IL7, *IL15*, *CXCL10*, and *CXCL11*. Furthermore, calprotectin induced downregulatory effects on TNFα-mediated gene expression of IL7, IL15, and CXCL10, hence validating our previous findings and emphasizing the *IL15*- and *CXCL10*-specific regulatory role of calprotectin.

3.3. Calprotectin-Dependent Modulatory Effects on IL27-Mediated Intracellular Protein Expression. To investigate the IL27-induced protein expression in HUVECs and to examine the potential regulatory role of calprotectin in this process, we used a label-free MS-based proteomic approach. HUVECs were stimulated with IL27 (30 ng/mL) ± calprotectin (1 μg/mL) for 6, 12, and 24 h. In total, the number of unique proteins detected for each time point was 1061 at 6 h, 994 at 12 h, and 886 at 24 h (Table 1). As shown in Figure S4 by a representation of q-values over p values derived from proteome data, a significance cut-off threshold corresponding to q-value <0.15 could be chosen. The data indicated the following unique differentially regulated proteins detected for each time step: 11 at 6 h, 143 at 12 h, and 193 at 24 h (Table 1). Furthermore, a time-dependent increase for significant expressed proteins for IL27- and calprotectin-stimulated HUVECs was observed. Interestingly, the costimulation revealed already highest protein expression number at 12 h suggesting a calprotectin modulatory role in IL27-mediated protein expression (Table 1). As shown in Figure 5, calprotectin time-dependent modulatory effects on protein expression were observed on 28 unique proteins. Figure 6 summarizes qualitatively the modulatory role of calprotectin in IL27-mediated protein expression. While calprotectin potentialized the IL27-dependent expression of NMT1 (12 h) and STAT1 (24 h) and upregulated STAT3 (6 h), it decreased the expression of GBP1 and WARS at 24 h and downregulated STAT1 at 6 h. Furthermore, calprotectin prevented the IL27-mediated downregulation of NID1 and TPM1, as well as the upregulation of 14 proteins: ARFGAP1, BASP1, CANX, COPE, GNB2, GP1, GSTP1, HMOX2, PKM, PEBP1, PDLIM5, RPL30, PSMA7, and YBX3. The results also showed that the costimulation of HUVECs with IL27 + calprotectin induced the downregulation of 5 proteins (FERMT3, H2B1C, KP2, PECAM1, and SRSF7) whereas 2 proteins (AHSG and S100A9) were upregulated. However, none of the proteins corresponding to the genes induced by IL27 and regulated by calprotectin were differentially expressed. These findings emphasize at the protein level the role played by calprotectin in the regulation of IL27-dependent protein expression.

3.4. Calprotectin-Dependent Modulatory Effects on IL27-Mediated STAT1/3 Signaling. The signal transducers and activators of transcription (STAT) 1 and 3 are both known to be part of the signaling downstream cascade of IL27 and are activated through phosphorylation [49, 50]. Our

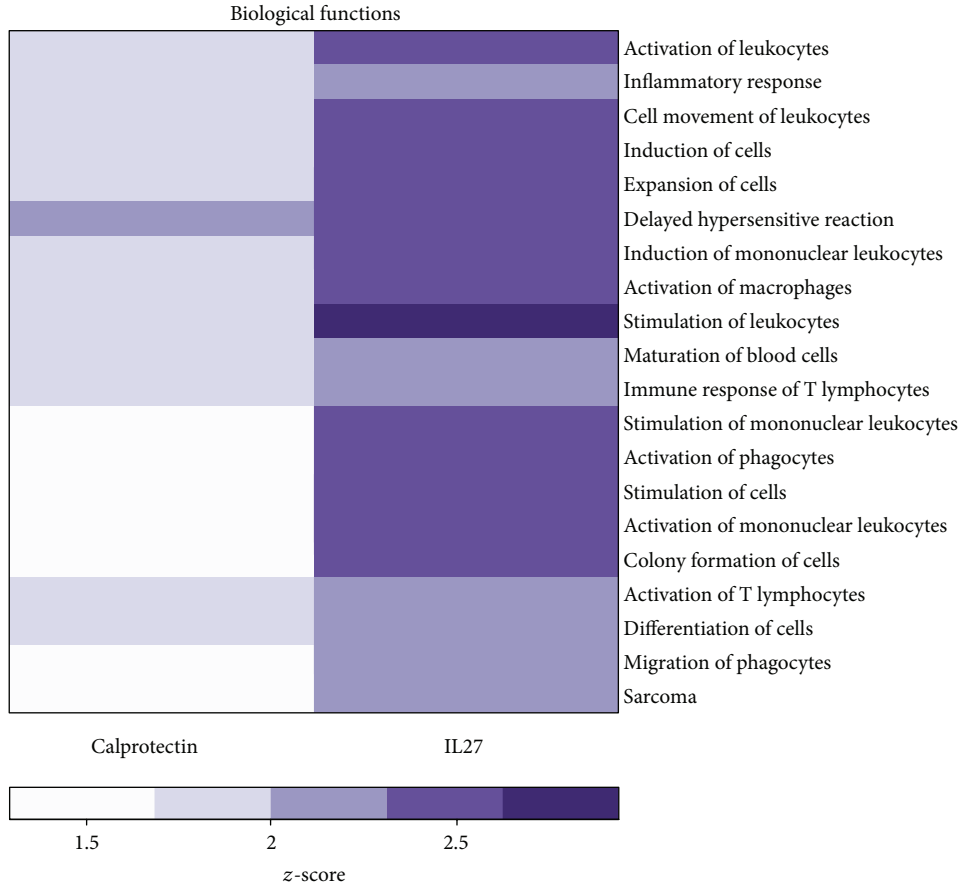

Figure 2: Top 20 biological functions from a comparative gene enrichment analysis of IL27 and calprotectin-mediated gene expression. The biological function gene enrichment analysis was carried out by IPA and represents the z-score (activation ≥ 2).

Table 1: Number of unique identified proteins without and with q-value cut-off of $q < 0.15$ of IL27 (30 ng/mL) ± calprotectin (1 μg/mL)-stimulated HUVECs for 6, 12, and 24 h.

	Time point		
	6 h	12 h	24 h
Unique proteins			
All identified	1061	994	886
q-value < 0.15	11	143	193
Condition (q-value < 0.15)			
IL27	0	55	132
IL27 + calprotectin	10	96	20
Calprotectin	1	17	66

proteomic results clearly indicate that calprotectin upregulated IL27-mediated STAT3 protein expression at 6 h whereas protein expression of STAT1 was downregulated at 6 h and upregulated at 24 h (Figure 5). We next analysed by western blotting the phosphorylation levels of STAT1 and STAT3 when HUVECs were stimulated with IL27 (30 ng/mL) ± calprotectin (1 μg/mL) for 3, 6, 12, and 24 h (Figures 7 and S5). Interestingly, the IL27 + calprotectin cotreatment clearly potentialized the IL27-mediated STAT1 phosphorylation at 12 and 24 h but had no effect on the STAT3 phosphorylation over the time, suggesting that calprotectin might act through the regulation of the STAT1 activity to modulate the IL27-dependent proinflammatory signaling.

4. Discussion

The function of the cytokine IL27 in the inflammatory mechanism leading to immune cell transmigration through the endothelium is controversially discussed. In this study, we investigated the potential IL27-dependent induced inflammatory responses regarding endothelial cell gene and protein expression in the context of vascular inflammation. In addition, we propose that calprotectin may be involved in the regulation of this process.

4.1. The Modulatory Role of Calprotectin in IL27-Mediated Inflammation. Animal and human studies revealed contradictory observations for the investigation of the atherogenic role of the IL27. Studies in animal models have shown an atheroprotective role whereas human studies demonstrated an important proatherogenic role of IL27 in cardiovascular disease [30, 51]. Our findings demonstrate that exposure of

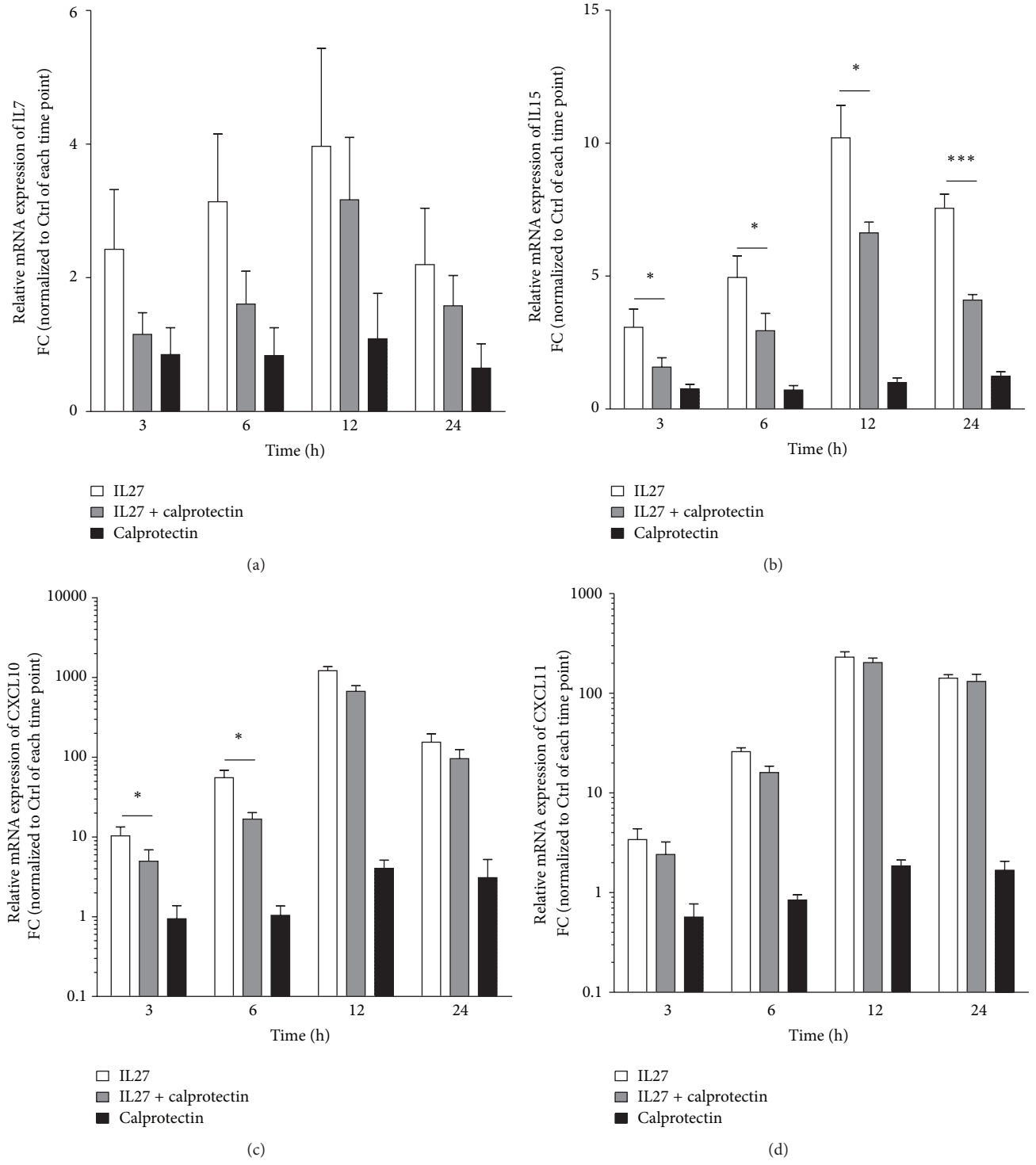

Figure 3: Effects of IL27, calprotectin, and IL27/calprotectin cotreatment on gene expression. Relative mRNA levels of IL7, IL15, CXCL10, and CXCL11 of IL27 (30 ng/mL) ± calprotectin (1 μg/mL)-stimulated HUVECs for 3, 6, 12, and 24 h are represented as mean ± SEM ($n = 6$). Indicated p values are corresponding to significant differences between IL27 and IL27 + calprotectin: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

HUVECs to different literature-based chosen IL27 concentrations results in differential gene expression of 19 out of 96 tested genes (Figure 1(a) and Table S1). Among these 19 genes, we observed 15 upregulated genes which are known to be involved in inflammation. Our pathway analysis revealed that the IL27-induced genes are highly involved in the activation of a variety of biological functions such as activation, stimulation, and migration of leukocytes (Figure 2). Since these functions are fundamental characteristics of the early steps of atherosclerosis development, we propose not only

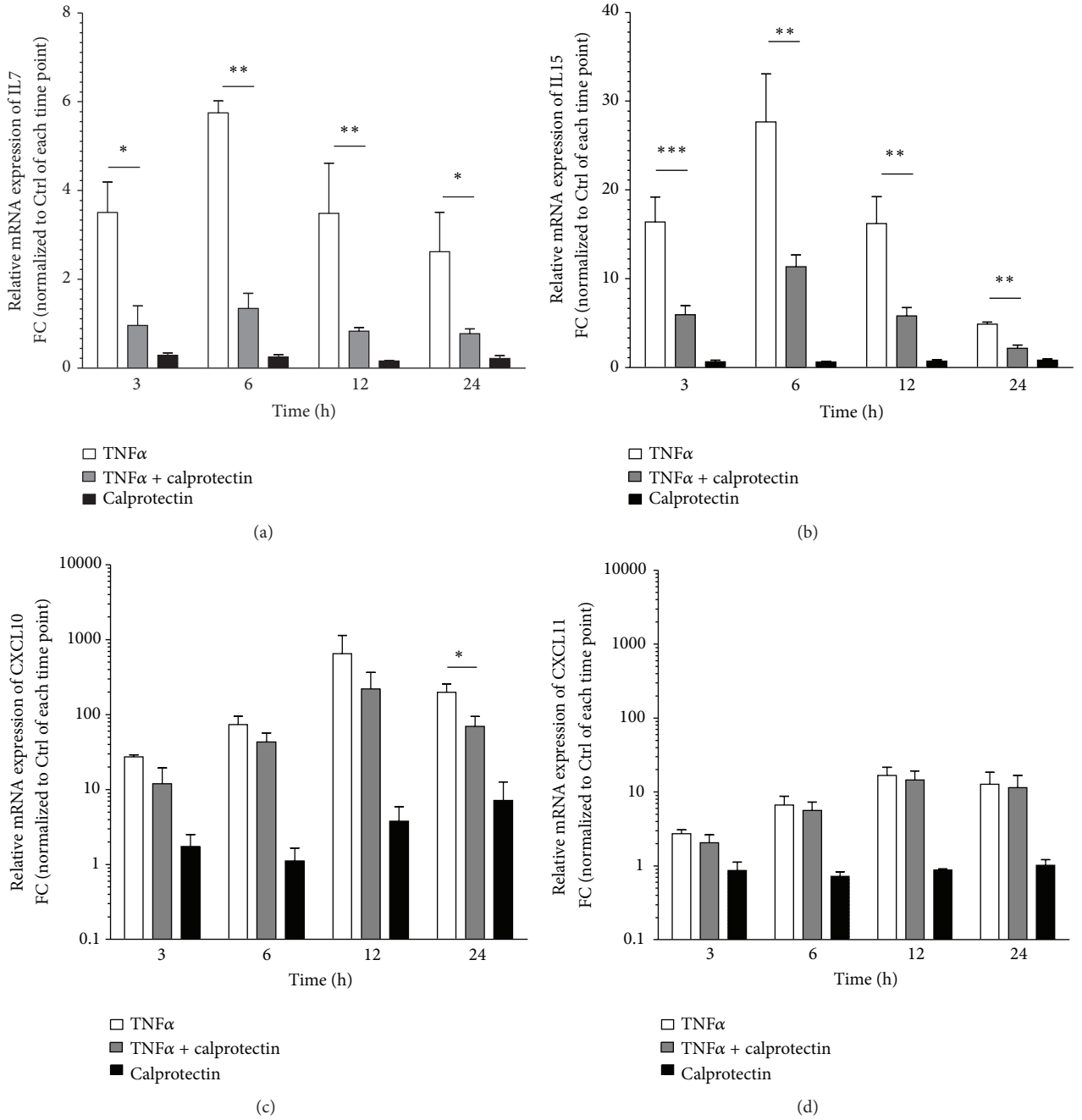

Figure 4: Effects of TNFα, calprotectin, and TNFα/calprotectin cotreatment on gene expression. Relative mRNA levels of IL7, IL15, CXCL10, and CXCL11 of TNFα (2 ng/mL) ± calprotectin (1 μg/mL)-stimulated HUVECs for 3, 6, 12, and 24 h are represented as mean ± SEM ($n = 3$). Indicated p values are corresponding to significant differences between TNFα and TNFα + calprotectin: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

a proinflammatory but also a proatherogenic role for IL27, which is in agreement with the pathway analysis of King and coworkers [30].

In human studies, the two calprotectin subunits S100A8 and S100A9 were detected in atherosclerotic plaques and elevated in serum of patients suffering from peripheral artery disease [52, 53]. In our study, stimulating HUVECs with different calprotectin concentrations revealed differential gene expression of 16 out of 96 tested genes (Figure 1(b) and Table S1). However, calprotectin-induced gene expression indicated poor regulatory effects in terms of expression level (only 2 genes with FC > 1.5). Our observations are in accordance with a study of Viemann and collaborators in which human microvascular ECs were stimulated with 200 μg/mL calprotectin for 6 h: although the calprotectin concentration used was elevated, only 16 genes with a FC > 1.7 were induced and proposed to be involved in promoting platelet aggregation, inflammation, and endothelial permeability [54].

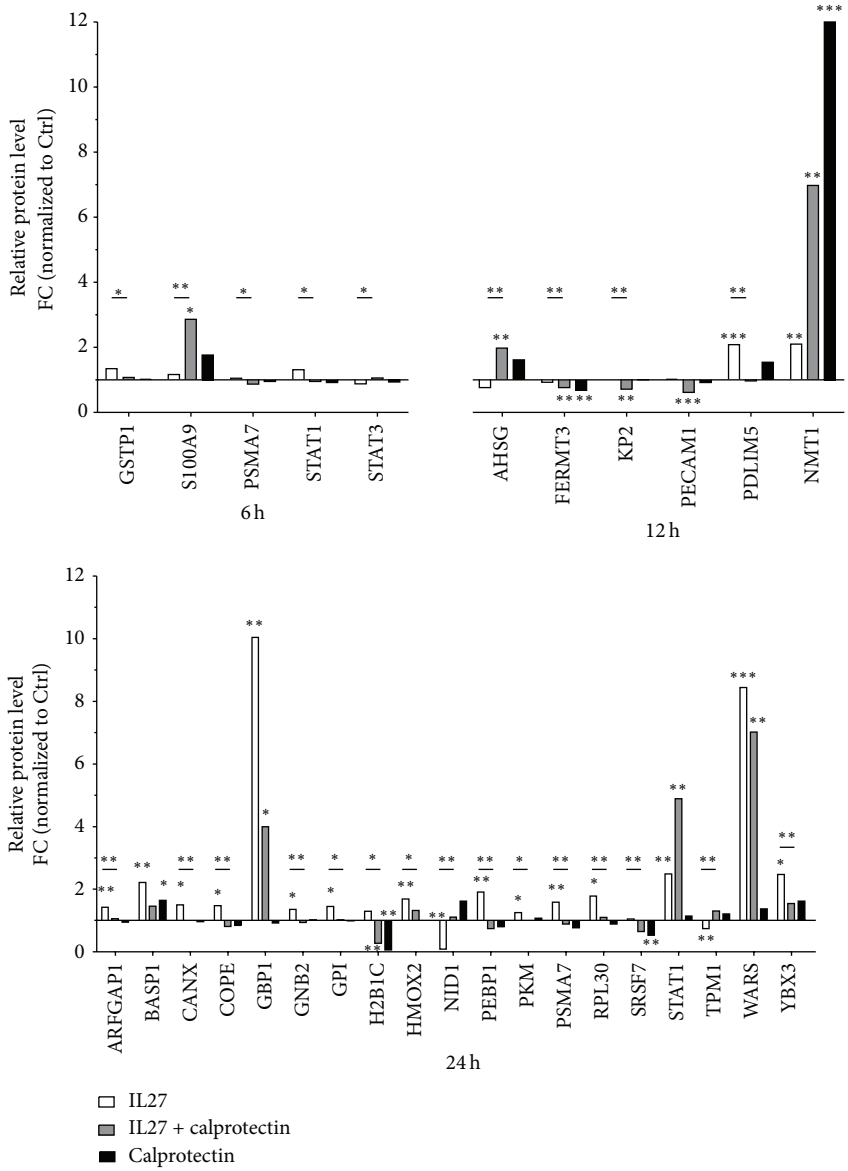

Figure 5: Label-free quantified proteins represented as relative protein levels. The calprotectin significant modulatory effects on mutual proteins in HUVECs stimulated with IL27 (30 ng/mL) ± calprotectin (1 μg/mL) are shown for 6, 12, and 24 h ($n = 9$). Significance is indicated by q-value: $^{*}q < 0.15$, $^{**}q < 0.10$, and $^{***}q < 0.01$. Significance is indicated for each stimulation by stars and between IL27 and IL27 + calprotectin stimulation by stars with a line. AHSG, alpha-2-HS-glycoprotein; ARFGAP1, ADP-ribosylation factor GTPase-activating protein 1; BASP1, brain acid soluble protein 1; CANX, calnexin; COPE, coatomer subunit epsilon; FERMT3, fermitin family homolog 3; GBP1, interferon-induced guanylate binding protein 1; GNB2, guanine nucleotide-binding protein, subunit beta-2; GPI, glucose-6-phosphate isomerase; GSTP1, glutathione S-transferase P; H2B1C, histone H2B type 1; HMOX2, heme oxygenase 2; KP2, importin subunit alpha-1; NID1, nidogen-1; NMT1, glycylpeptide N-tetradecanoyl transferase 1; PDLIM5, PDZ and LIM domain protein 5; PEBP1, phosphatidylethanolamine-binding protein 1; PECAM1, platelet/endothelial cell adhesion molecule; PSMA7, proteasome subunit alpha type-7; RPL30, 60S ribosomal protein L30; S100A9, S100 calcium binding protein A9; SRSF7, serine/arginine-rich splicing factor 7; STAT, signal transducers and activators of transcription; TPM1, tropomyosin 1; WARS, tryptophan tRNA ligase, cytoplasmic; YBX3, Y-box-binding protein 3.

In order to enlighten the role of calprotectin in our experimental setup, our pathway analysis indicated that the calprotectin-regulated genes were not implicated in the typical activation and migration of leukocytes but solely involved in the delayed hypersensitive reaction (Figure 2). Based on this pathway analysis, we hypothesized a possible calprotectin modulatory activity in inflammatory response of HUVECs. In order to clarify the inflammatory role of calprotectin, we investigated their potential synergistic, additive, or antagonistic effects on transcriptome and proteome levels. We first analysed the impact of calprotectin on HUVEC gene expression level by focussing on the 4 most upregulated genes upon IL27 stimulation (*IL7*, *IL15*, *CXCL10*, and *CXCL11*) (Figure 3).

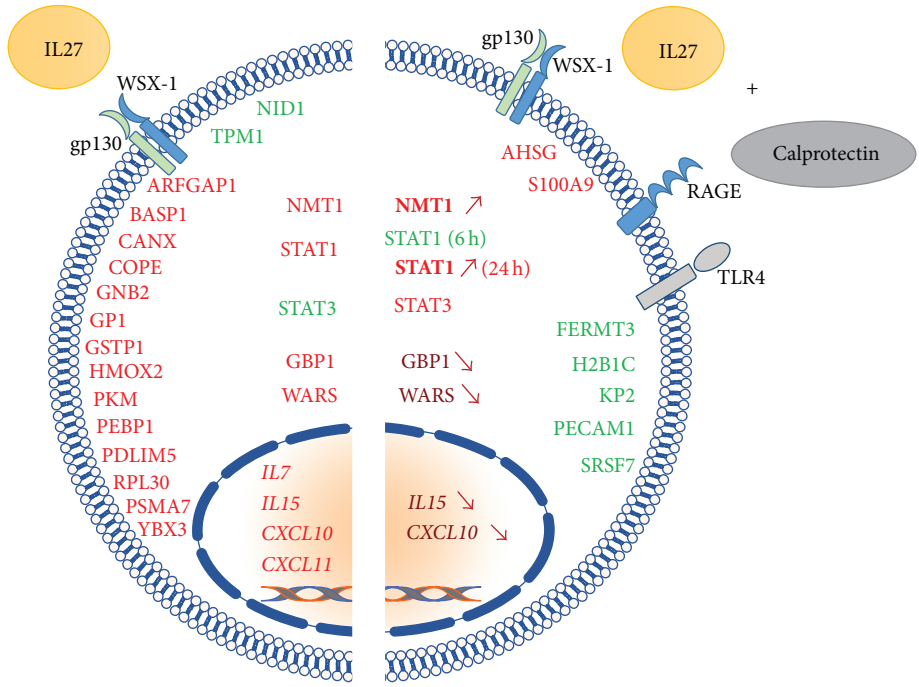

FIGURE 6: Qualitative representation of the calprotectin modulatory effects on IL27-mediated protein and gene expression in HUVECs. Colour code: red: upregulation, bold red (arrow up): increased upregulation compared to IL27 stimulation only, dark red (arrow down): decreased upregulation compared to IL27 stimulation only, and green: downregulation. IL27 receptor: gp130/WSX-1 and calprotectin receptors: RAGE (advanced glucated end product receptor) and TLR4 (Toll-like receptor 4).

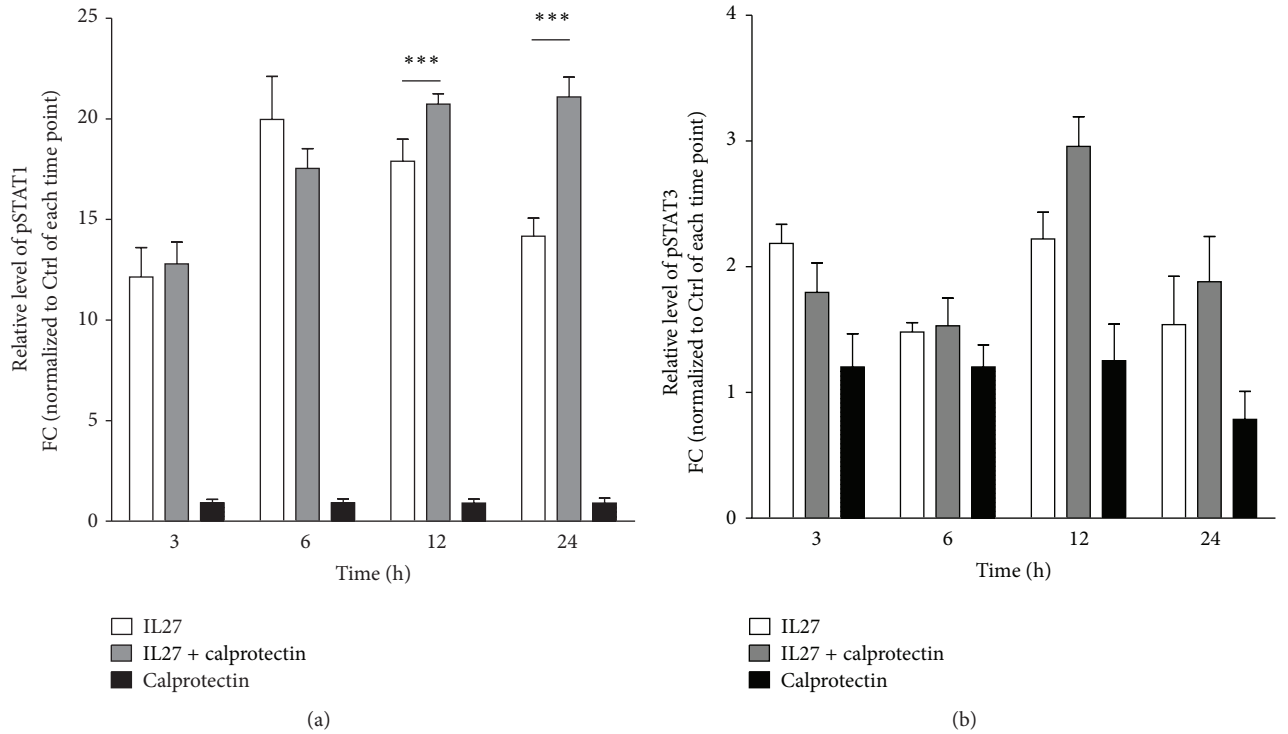

FIGURE 7: Effects of IL27, calprotectin, and IL27 + calprotectin cotreatment on STAT1/3 phosphorylation. Relative protein levels of Tyr701-phosphorylated pSTAT1 (a) and Tyr705-pSTAT3 (b) in IL27 (30 ng/mL) ± calprotectin (1 μg/mL)-stimulated HUVECs for 3, 6, 12, and 24 h performed by western blot. Values are represented as mean ± SEM. Normalization was performed against tubulin. Presented statistical significances are between IL27 and IL27 + calprotectin and the indicated p values correspond to $^{***}p < 0.001$.

We observed clear calprotectin modulatory effects on IL27-mediated *IL15* and *CXCL10* gene expression (Figures 3(b) and 3(c)). Interestingly, the stimulation with calprotectin reduced the IL27-mediated gene expressions of *IL15* over the entire studied time range and of *CXCL10* at the early time points (Figures 3(b) and 3(c)). Based on our observations, we propose an anti-inflammatory role of calprotectin in the IL27-mediated *IL15* and *CXCL10* gene expression. But the cytokine IL27 is known to have pleiotropic functions with pro- and anti-inflammatory capacities [20], which could lead to misinterpretation of the anti-inflammatory function proposed for calprotectin. Thus, we also stimulated HUVECs with the potent proinflammatory TNFα in the presence of calprotectin in the same conditions. Interestingly, similar calprotectin reducing effects were observed on TNFα-mediated gene expression (Figure 4), which highly supports our hypothesis about the anti-inflammatory capacity of calprotectin on IL27-stimulated HUVECs. Analysing only the genome is not sufficient to completely understand a phenotype or a disease development process. Therefore we further analysed the intracellular proteome of whole cell lysates of IL27 ± calprotectin-stimulated HUVECs by a shot-gun label-free LC-MS/MS approach to gain greater insight into our proposed anti-inflammatory capacity of calprotectin in the context of endothelial inflammation. The label-free quantification revealed a number of identified and quantified proteins similar to the one of Gautier et al. [55], who identified 725 unique proteins in untreated HUVEC samples. Another shot-gun proteomic approach combined with label-free quantification was also successfully applied to HUVECs treated with a clinical phase III candidate [56]. In order to further proceed with our data, we used the positive false discovery rate also known as q-value, which controls the false discoveries and corrects for multiple testing to calculate the significance [57]. Indeed q-values were shown to provide a more direct way of interpreting significance than the p value in the context of quantitative proteomics [45]. In our study, the direct comparison between p values and q-values using the well accepted significance threshold for p value <0.05 revealed a q-value threshold of maximum 0.175 (Figure S4). Based on this result, we chose a significance threshold of q-value <0.15, meaning that less than 15% of our significant regulated proteins are false discoveries. This finally revealed the numbers shown in Table 1 for significant regulated proteins.

Our proteomic approach revealed that calprotectin modulated 28 unique proteins (Figure 5). Unfortunately, the sample complexity of our whole HUVEC cell lysates did not allow the determination of low abundant proteins such as cytokines. Thus, the calprotectin inhibitory effect on IL27-mediated gene expression of *IL15* and *CXCL10* could not be examined on protein level by shot-gun LC-MS/MS. However, higher protein levels of CXCL10 as well as CXCL11 were detected in human and carotid atherosclerotic tissues whereas none of them were detected in normal vessel walls, highlighting that both proteins are playing an important role in the development of atherosclerosis [58].

Regarding our results, we therefore propose a model of a calprotectin reducing effect on IL27-mediated inflammation of the vascular endothelium in the context of atherosclerosis. Figure 8 describes the possible mechanism of IL27-induced vascular inflammation and the potential atheroprotective role of calprotectin in this context. IL27 induced the secretion of the chemokines CXCL10 and CXCL11 which are known to attract monocytes as well as T cells. In more detail, CXCL10 and CXCL11 mainly recruit CD4 or CD8 T cells, which also represent the main T cell subsets found in atherosclerotic plaques [58–60]. CXCL10 and CXCL11 can bind via their cognate receptor CXCR3 which is highly expressed on monocytes, T cells, and NK cells [61]. In the atherosclerotic context, endothelial cells do not express CXCR3 but use a very defined system [62]. CXC chemokines such as CXCL10 and CXCL11 can also bind, for example, to heparan sulfate proteoglycans, which are present at the cell surface of endothelial cells. This binding of CXCL10 and CXCL11 can facilitate the rolling of monocytes and T cells on the vascular endothelium [62, 63]. Furthermore, it can facilitate the adhesion to other proteins such as the transpresented IL15 at the surface of endothelial cells. T cells and monocytes express the counterparts IL2Rα and IL2R for IL15R to form the tridimeric IL15 receptor [64]. Independent studies have shown the transendothelial migration of T cells and monocytes through IL15 expression on endothelial cells [64, 65].

Our proteome analysis revealed an IL27-induced regulation of a variety of proteins which are involved in the inflammatory state of the endothelium as well as the development of atherosclerosis. For example, the extracellular matrix glycoprotein nidogen-1 (NID1) was highly downregulated by IL27. NID1 belongs to the basement membrane and is an important linker between the extracellular matrix components collagen and laminin [66]. It could be shown that suppression of NID1 can influence the cell morphology from a flat to a round shape by losing contact to the underlying basement membrane. Thus, an important functional implication of NID1 was suggested in the blood-vessel tissue barrier [67]. Furthermore, NID1 may be involved in the defense against infiltration of cancer cells [68]. Another highly downregulated protein by IL27 was tropomyosin 1 (TPM1), which is known as a tumour suppressor. Suppression of TPM1 can lead to the destabilization of the cytoskeleton [69]. It was described that the initiation and growth of atheroma can occur due to the loss of integrity of an intact endothelial monolayer [70]. In the context of atherosclerosis, a potential downregulator of TPM1 could be the small noncoding microRNA miR21 [71], as miR21 was the highest upregulated miRNA in a study of human atherosclerotic plaques [72]. In our study, IL27 induced the upregulation of GBP1 which was also observed in IFNγ-treated HUVECs [73], leading to enhanced adhesion of monocytes. Similar results of increased adherence of monocytes were demonstrated in stimulated Human Dermal Microvascular Endothelial Cell (HDMEC) and Human Coronary Artery Endothelial Cells (HCAEC) [74].

Based on our data and our hypothetical model, IL27-induced endothelial inflammation is involved in the recruitment, adhesion, and infiltration of monocytes and of T cells. Thus, we propose an important proatherogenic role for IL27 in the early steps of atherosclerosis. Furthermore,

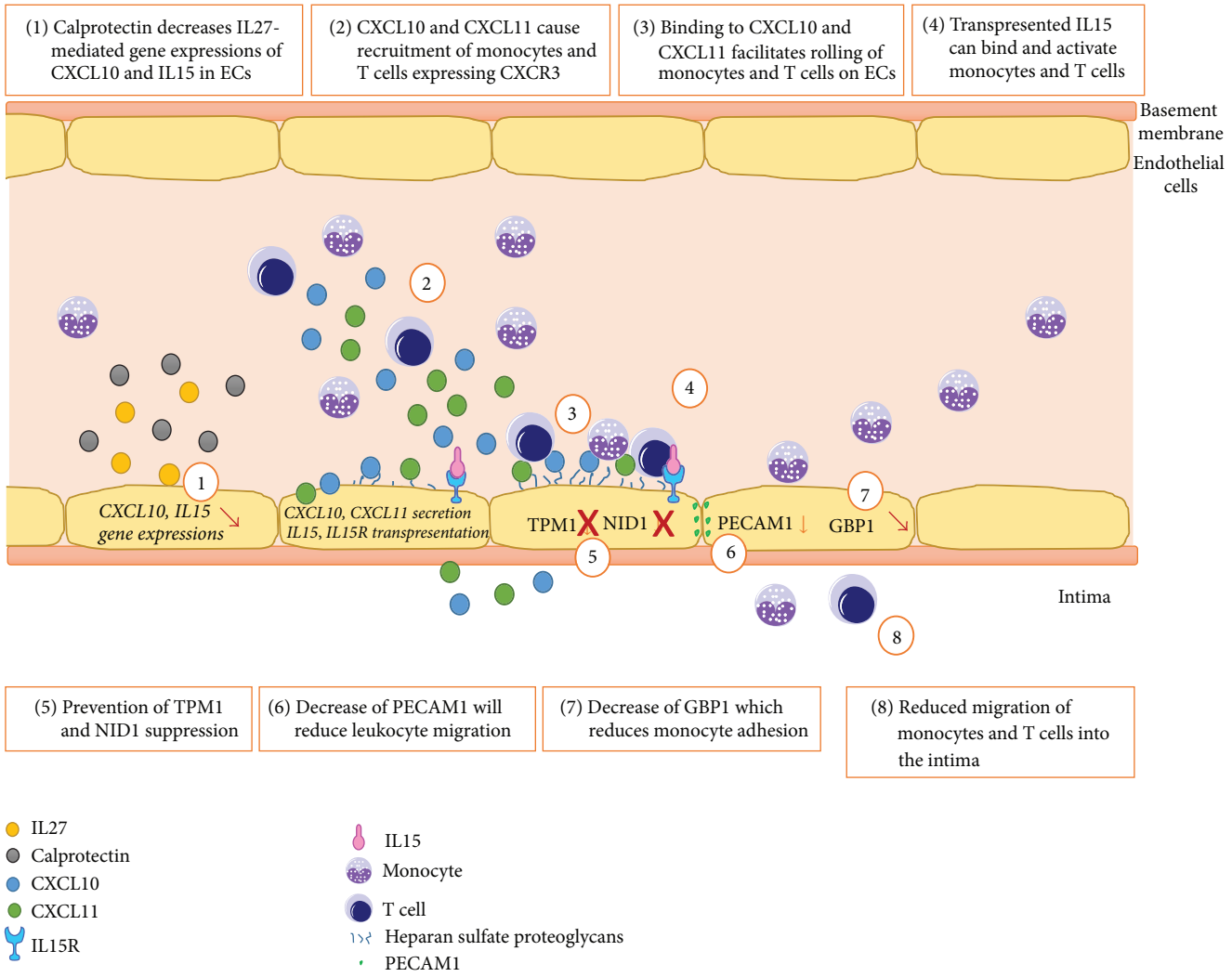

FIGURE 8: Hypothetical model of the calprotectin modulatory effects on IL27-mediated gene and protein expression based on our experimental results and literature data. GBP1, guanylate binding protein 1; NID1, nidogen-1; PECAM1, platelet endothelial cell adhesion molecule; TPM1, tropomyosin 1.

we observed IL27-induced upregulation of PEBP1 which is consistent with elevated PEBP1 levels found in atherosclerotic apoE$^{-/-}$ mice [75]. We also propose an atheroprotective role for calprotectin, as illustrated in Figure 8. Stimulation with calprotectin prevented the IL27-mediated upregulation of NID1 and TPM1, suggesting that no impairment of the underlying basement membrane is occurring. In addition, calprotectin reduced IL27-mediated upregulation of the GBP1 which may lead to reduced monocyte adhesion to the endothelium. Interestingly, calprotectin downregulated the protein expression of PECAM1, leading to the assumption of reduced transendothelial migration: PECAM1 is indeed known as an important contributor for transendothelial migration of leukocytes into the vessel wall [76].

Comparing the stimulations by IL27 and by IL27 + calprotectin by IPA pathway analysis (Table S2), we identified that, regarding the costimulation, calprotectin was responsible for the inhibition of mTOR as well as of EIF2 signaling pathways. Different groups have observed in animal and human models that inhibition of mTOR can result in antiatherosclerotic effects causing prevention or delay of the pathogenesis of atherosclerosis [77, 78]. Based on our results, we propose an atheroprotective role for calprotectin through its IL27-dependent effect on the transendothelial migration of leukocytes into the vessel wall.

4.2. Hypotheses Regarding the Immunoprotective Role of Calprotectin. It has been demonstrated *in vivo* that calprotectin reduced LPS-mediated inflammation by binding directly to LPS but also to other cytokines such as IL1β, IL6, and TNFα [37]. Another *in vivo* animal study revealed a reduction of severe infiltration of inflammatory cells after calprotectin administration and suggested a scavenger activity for calprotectin [38]. Regarding our experimental setup, based on the knowledge that calprotectin can bind to TNFα, and since we showed that calprotectin similarly reduced both TNFα- and IL27-mediated gene expression, we also suggest a potential protein-protein interaction between calprotectin and IL27.

This scavenger function does not directly imply a prevention of receptor binding and subsequent activation of the signaling pathway, as it depends on the availability of free binding sites of IL27. Whether calprotectin can prevent the binding of IL27 to its receptor requires more investigations. Besides, it is also well documented that IL27 receptor activation occurs through Janus kinases and STATs. Interestingly, a study identified that excretory/secretory (ES) products from a parasite reduced IFNγ-mediated *CXCL10* gene expression without any suppression of the phosphorylation-dependent activation of the upstream regulator STAT1 [79]. However, no decrease of *CXCL9* mRNA level was observed, which may be explained by the presence of different transactivating cofactors required for gene transcription. Whether this regulation process could be extrapolated to the observed different modulatory effects of calprotectin on IL27-mediated STAT-dependent *CXCL10*/*CXCL11* gene expression would require further investigations.

5. Conclusion

In conclusion, our findings clearly demonstrate that the cytokine IL27 plays an important role as a proinflammatory mediator by regulating the expression of endothelial genes and proteins, which may be mainly involved in the early stages of the atherosclerosis mechanism. More importantly, we show for the first time that calprotectin acts as a modulator of this process. Our main findings are the identification of a role of calprotectin in the regulation of IL27-mediated gene expression (*IL15*, *CXCL10*), protein expression (TPM1, NID1, PECAM1, and GBP1), and signaling activation (STAT1). Based on these observations, we suggest that, in the context of the IL27-induced vascular inflammation, calprotectin might be a novel attractive candidate as a regulator of monocyte recruitment to early atherosclerotic lesions, hence preventing the progression of inflammation and atherosclerosis.

Conflict of Interests

The authors declare no conflict of interests regarding the publication of this paper.

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Research Article

Early Dynamics of Cerebrospinal CD14+ Monocytes and CD15+ Granulocytes in Patients after Severe Traumatic Brain Injury: A Cohort Study

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In traumatic brain injury (TBI) the analysis of neuroinflammatory mechanisms gained increasing interest. In this context certain immunocompetent cells might play an important role. Interestingly, in the actual literature there exist only a few studies focusing on the role of monocytes and granulocytes in TBI patients. In this regard it has recently reported that the choroid plexus represents an early, selective barrier for leukocytes after brain injury. Therefore the aim of this study was to evaluate the very early dynamics of CD14+ monocytes and CD15+ granulocyte in CSF of patients following severe TBI with regard to the integrity of the BBB. Cytometric flow analysis was performed to analyze the CD14+ monocyte and CD15+ granulocyte population in CSF of TBI patients. The ratio of CSF and serum albumin as a measure for the BBB's integrity was assessed in parallel. CSF samples of patients receiving lumbar puncture for elective surgery were obtained as controls. Overall 15 patients following severe TBI were enrolled. 10 patients were examined as controls. In patients, the monocyte population as well as the granulocyte population was significantly increased within 72 hours after TBI. The BBB's integrity did not have a significant influence on the cell count in the CSF.

1. Introduction

Traumatic brain injury (TBI) is especially prevalent in young adults [1] and represents one of the leading causes of death and of persistent damage of neurocognitive functions. The outcome is primarily determined by the initial trauma resulting from the physical impact and secondarily determined by the extent of secondary injury to the brain in terms of brain edema, increased intracranial pressure, and delayed cell destruction [2]. These secondary injury mechanisms could be responsible for the development of neurological deficits after TBI evolving minutes to days or even months after the primary mechanical injury [3]. The delayed incidence of the secondary injury mechanisms indicates that there might be a time window for therapeutic interventions to reduce brain tissue damage and improve the functional neurological outcome [3]. Therefore improved understanding of the complex processes following TBI [3] is crucial for the development of an effective neuroprotective treatment. Although the key role of the systemic cellular immune response in patients following multiple trauma has been emphasized by several authors, there is only a limited number of studies analyzing the cellular response of the key inflammatory cells such as monocytes and granulocytes in the cerebrospinal fluid (CSF) of patients following TBI [4–6]. Monocytes are characterized by CD14, a 56 kDa cell membrane anchored

protein [7, 8]. In parallel, the carbohydrate antigen CD15 (the carbohydrate antigen 3-fucosyl-N-acetyl-lactosamine) with an approximate molecular mass of 165 and 105 kDa is expressed on membrane glycoproteins of neutrophil granulocytes [9, 10]. Under physiologic conditions the CSF is separated from peripheral and cerebral blood flow by the blood brain barrier (BBB). In analyzing the dynamics of monocytes and granulocytes in CSF of patients after TBI, the question arises whether potential changes of cellular contents occur due to a disrupted BBB or by a certain mechanism still to be explained. It is well known that the leukocyte count of the CSF is far lower compared to peripheral blood. Therefore a disrupted BBB potentially leads to an increase of leukocytes in the CSF following cell leakage due to disrupted blood vessels.

Therefore the aim of the present study was to evaluate the fraction of CD14+ monocytes and CD15+ granulocytes in CSF of patients following TBI beginning at the time of admission until 72 hours (hrs) after TBI. The influence of the BBB integrity on the number of monocytes and granulocytes in CSF was also assessed in this context.

2. Patients and Methods

2.1. Study Design and Patient Collective. The study protocol was approved by the university's board of ethics (reference number 330/03). Inclusion criteria for prospective enrolment were presence of isolated closed TBI, initial Glasgow Coma Score (GCS) ≤ 8 points (i.e., severe brain injury), proof of intracranial bleeding (ICB) on the initial cranial computed tomography scan (CCT; performed within 90 minutes after TBI), and the indication for placing an external ventricular drainage (EVD) catheter. Exclusion criteria were a history of preexisting neurological, malignant, or chronic inflammatory disease. Written informed consent was obtained when the patient regained consciousness. In case of remaining unconscious, a next of kin or a legal representative was asked for the presumed consent.

2.2. Clinical and Surgical Procedures. An external ventricular drainage (EVD) catheter (TraumaCath, Integra Neurosciences, Plainsboro, USA) was placed in the frontal horn of the lateral ventricle using CT fluoroscopy guidance to continuously monitor the intracranial pressure (ICP) and to drain CSF [11]. After a CT scan performed to control the correct placement of the drainage, the patients were referred to the intensive care unit (ICU) and treated according to the guidelines of the Brain Trauma Foundation [12]. If the ICP remained under 15 mmHg for at least 72 hrs without mannitol administration or CSF drainage, the EVD was removed.

2.3. Sampling Procedures. The first sampling took place immediately after the insertion of the EVD (90 ± 45 minutes after admission to the hospital). Further samples were obtained 12, 24, 48, and 72 hrs after TBI. At every sampling time point 4 mL of drained CSF, 5 mL of peripheral serum blood, and 5 mL of EDTA blood were collected. 500 μL of CSF was sent to the institute of laboratory medicine of our university hospital for the determination of the total cell counts and albumin levels in CSF. The serum blood was used for the determination of the albumin levels in peripheral blood by the institute of laboratory medicine. Albumin levels were necessary for the evaluation of the blood brain barrier (BBB). The EDTA blood was sent to the institute of laboratory medicine and was used for peripheral differential blood counts.

2.4. Fluorescence Labeling. For the fluorescence activated cell sorting (FACS) analysis, 3 mL of the cerebrospinal fluid samples was centrifuged with 1200 G at 4°C for 10 minutes to remove the supernatant and processed immediately. The cell pellet was resuspended in 200 μL FC buffer suspension (0.01 M phosphate buffered saline, pH 7.2, 1% bovine serum albumin, and 5% fetal calf serum (FCS; 0.02% NaN_3)).

20 μL of the resuspension was incubated for 20 minutes with 3 μL fluorochrome-labeled antibodies in the dark on ice. All antibodies CD14 TC and CD15 FITC (Caltag Laboratories, Hamburg, Germany) were used in single-stain arrays. For lysing of the erythrocytes, 500 μL FACS lysing solution (Caltag Laboratories, Hamburg, Germany) was used and afterwards the suspension was incubated for another 10 minutes. After washing with 500 μL PBS (5% fetal calf serum (FCS; 0.02% NaN_3, Pettenkofer, Munich, Germany)) twice, the suspension was centrifuged again for 10 minutes with 1200 rounds per minute at 4°C.

2.5. Flow Cytometric Analysis. The FACS analysis was performed using a four-channel Epics XL MCL flow cytometer (Beckman-Coulter, Krefeld, Germany) with an air-cooled 15 mW 488 nm argon ion laser. A 530 nm filter for FITC (fluorescein isothiocyanate) and a >650 nm filter for TC (Tri-Color, Caltag Laboratories, Hamburg, Germany) were applied. Data acquisition was obtained using the Expo 32 Software (Beckman-Coulter, Krefeld, Germany). Spectral compensations and instrument settings were appropriately adjusted. Fluidics and Instruments were regularly checked using FlowCheck beads (Beckman-Coulter, Krefeld, Germany). For a respective analysis, the cytometer counted 5000 events (cells) in a list mode. The free WinMDI Software (Version 2.8, Bio-Soft Net [13]) was used for data analysis. Antibody-positive cells were determined as the most positive on side scatter versus TC (CD14) and FITC (CD15) positive cells, respectively. Antibody-positive cells were counted as percentage of the total cell count.

2.6. Assessment of the Blood Brain Barrier Function. In order to determine whether CD14+ monocytes' and CD15+ granulocytes' dynamics correlate to posttraumatic BBB disruption, the ratio of CSF and serum albumin (Q_{alb}) was calculated at each sampling time point. Albumin levels were evaluated by using standardized turbidimetric assays (Cobas Integra Albumin, Roche Diagnostics, Mannheim, Germany). According to Reiber and Felgenhauer, Q_{alb} is a sensitive parameter for the analysis of the BBB [14]. In this study, the disturbance of the BBB was assessed as follows: Q_{alb} values below 0.007 were considered as normal, values between 0.007 and 0.01 as mild dysfunction, values between 0.01 and 0.02 as moderate, and levels above 0.02 as severe dysfunction

of the BBB. Normal and mild dysfunction were defined as intact BBB whereas moderate and severe dysfunction were defined as disrupted BBB. To assess the influence of the BBB's function on the dynamics of the monocytes, the enrolled patients were divided into two groups: group I presenting with an intact BBB and group II with a defect BBB.

2.7. Data Analysis. Data are given in mean ± standard error of the mean (SEM). For comparing patient values with control values or the patient value at the time of admission, the data was analyzed using ANOVA (analysis of variance on ranks) for nonparametric data according to Kruskal-Wallis. For analysis of significant differences over the course of time ANOVA was performed using the Student-Newman-Keuls test. For determination of specific differences Dunn's test was performed. The level of significance was set to ≤0.05. For all statistical analysis the Sigma Stat 3.0 software package (SPSS Inc., Chicago, USA) was used.

3. Results

3.1. Patients and Demographic Data. In total, 23 patients with isolated severe TBI were enrolled (8 females and 15 males; age 46 ± 5 years). Eight patients died within 24 hrs after TBI, because of therapy resistant brain swelling and/or brain stem herniation and thus had to be excluded. CSF was obtained from 10 control patients (5 females and 5 males; age 44 ± 9 years) who underwent spinal anesthesia for an elective orthopedic procedure of the lower extremity.

3.2. Dynamics of CD14+ Monocytes. The baseline value of the control group monocyte population was 2.6 ± 0.6%. At admission the monocyte count was 2.32 ± 0.6% in the TBI patient group, not significantly different to the control group. The monocyte population increased to 4.10 ± 1.0% at 12 hrs, to 4.61 ± 1.4 % at 24 hrs, and to 5.26 ± 1.0% at 48 hrs. Although a continuous increase was observed, there was no statistically significant difference in comparison to the admission level. In addition, the monocyte populations at 12, 24, and 48 hrs were not significantly different in comparison to the baseline level of healthy controls. However, 72 hrs after TBI the monocyte population accounted for 6.48 ± 0.8% and was thereby significantly higher in comparison to the value at admission. Furthermore the monocyte population was significantly higher in comparison to the baseline level of the healthy control group (see Figure 1).

3.2.1. Group I (Intact BBB, n = 9). At admission the CD14+ monocyte count of patients with intact BBB was 2.61 ± 0.9%, which was not significantly different in comparison to the baseline level of the control group. The fraction increased to 4 ± 1.5% and 4.34 ± 1.4% at 12 hrs and 24 hrs after TBI, respectively. The cell counts were still not significantly different in comparison to the cell level at admission. In addition, there was no significant difference in comparison to the baseline level of the control group. At 48 hrs after TBI the population accounted for 6.40 ± 1.5%, which was significantly higher than the baseline level of the control group ($p < 0.05$). At 72 hrs the CD14+ monocytes further increased to a value of 6.91 ± 1.0%. This was significantly higher than the baseline value of the control group and significantly elevated compared to the admission value.

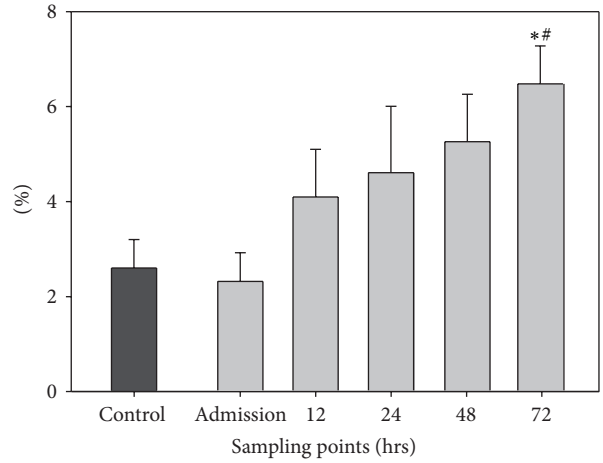

Figure 1: Dynamics of CD14+ monocytes. The graph demonstrates the number of CD14+ monocytes in percent (%) of all CSF cells at the different sampling time points. Data are given in mean ± standard error of the mean. $^{*}p < 0.05$ versus control group; $^{\#}p < 0.05$ versus admission.

3.2.2. Group II (Disrupted BBB, n = 6). At the time of admission the monocyte population accounted for 1.89 ± 0.9 and it was only slightly but not significantly higher at 12 hrs after TBI (4.25 ± 1.6%). At 24 hrs the population dropped to a level of 3.53 ± 1.1% and there was still no significance compared to the value at the time of admission (0 hrs). In addition, the monocyte population at the time of admission, 12 hrs, and 24 hrs after TBI was not significantly different in comparison to the baseline level of the control group. At 48 hrs and at 72 hrs after TBI the populations were 5.02 ± 3.2% and 5.83 ± 1.4%, respectively; both values were significantly higher than the value at the time of admission as well as the baseline level of the control group.

3.2.3. BBB Evaluation. Regarding the evaluation of the BBB there were no significant differences between group I with intact BBB and group II with disrupted BBB at the defined sample points (0, 12, 24, 48, and 72 hrs after TBI; see Figure 2).

3.3. Dynamics of CD15+ Granulocytes. The control group value accounted for 0.98 ± 0.2%. The population of the CD15+ granulocytes was already significantly elevated at admission (9.15 ± 1.6%) compared to the baseline level of controls. At 12 hrs the value increased to 24.65 ± 1.6% and was therefore significantly higher in comparison to the admission time point. The granulocyte populations were 25.9 ± 3.1, 34.3 ± 5.0, and 25.5 ± 3.5 at 24, 48, and 72 hrs, respectively, so that the values remained significantly elevated compared to the admission levels as well as the baseline level of controls (see Figure 3).

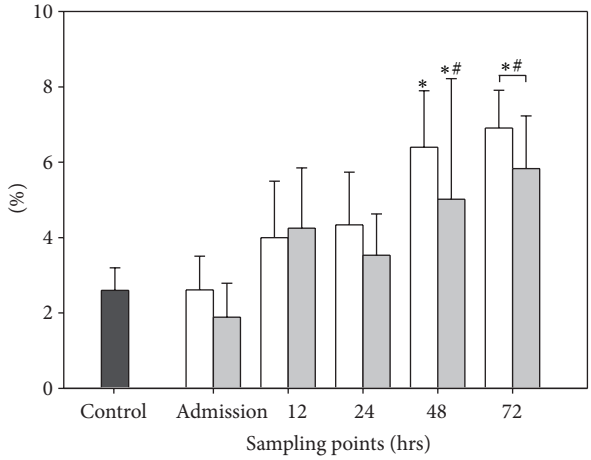

Figure 2: Dynamics of CD14+ monocytes and function of the blood brain barrier. The graph demonstrates the number of CD14+ monocytes in percent (%) of all CSF cells. Graphs in white demonstrate data of patients with intact blood cerebrospinal fluid barrier ($n = 9$), whereas graphs in grey demonstrate data of patients with disrupted blood cerebrospinal fluid barrier ($n = 6$). Data are given in mean ± standard error of the mean. $^{*}p < 0.05$ versus control group; $^{\#}p < 0.05$ versus admission.

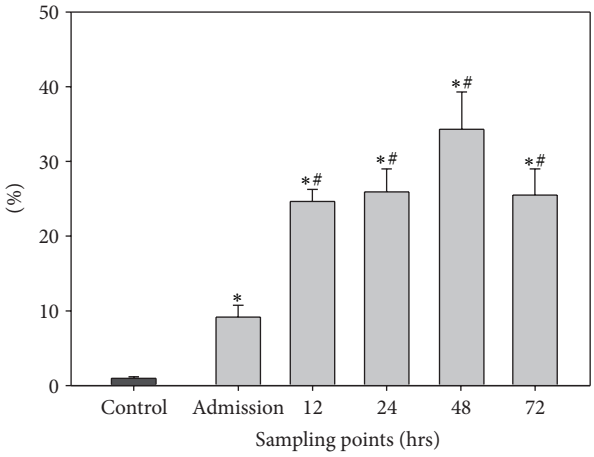

Figure 3: Dynamics of CD15+ granulocytes. The graph demonstrates the number of CD15+ granulocytes in percent (%) of all CSF cells at the different sampling points. Data are given in mean ± standard error of the mean. $^{*}p < 0.05$ versus control group; $^{\#}p < 0.05$ versus admission.

3.3.1. Group I (Intact BBB, n = 9). The population of granulocytes was already significantly elevated (8.61 ± 1.3%) at admission compared to the baseline level of the control group (0.98 ± 0.2%). At 12 hrs after TBI the population accounted for 26.96 ± 2.6%, which was significantly higher in comparison to admission. The granulocytes were 25.42 ± 4.6%, 33.38 ± 7.0%, and 22.27 ± 4.6% at 24 hrs, 48 hrs, and 72 hrs, respectively. These populations remained significantly higher in comparison to admission. In addition the granulocytes in group I were significantly higher compared to the baseline level of the control group at every sampling time point.

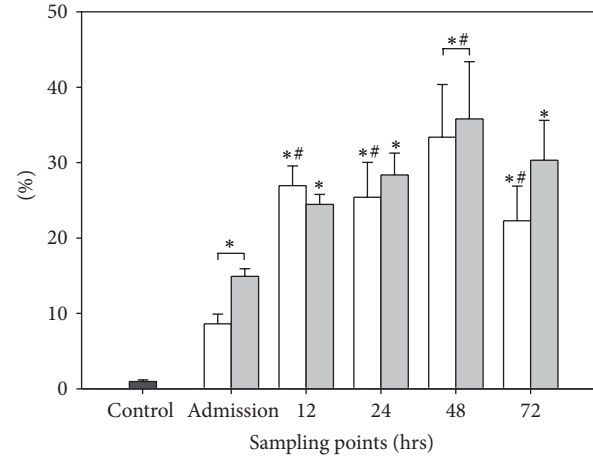

Figure 4: Dynamics of CD15+ granulocytes and function of the blood brain barrier. The graph demonstrates the number of CD15+ granulocytes in percent (%) of all CSF cells. Graphs in white demonstrate data of patients with intact blood cerebrospinal fluid barrier ($n = 9$), whereas graphs in grey demonstrate data of patients with disrupted blood cerebrospinal fluid barrier ($n = 6$). Data are given in mean ± standard error of the mean. $^{*}p < 0.05$ versus control group; $^{\#}p < 0.05$ versus admission.

3.3.2. Group II (Disrupted BBB, n = 6). The population of granulocytes accounted for 14.92 ± 1.0% at admission and was therefore significantly higher in comparison to the control group (0.98 ± 0.2%). At 12 hrs and at 48 hrs the values increased to 24.48 ± 1.3% and 28.37 ± 2.9%, respectively. At 48 hrs granulocytes increased to 35.80 ± 7.6% and were therefore significantly higher in comparison to admission. At 72 hrs granulocytes dropped to 22.27 ± 4.6%, so that there was no further significant difference in comparison to admission levels. The populations of granulocytes in group II were significantly higher in comparison to the baseline level of the control group for every sampling time point.

3.3.3. BBB Evaluation. Regarding the evaluation of the BBB there was no significant difference between group I with intact BBB and group II with disrupted BBB (see Figure 4).

3.4. CSF Total Cell Count. The absolute level of cells (total cell count) as well as the percentages of monocytes and granulocytes at the various sample points is given in Tables 1, 2, and 3. There was no significant difference found comparing the sampling points to base line levels at admission. There was also no difference comparing patients with intact and disrupted BBB.

3.5. Peripheral Blood Monocyte and Granulocyte Count. The populations of monocytes and granulocytes in peripheral blood are provided in Table 4. There were no significant changes over the course of time.

4. Discussion

In the present study the dynamics of CD14+ monocytes and CD15+ granulocytes in the cerebrospinal fluid of patients

Table 1: Total cell count in the CSF of patients.

Cells/μL	Admission	12 hrs	24 hrs	48 hrs	72 hrs
All patients ($n = 15$)	30.7 ± 5.6	17.8 ± 2.5	16.9 ± 3.0	24.6 ± 4.2	27.8 ± 4.9
Intact BBB ($n = 9$)	31.4 ± 7.8	12.75 ± 1.8	11.0 ± 1.2	17.5 ± 2.3	25.4 ± 5.9
Disrupted BBB ($n = 6$)	29.8 ± 8.7	24.5 ± 4.1	24.7 ± 5.9	34.2 ± 8.5	31.0 ± 9.0

Total numbers of CSF cells. Data given as mean ± SEM.
BCSFB: blood cerebrospinal fluid barrier.
CSF: cerebrospinal fluid.
TBI: traumatic brain injury.

Table 2: Monocyte count in the CSF of patients.

Monocyte count (%)	Admission	12 hrs	24 hrs	48 hrs	72 hrs
All patients ($n = 15$)	5.3 ± 0.4	6.9 ± 0.6	6.3 ± 0.4	7.6 ± 0.9	5.7 ± 0.5
Intact BBB ($n = 9$)	5.6 ± 0.6	6.7 ± 0.7	6.1 ± 0.5	7.4 ± 0.7	5.9 ± 0.76
Disrupted BBB ($n = 6$)	4.9 ± 0.4	7.4 ± 0.9	6.6 ± 0.6	7.8 ± 1.8	5.2 ± 0.7

Percentages of monocytes. Data given as mean ± SEM.
BCSFB: blood cerebrospinal fluid barrier.
CSF: cerebrospinal fluid.
TBI: traumatic brain injury.

Table 3: Granulocyte count in the CSF of patients.

Granulocyte count [%]	Admission	12 hrs	24 hrs	48 hrs	72 hrs
All patients ($n = 15$)	63.9 ± 3.7	69.9 ± 3.2	70.6 ± 3.4	66.2 ± 5.5	65.9 ± 3.6
Intact BBB ($n = 9$)	63.9 ± 4.1	71.4 ± 2.8	71.9 ± 3.2	64.7 ± 8.4	64.4 ± 4.3
Disrupted BBB ($n = 6$)	64 ± 6.0	67.7 ± 6.1	68.5 ± 6.4	68.5 ± 5.1	68 ± 5.2

Percentages of granulocytes. Data given as mean ± SEM.
BCSFB: blood cerebrospinal fluid barrier.
CSF: cerebrospinal fluid.
TBI: traumatic brain injury.

Table 4: Peripheral blood monocytes and granulocytes.

Cells [%]	Admission	12 hrs	24 hrs	48 hrs	72 hrs
Monocytes ($n = 15$)	5.3 ± 0.4	7.0 ± 0.6	6.3 ± 0.4	7.6 ± 1.0	5.7 ± 0.5
Intact BBB ($n = 9$)	5.6 ± 0.6	6.7 ± 0.8	6.1 ± 0.6	7.4 ± 0.8	6.0 ± 0.7
Disrupted BBB ($n = 6$)	4.9 ± 0.5	7.4 ± 1.1	6.6 ± 0.7	7.8 ± 2.2	5.2 ± 0.8
Granulocytes ($n = 15$)	64.0 ± 3.7	69.9 ± 3.2	70.6 ± 3.4	70.9 ± 2.9	65.9 ± 3.6
Intact BBB ($n = 9$)	64.0 ± 4.8	71.4 ± 3.2	71.9 ± 3.6	72.5 ± 3.4	64.4 ± 4.9
Disrupted BBB ($n = 6$)	64.0 ± 6.6	67.7 ± 6.7	68.5 ± 7.0	68.5 ± 5.5	68 ± 5.7

Percentages of cells. Data given as mean ± SEM.
BCSFB: blood cerebrospinal fluid barrier.
TBI: traumatic brain injury.

suffering from traumatic brain injury were evaluated in the direct posttraumatic course over 72 hours for the first time. CD14+ monocyte and CD15+ granulocyte cell populations were significantly elevated within the first 72 hrs after TBI compared to baseline levels at admission to hospitals as well as in comparison to healthy controls. Regarding a potential dysfunction of the BBB no significant difference was found between TBI patients with intact BBB and TBI patients with disrupted BBB, respectively.

Only patients with isolated TBI were included to avoid an increase of monocytes and granulocytes in the peripheral blood not resulting from TBI and a potential consecutive bias. In addition the inclusion criteria in terms of severe TBI (GCS $\leq$ 8) and signs of intracerebral hemorrhage were established to ensure that an adequate trauma to the brain had happened possibly resulting in detectable and significant changes of the CSF on cellular level. In conclusion the inclusion criteria lead to enrollment of patients in critical conditions, so that research as conducted in this study should form the basis of future research regarding especially a potential modification of therapeutic options after TBI to avoid secondary brain damage.

In general the effect of moderate (and even mild) TBI on the dynamics of monocytes and granulocytes would be

additionally interesting for the understanding of TBI and the processes happening in the brain on cellular level. However harvest of CSF is quite difficult to account for in patients who are not considered to get an EVD placed. In the current literature several studies report detection of pro- and anti-inflammatory mediators such as cytokines or interleukins in the CSF of patients [15]. However, there is also evidence in the literature that secondary brain damage may also develop without an increase of interleukins in CSF [16]. In this context a functional involvement of immunocompetent cells like monocytes [17–21] and granulocytes [22, 23] in the formation of secondary brain damage was described.

In the present study the population of CD14+ monocytes and CD15+ granulocytes increased significantly within the first 72 hrs after TBI compared to the corresponding populations of the control group and even to the cell populations at the time of admission. Consecutively the question arose whether this was due to a nonmodulated influx of these cells from the peripheral blood into CSF because of a disrupted BBB or whether the cells were transported across the BBB via certain mechanisms to be described. In this context the presented results demonstrate that the integrity of the BBB had no significant influence on the populations of the CD14+ monocytes and CD15+ granulocytes in the time course of the first 72 hours after TBI. In addition there was no significant drop of monocyte or granulocyte populations detected in the peripheral blood. Therefore our data does not support the theory of a nonmodulated influx of cells from the peripheral blood. However, subdividing into groups with a rather small number of $n < 10$ has to be critically interpreted from the statistical point of view. In this context recent publications demonstrated a monocyte trafficking across the BBB using a transmission electron microscopy in a rat TBI model [24, 25]. In this regard Schwartz and Baruch recently reported that the choroid plexus represents an early entry site for leukocytes after injury of the CNS (Central Nervous System) [26]. Shechter et al. also recently found that monocytes primarily traffic through the choroid plexus after CNS injury [27]. These recently reported findings possibly help to explain the significantly elevated populations of CD14+ monocytes and CD15+ granulocytes in the CSF in the early phase of TBI patients in the present study.

The CD15+ granulocytes were already significantly and nearly tenfold increased at the time of admission indicating a very fast response of the quantity of granulocytes after TBI. In addition the granulocyte population further increased; 12 hrs after TBI the population was already significantly higher than at the time of admission. These data show that the granulocytes are fast-responder and available for migration into the CSF in sufficient numbers. Also the data about the dynamics of the cells provide initial evidence that the granulocytes could play an important role in the very early posttraumatic phase after severe TBI.

The increase of CD14+ monocytes did not lead to a significantly higher level of the monocyte population until 72 hrs after TBI. The comparably slower increase of monocytes could mean that these cells are not as much involved in the early response after TBI as granulocytes. This could however just indicate that monocytes are far more immobile than granulocytes or just not in an equal number available for migration into the CSF. In this regard it would be very interesting to determine the population of activated cells among the overall CD14+ monocytes. Future work is therefore necessary to further understand the role of monocytes in TBI.

A limitation of this study to be mentioned is that only dynamics of CD14+ monocytes and CD15+ granulocytes were analyzed and that the distinct cause of the resulting increase was not determined. In this regard it has to be stated that the placement of an EVD itself can be considered as some kind of brain injury. Hence, the inflammatory response in the CSF could potentially be caused by placement of the EVD. This is a potential bias, which is not excluded by the control CSF group. In the control group CSF was obtained via lumbar puncture for spinal anesthesia which is a completely different injury model concerning the CSF collection. However our study was initiated to measure the effect of immunocompetent cells in TBI in the early posttraumatic phase. Strictly speaking this study compared the inflammation response after two known TBI events (the first event(s) up to 90 minutes previous to the second TBI event considering placement of the EVD as TBI event) to a control group with a minor spinal injury in terms of spinal anesthesia. This should be kept in mind when interpreting the results. Therefore a further limitation of the study is that a second control group with patients needing EVD or a ventricular shunt for other reasons than TBI (e.g., hydrocephalus) could have also been included possibly allowing for recognition of potential effects caused by placing an EVD. We were not able to provide data from such appropriate control patients in our academic university trauma department. Therefore a multicenter study should be considered for future investigation. However this study analyzed the dynamics of monocytes and granulocytes after severe TBI events which was an important first step that future research can build on.

In conclusion the populations of CD14+ monocytes and CD15+ granulocytes significantly increased compared to the cell populations of the control group and even to the cell populations at the time of admission in the patient group, but it remained unclear how this increase occurred and whether the increase influenced the outcome of TBI patients. Further research is necessary and is the focus of our study group's current work.

Conflict of Interests

The authors declare that they have no competing interests.

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Review Article

Crossing the Vascular Wall: Common and Unique Mechanisms Exploited by Different Leukocyte Subsets during Extravasation

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Leukocyte extravasation is one of the essential and first steps during the initiation of inflammation. Therefore, a better understanding of the key molecules that regulate this process may help to develop novel therapeutics for treatment of inflammation-based diseases such as atherosclerosis or rheumatoid arthritis. The endothelial adhesion molecules ICAM-1 and VCAM-1 are known as the central mediators of leukocyte adhesion to and transmigration across the endothelium. Engagement of these molecules by their leukocyte integrin receptors initiates the activation of several signaling pathways within both leukocytes and endothelium. Several of such events have been described to occur during transendothelial migration of all leukocyte subsets, whereas other mechanisms are known only for a single leukocyte subset. Here, we summarize current knowledge on regulatory mechanisms of leukocyte extravasation from a leukocyte and endothelial point of view, respectively. Specifically, we will focus on highlighting common and unique mechanisms that specific leukocyte subsets exploit to succeed in crossing endothelial monolayers.

1. Introduction

The inflammatory response is critical for fighting infections and wound healing and is thus indispensable for survival [1, 2]. However, continuously active immune responses precede chronic inflammatory disorders and other pathologies. Thus, the immune response to injury and infection needs to be tightly controlled. In order to specifically interfere with excessive leukocyte transendothelial migration (TEM), a detailed understanding of the regulation of this multistep process is required. Butcher and Springer proposed in timeless reviews a multistep model for the process of TEM [3, 4]. Currently, this proposed model is still valid; however, over time some additional steps have been added to the sequence of events during TEM [2]. The inflammatory response starts with secretion of proinflammatory mediators such as histamine or cytokines that induce the opening of endothelial cell (EC) contacts in postcapillary venules to allow for passage of blood molecules, for example, complement factors. Inflammation also involves surface expression of endothelial adhesion molecules, actin remodeling, and activation of leukocyte integrins that enable leukocyte adhesion onto the endothelium within the vascular wall and subsequent diapedesis [5–8]. The sequence of adhesive interactions of leukocytes with EC is termed leukocyte extravasation cascade and involves a series of adhesive interactions that allow first tethering, rolling, and slow rolling, followed by firm adhesion, crawling, and transmigratory cup formation on the apical endothelial surface (Figure 1). Next is the actual TEM of leukocytes (also termed diapedesis) that can occur by crossing either EC contacts (paracellular) or the body of EC (transcellular). Both ways exist and it is known that the strength of endothelial

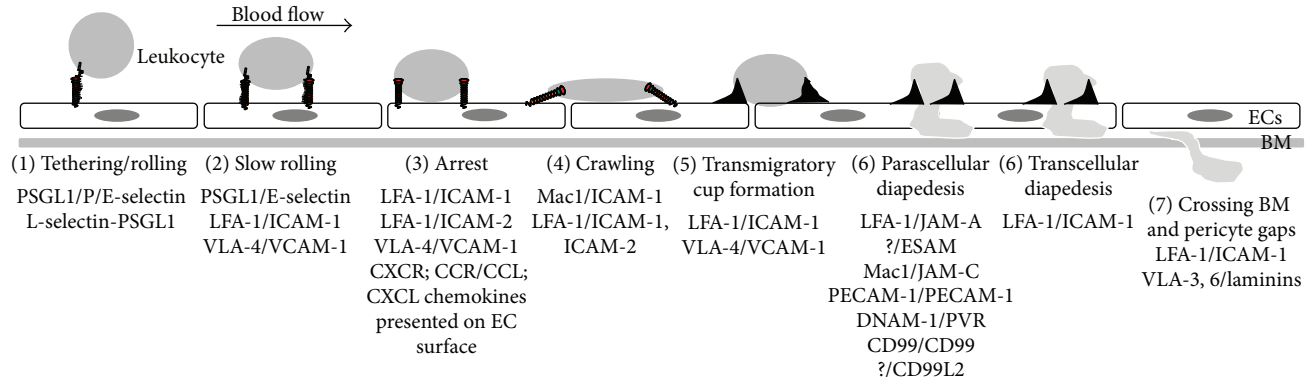

Figure 1: General scheme of the leukocyte extravasation cascade. The different steps of leukocyte interactions with endothelial cells during adhesion and transmigration are depicted. The known adhesion receptor interactions are listed for each step with the leukocyte receptor being named first. Unknown ligands are represented by question marks. During rolling, secondary rolling of leukocytes on already adherent leukocytes can occur that involve interactions of leukocyte L-selectin with leukocyte PSGL1 (not depicted). All receptors are connected to the actin cytoskeleton via actin-binding proteins to facilitate the extensive actin remodeling required for the morphological changes and movement of both cell types involved (not depicted). For details, see text.

junctions controls route preference [9] but the exact underlying mechanisms remain elusive. After crossing the endothelium, leukocytes also have to cross the pericyte layer and the basement membrane (BM) to reach the inflamed tissue and contribute to clearance of infection and wound healing [10]. Different types of leukocytes are being recruited to sites of inflammation including neutrophils, monocytes, and lymphocytes. In response to an inflammatory stimulus, neutrophils are generally among the first leukocytes to exit the blood stream, and, after degranulation, they contribute to a second wave of transmigration by mainly monocytes [11]. The reverse case has also been observed, in which the presence of monocytes and monocyte-derived neutrophil chemoattractants were required for neutrophil recruitment to sites of sterile inflammation [12]. Recruitment of all of these leukocyte subsets is compulsory for a proper immune response since all fulfill different functions once recruited to the inflamed tissue [13]. All these leukocyte types follow the sequential steps of the extravasation cascade in general, but differences in responsiveness to certain chemokines and in expression/activation of adhesion molecules to mediate interactions with EC have been described [8, 14]. Several mechanisms during the leukocyte extravasation cascade such as certain receptor-ligand interactions or signaling pathways have been confirmed as being exploited by all leukocyte subsets. However, other mechanisms have so far only been described for a single type of leukocyte. Whether these mechanisms are indeed unique for a given leukocyte subset or whether it has just not been studied yet in other leukocyte subsets is an important question to be answered in the future. A plethora of reviews have been published that summarize several aspects of leukocyte recruitment but in a generalized form that speaks only of "leukocytes." In this review, we summarize current knowledge on common and unique mechanisms that different leukocyte types such as neutrophils, monocytes, and lymphocytes exploit during extravasation (Table 1). This includes signals induced within each leukocyte subset as well as differential signals that each leukocyte subset induces in EC to facilitate transmigration.

2. Mechanisms Exploited by Neutrophils to Achieve Extravasation

Representing ~40–60% of circulating leukocytes in the blood of humans, released at a rate of ~1-2 $\times$ 10^{11} cells per day into the blood stream and with a lifespan of only 1–5 days [15], neutrophils are among the first leukocytes to be recruited at sites of inflammation and/or injury. Migration of these unique leukocytes through blood vessel walls is a tightly regulated process for which some of the molecular interactions with the different components of the vessel wall (e.g., endothelium, pericyte sheath, and the venular BM) have been relatively well described in the literature [5, 14, 16]. There are now 5 major steps considered for the recruitment of neutrophils, namely, (1) capture and rolling along the luminal side of the endothelium, (2) firm adhesion and crawling toward the site of TEM, (3) TEM (and its variations), (4) subendothelial crawling along the pericytes processes, and (5) exit into the extravascular space through pericyte gaps and specific regions within the vascular BM. For many decades, it was assumed that chemokines and other soluble chemoattractants were responsible for the specificity of recruitment of leukocyte subsets due to a unique repertoire of G-protein coupled receptors present on their surface [17–19]. However, recent compelling *in vivo* evidences have challenged this idea and demonstrated a role for many adhesion molecules present on the EC surface specifically instructing the neutrophil to extravasate [20–22].

2.1. Capture and Rolling. Free flowing neutrophils are isolated from the endothelium by a dense, 0.5 to 5 μm thick, network of negatively charged proteoglycans, glycosaminoglycans,

Table 1: Overview of some mechanisms that regulate extravasation of leukocyte subtypes in the order of events during the leukocyte extravasation cascade.

TEM step	Regulatory proteins	Cell	Function	Reference
Tethering/rolling/slow rolling	L-selectin, PSGL-1	EC/monos	L-selectin interacts with PNAd and PSGL-1 with P- and E-selectin to mediate proper rolling	[80]
	P-selectin, Mac1	EC/monos	Rolling and adhesion on ECM-bound platelets under flow	[84]
	P-selectin, PSGL-1, and CD44	EC/monos	Mediate rolling during monocyte recruitment to lymphoid tissues during inflammation	[79]
	CD44	Neutrophils/T cell	CD44 interacts with E-selectin in cooperation with PSGL-1 to mediate rolling	[141]
	TIM-1	T cell	TIM-1 interacts with PSGL-1 to mediate rolling	[140]
	CD43	T cell	CD43 interacts with E-selectin in cooperation with PSGL-1 to mediate rolling	[142, 143]
	P-selectin, PSGL1/PSGL-1 CD44, and L-selectin	ECs/neutrophils	Mediate rolling during recruitment of neutrophils in cremasteric postcapillary venules	[21, 22]
	PSGL-1, LFA-1/P-selectin, and ICAM-2	Neutrophil/ECs	Mediate sling formation and slow rolling	[32, 37]
Arrest/adhesion	VLA-4	Monos	PLC-, Ca-, and calmodulin-dependent arrest in response to chemokines	[86]
	VLA-4, GDF-15	Monos	GDF-15 reduces VLA-4 activation and monocyte adhesion	[87]
	LFA-1/ICAM-1	Neutrophils	Mediate neutrophil arrest	[42]
	EphA2	EC	Reduction of VCAM-1 expression and monocyte adhesion	[89]
	DARC	EC	CCL2 transport to the apical EC surface to induce monocyte activation and recruitment	[85]
	SIRPα/CD47	Monos/EC	Negatively regulates β2-integrin-mediated monocyte adhesion and transmigration	[91]
	CD47	T cell	Mediates integrin-dependent arrest on VCAM-1 and ICAM-1 and T cell recruitment *in vivo*	[145]
	Kindlin 3	T cell	Reinforces T cell adhesion	[146]
	CXCR4	Monos/B cell	CXCL12-dependent adhesion and diapedesis	[159]
	VCAM-1	B cell	VCAM-mediated arrest without rolling	[160]
Crawling	LFA-1, Mac1	Monos	Locomotion in search for the nearest suitable junction to start diapedesis	[92]
	LFA-1, Mac1	Monos	Crawling in unstimulated cremaster venules LFA-1-dependent that becomes Mac1-dependent after TNF-α-stimulation	[93]
	LFA-1; CCL3/CXC3CL1	Monos	Patrolling of resident monocytes and recruitment into noninflamed tissues	[72]
	Mac1/ICAM-1	Neutrophils	Control the luminal crawling of neutrophils on endothelial ICAM-1	[42]
	Mac1/ICAM-2	Neutrophils	Control the directionality and speed of crawling of neutrophils on endothelium	[45]
Cup formation	LFA-1/ICAM-1 VLA-4/VCAM-1 ALCAM-1	All	Clustering of these receptor-ligand pairs around adhering leukocytes causes GTPase activation, actin adaptor molecule recruitment, actin remodeling, and protrusion formation to engulf and support the adherent leukocyte	[49, 50, 94–100]

Table 1: Continued.

TEM step	Regulatory proteins	Cell	Function	Reference
TEM	JAM-A, JAM-L, JAM-C, PECAM-1, DNAM-1, CD155, and CD99	All	Serve as counterreceptors for leukocyte-EC interactions during the passage through interendothelial cell contacts	[105, 108–112]
	Mac1, NE/JAM-C	Neutrophils/ECs	Control the directionality of neutrophil transendothelial migration. Cleavage of JAM-C induces aberrant transendothelial migration	[57]
	VAP-1	T cell	Together with ICAM-1 and CLEVER-1 specifically regulates T cell TEM	[156, 157]
		Monos	Support CX_3CL1-dependent monocyte transmigration across hepatic sinusoidal EC	[161]
		Neutrophils	Blocking enzymatic activity of VAP-1 reduces neutrophil diapedesis and accumulation in lungs	[162, 163]
	Occludin	EC	Methamphetamine-induced Arp2/3 activation induces occludin internalization and monocyte transmigration	[113]
	JAM-A	Monos Neutrophils	Blocking JAM-A interaction with LFA-1 reduces recruitment of monocytes and neutrophils into the brain after ischemia/reperfusion injury	[107]
After TEM	CXCL1/ICAM-1 Mac1/LFA-1	Pericytes/neutrophils	Abluminal crawling along pericyte processes	[63]
	VLA-3 VLA-6/collagen, laminin	Neutrophils/venular BM	Control the migration of neutrophils through venular basement membrane and exit through LERs	[10]
Interstitial motility	LFA-1 VLA-3	All	Interaction with abluminal ICAM-1 enables uropod extension while VLA-3 mediates movement of the leading edge in the BM	[68]
	ICAM-1	Pericytes	$NG2^+$-pericytes secrete chemokines and express ICAM-1 to attract/bind transmigrated leukocytes	[114]
	DDR1α	Monos	Expressed after transmigration *in vivo* to support migration within collagen-rich ECMs	[118]
	JAM-A	Neutrophils	Controls polarized interstitial migration	[55]
	RhoA	Monos	Active RhoA required for tail retraction to complete diapedesis	[115]

and glycoproteins called the EC glycocalyx [23]. This structure acts as a formidable barrier for emigrating leukocytes and exceeds the dimensions of cellular adhesion molecules involved in neutrophil recruitment. Alterations of the EC glycocalyx are therefore a prerequisite for the first steps of neutrophil extravasation [24, 25]. Expression of positively charged molecules such as MPO on the surface of neutrophils [26] as well as shedding of the EC glycocalyx by heparinase [27], release of neutrophil-derived reactive oxygen species (ROS) [28], and matrix metalloproteinases (MMP) [29] contribute to facilitation of neutrophil-EC contacts. Once the EC glycocalyx is removed, neutrophils can reach the endothelial surface via a specific class of 3 closely related glycoproteins called the selectins and their glycoconjugate ligands (P-selectin glycoprotein ligand-1 (PSGL-1), CD44, and E-selectin ligand-1 (ESL-1)) [21, 30, 31]. L-selectin is constitutively expressed on the surface of neutrophils, whereas P- and E-selectins are more specific to EC. P-selectin is constitutively stored in distinct EC granules called Weibel-Palade bodies that are rapidly mobilized to the EC surface where P-selectin gets homogeneously distributed on the cells. By contrast, E-selectin is synthetized *de novo* during activation and concentrated mainly at EC junctions. Interestingly, a new study from Zuchtriegel and colleagues [22] demonstrated that neutrophils mainly use P-/L-selectin and PSGL-1/CD44 but not E-selectin to tether and roll along the endothelium *in vivo*. When blocked, these interactions affected not only the flux of rolling neutrophils but also their subsequent firm adhesion, crawling, and TEM. By contrast, inflammatory monocytes additionally need to interact with

E-selectin for proper transmigration across the vessel wall, thus highlighting a new singular difference in the molecular interactions between different subsets of leukocytes and EC during this first stage of transmigration.

Despite the weak and transient nature of the molecular interactions between selectins and their ligands, neutrophils roll even under high shear stress within blood vessels. Sundd and colleagues have recently made some interesting observations on how these neutrophils maintain contact with EC during rolling [32]. During the initial contacts with EC through selectins/selectin ligands, the structure of the neutrophil cell membrane is modified by reorganization of both cytoskeleton and surface adhesion molecules leading to the formation of an extended protrusion called sling [33]. This structure is formed from a membrane tether at the back of the rolling neutrophil like an anchor before it is wrapped around the rolling leukocyte and swings to the front of the cell to recontact the EC. Such slings contained heterogeneous patches of PSGL-1 conferring intermittent adhesive structures to the EC surface but are also rich in the β2-integrin lymphocyte function-associated antigen-1 (LFA-1). Furthermore, the binding of PSGL-1 to P-/L-selectin during the rolling step leads to conformational changes in the neutrophil β2-integrin LFA-1 through outside-in signalling [34–36]. This response allows for binding of LFA-1 to its ligands on EC supporting slow rolling and eventually transition to firm adhesion of the neutrophil [37].

2.2. Firm Adhesion and Crawling. Strengthening of neutrophils-EC interactions occurs mainly through the binding of the leukocyte β2-integrins LFA-1 and macrophage-antigen-1 (Mac1) to their cognate receptor intercellular adhesion molecule-1 (ICAM-1) expressed on activated EC [38]. These interactions enable the neutrophils to firmly adhere to the surface of the endothelium. In parallel to β2-integrins/ICAM-1 adhesive complexes, the neutrophil β1-integrin very late antigen-4 (VLA-4) and its EC binding partner vascular cell adhesion molecule-1 (VCAM-1) can contribute to the arrest of leukocytes in specific inflammatory conditions in humans [39]. This strengthened adhesion is completed by the sensing of chemotactic molecules such as chemokines (e.g., CXCL1/2), lipid mediators (e.g., LTB4, PAF), and complement proteins (e.g., C5a) by G-protein-coupled receptors (GPCRs) on the surface of the neutrophils that further signal through the cytoskeleton to induce full activation of the integrins and firm adhesion [37]. Following this firm adhesion, neutrophils crawl perpendicular to or even against the flow of the bloodstream, toward chemotactic [40] (e.g., chemokines) or haptotactic (e.g., ICAM-2) gradients. The mechanism of this luminal crawling is strictly ICAM-1/Mac1-dependent [41, 42] as blockade of these two molecules *in vivo* resulted in neutrophils failing to both crawl and migrate through EC junctions without affecting neutrophil adhesion. It has been suggested that the transition between LFA-1-dependent firm adhesion and Mac1-dependent crawling of neutrophils occurs via inside-out signalling through LFA-1 and the activation of the guanine exchange factor Vav-1 [43] that consequently activates Mac1 [44]. Recently, another member of the CAM family, ICAM-2, has been shown to play a role in neutrophil crawling dynamics toward EC junctions prior to TEM [45]. In mice exhibiting genetic deletion of this molecule as well as in WT animals treated with a blocking antibody against ICAM-2, neutrophils exhibited an increase in crawling duration and reduced crawling speed, leading to neutrophils lingering longer along the luminal surface of EC and delaying their migration through endothelial junctions.

2.3. TEM and Its Variations. TEM is the most rapid response of the migration cascade of neutrophils, lasting 5–10 min depending on the inflammatory scenario. Several molecular interactions between neutrophils and EC have been described for this step in the literature [5, 14, 16]. The penetration of EC by neutrophils occurs via two routes: through EC-EC intercellular junctions (i.e., paracellular migration) or through the body of the EC (i.e., transcellular migration). Recent *in vivo* evidence showed the predominance of the paracellular route (90% of transmigration events) over the transcellular migration [46]. Genetically modified mice in which the adherens junctions and more particular the VE-cadherin-catenin/VE-PTP complex are stabilized showed that the blood vessel wall became impermeable to macromolecules and neutrophil infiltration [47, 48]. By contrast, mice deficient for the actin-binding protein cortactin showed reduced clustering of ICAM-1 around adherent neutrophils due to defective activation of the GTPase RhoG in EC leading to strongly reduced adhesion and transmigration [49, 50]. Numerous adhesion molecules enriched at EC-EC junctions such as PECAM-1, JAM family members, ICAM-2, CD99, ESAM, and CD99L2 are involved in the process of neutrophil TEM. These molecules are also detected in subcellular structures called the lateral border recycling compartment (LBRC) that play a key role in neutrophil TEM [51, 52]. In basal conditions, these adhesion molecules contribute to the maintenance of EC junctions; however, during inflammation they engage with their counter-receptors on neutrophils (e.g., β2-integrins LFA1 and Mac1 and through homophilic interactions of PECAM-1, JAM-A, or CD99 that are also expressed on leukocytes) to allow for crossing of the junctions in a sequential manner [16, 53, 54]. The binding of adhesion molecules between neutrophils and EC can also mediate polarization signals in the neutrophils allowing them to correctly migrate from the luminal to abluminal sides of the EC. This is particularly true for JAM-A [55] and JAM-C [56]. Two recent publications demonstrated *in vivo* the presence of abnormal transendothelial migratory events [46, 57] characterized by the neutrophil partially migrating through the junction with oscillatory movements in the junction (i.e., hesitant migration) or even returning back to the circulation in an abluminal-to-luminal direction (reverse migration) following ischemia-reperfusion injury or leukotriene B4- (LTB4-) induced inflammation. This abnormal transmigration can represent up to 20% of total TEM events. This response could be reproduced or even enhanced in other inflammatory conditions in the absence or by blockade of JAM-C [46]. Abnormal transmigration indeed

involves the removal of JAM-C from the junction via cleavage by neutrophil elastase [57] following its translocation from azurophilic granules to the surface of the leukocyte in a complex with the integrin Mac1 upon direct stimulation of the neutrophil by LTB4 [58]. Genetic deletion or pharmacological inhibition of NE could significantly restore the presence of JAM-C at junctions and reduce the abnormal transmigration events. On the other hand, exogenous injection of NE in inflammatory models not known for exhibiting abnormal TEM of neutrophils increased the number of these events. This specific abnormal TEM response was associated with the presence of soluble JAM-C in the serum and an increase in secondary organ damage, two key features regularly observed in patients with trauma or ischemia-reperfusion injury.

2.4. Abluminal Crawling. Earlier observations of migration events showed that, after TEM, the vessel wall was thickening and neutrophils could only be detected in the tissue more than 20–40 min after TEM had occurred. For many decades, nothing was known about what was happening to the neutrophil during this period of time. Once migrated through the EC, the abluminal neutrophil faces the second cellular component, that is, the pericytes, and its tight matrix, the venular basement membrane (BM), in which they are embedded [59]. Many of the studies of transmigration events have neglected these two components of blood vessel walls due to the difficulty to reproduce the complete structure *in vitro* or to visualize it *in vivo*. However, recent developments of new advanced microscopy techniques and the generation of genetically fluorescent animals have shed new lights on the role of pericytes in the recruitment of neutrophils *in vivo*. Pericytes express adhesion molecules and chemokines such as ICAM-1, VCAM-1, and CXCL1 upon inflammation both *in vivo* and *in vitro* [60–63]. This response was correlated with the observations that, after TEM, neutrophils were found crawling along pericyte processes away from their site of TEM in an ICAM-1/Mac1- (and to a lesser extent LFA-1) dependent manner before fully breaching the venular wall [63]. Blocking those molecular interactions with blocking antibodies could suppress both neutrophil abluminal motility and their entry into the interstitial space.

2.5. Exit from the Vessel Wall. Following abluminal crawling, neutrophils exit the vessel wall through specific enlarged gaps between adjacent pericytes. The role of pericyte gap enlargement is still unclear, but, interestingly, less than 10% of the gaps were used by migrating neutrophils and most of the time hot spots of transmigration could be observed where more than 2-3 neutrophils exited via the same pericyte gaps. It has been suggested that potential enrichment in adhesion molecules and chemokines around specific pericyte gaps [63] as well as the release/generation of chemoattractants by the leading neutrophils from their granules [11] and/or from the cleavage of BM proteins into chemotactic fragments [64] could pave the way for subsequent neutrophils.

The venular BM (generated by both the EC and the pericytes) is the final interactive matrix (but also barrier) for emigrating neutrophils. This structure is composed of tight networks of matrix proteins such as collagen type IV and laminins [65]. Interestingly, blocking the interactions between leukocyte integrins VLA-3 and VLA-6 (receptors for collagen and laminin, resp.) and the venular BM using blocking antibodies could inhibit the migration of neutrophils through this layer [66–68]. Another unique characteristic of neutrophil interaction with the venular BM is the discovery of low expression regions (LERs) within the BM that are preferred sites for neutrophil migration [10, 59]. These sites contain low quantities of matrix proteins, are associated with gaps between adjacent pericytes, and are being used and enlarged during neutrophil, but not monocyte, migration [69]. In fact, it will take another 10 to 20 minutes more for the neutrophils to migrate through pericyte gaps and LERs as observed *in vivo*, with many oscillatory movements by the neutrophils [63]. However, the duration of LER/pericyte gap penetration and oscillatory movements are reduced for the subsequent neutrophils following the same hot spot of migration. Though the mechanism of the remodelling of such permissive sites in the BM of venular walls is not fully understood, it has been suggested that proteolytic cleavage by neutrophil enzymes [10, 59] and/or reversible disassembly of collagen fibres [65] could be involved in this process, thus allowing the neutrophil to finally access the interstitial space.

3. Mechanisms Exploited by Monocytes to Achieve TEM

3.1. Monocyte Populations. Monocytes are heterogeneous cells that circulate in the blood in distinguishable populations termed resident (or patrolling) and inflammatory monocytes according to the expression profile of certain chemokine receptors and adhesion molecules [70–72]. While resident monocytes are associated with immune surveillance and wound healing, inflammatory monocytes are connected to induction and maintenance of inflammatory immune responses [73]. On the other hand, monocytes give rise to dendritic cells and macrophages to promote inflammatory responses [74, 75]. Monocytes are actively being recruited from the bone marrow via the blood stream to inflamed tissues in a largely CC-chemokine receptor 2/CC-ligand-2-(CCR2/CCL2) dependent fashion. Very recently, an elegant intravital imaging study reported a phenotypic conversion of monocyte subsets at sites of sterile liver injury [76]. First, inflammatory monocytes were rapidly recruited and stayed around the injured site for about 48 h before a conversion to a resident monocyte phenotype and entry into the injured area occurred to induce wound healing. This previously unrecognized monocyte plasticity highlights the importance of monocytes for resolution of inflammations. Furthermore, a targeted silencing approach using nanoparticles containing CCR2-specific siRNA has been described in mice that prevented accumulation of inflammatory monocytes at sites of inflammation and ameliorated various pathological conditions in which inflammatory monocytes have been implicated [77]. This is a promising approach to specifically target inflammatory monocytes without affecting other immune cells during inflammation; however, it remains to be proven whether such an approach is applicable to humans.

3.2. Rolling and Slow Rolling. TEM of monocytes occurs according to the paradigm of the leukocyte extravasation cascade as described above [78]. In addition to the well-established role of PSGL-1 in all leukocyte rolling, monocyte rolling during recruitment to lymphoid tissues also depended on L-selectin and CD44 [79]. In the infected skin, proper monocyte rolling and subsequent recruitment depended on monocyte PSGL-1 interaction with endothelial E- and P-selectin, whereas monocyte L-selectin interacted with endothelial peripheral node addressin (PNAd) [80]. L-selectin shedding is required at later stages of transmigration to ensure a regulated and polarized conclusion of transmigration [81]. On EC expressing high amounts of VCAM-1, for example, in atherosclerotic lesions, monocyte rolling and transition to slow rolling and arrest strongly depended on the β1-integrin VLA-4 [82, 83]. However, on ECM-bound platelets, monocyte rolling rather depended on the β2-integrin Mac1 and P-selectin [84].

3.3. Firm Adhesion. Once slowed down, leukocytes recognize chemokines presented on the endothelium that lead to GPCR-mediated inside-out signaling, full integrin activation, and the subsequent arrest of leukocytes. The endothelial Duffy antigen receptor for chemokines (DARC) has recently been reported to transport CCL2 across the endothelium to the apical side where it contributed to proper monocyte activation and recruitment [85]. In monocytes, full VLA-4 activation after GPCR stimulation depended on a signaling axis including phospholipase C (PLC), inositol-1,4,5-triphosphate (IP3), Ca2+-flux, and calmodulin but not on PI3K [86], which is in contrast to VLA-4 activation in neutrophils. Although the adhesion cascade has been best studied in neutrophils (reviewed in [8]), a common denominator regulating the transition from rolling via slow rolling to arrest in all leukocytes is the activation of PLC. However, depending on the stimulus, the primary integrin inducing firm adhesion/arrest varies among leukocyte subsets. In the case of monocytes, it seems to be VLA-4. Recently, growth differentiation factor-15 (GDF-15) has been identified as an endogenous inhibitor of VLA-4 activation that prevented monocyte binding to VCAM-1 and could thus serve as a local inhibitor of inflammation [87]. VCAM-1 expression and thus monocyte adhesion are increased by the endothelial receptor protein tyrosine kinase EphA2 [88, 89]. Other examples of endogenous monocyte integrin inhibitory molecules are the endothelial matrix protein developmental endothelial locus-1 (Del-1) and endothelial CD47 interacting with monocyte signal regulatory protein-α (SIRP-α) [90, 91]. Thus, monocyte-endothelial interactions seem to be regulated at different levels and most likely other regulation mechanisms will be unraveled in the near future.

3.4. Crawling. After firm adhesion, leukocytes spread and crawl on the endothelial surface to find a suitable spot for transmigration. This process has first been observed with monocytes and was called locomotion [92]. Such directional movement of monocytes preceding transmigration could be blocked by antibodies against LFA-1 or Mac1 strongly suggesting dependence on β2-integrins. In the case of patrolling monocytes, antibodies against LFA-1 but not Mac1 detached crawling monocytes, an effect that was also observed in mice lacking CX3CR1 [72]. In unstimulated cremaster venules, more monocytes (compared to neutrophils) adhered and crawled for longer distances in an LFA-1-dependent manner. Once stimulated by tumor necrosis factor-α (TNF-α), monocytes reduced their crawling distance that now became Mac1-dependent and more neutrophils crawled in a strictly Mac-1-dependent fashion [93]. In this study, Mac1 blockage was more efficient in reducing both monocyte and neutrophil extravasation compared to LFA-1 blockage. While crawling of monocytes can be both LFA-1-dependent and Mac1-dependent, neutrophil crawling is strictly Mac1-dependent [42], thus marking a striking difference between these leukocyte subsets during extravasation.

3.5. Docking Structures or Transmigratory Cups. During inflammatory leukocyte recruitment, the activated endothelium supports neutrophils by forming clusters around adhering leukocytes that are enriched in LFA-1/ICAM-1 and VLA-4/VCAM-1. These structures first appear as ring-like structures that surround adherent leukocytes and later engulf leukocytes as docking structures or transmigratory cups that are enriched in actin and various adaptor molecules such as cortactin, ezrin, radixin, moesin- (ERM-) proteins, and filamin, and signaling molecules such as RhoG and Rac1 [49, 50, 94–100]. Another endothelial adhesion receptor found in docking structures is activated leukocyte cell adhesion molecule-1 (ALCAM-1) that supports monocyte recruitment into the central nervous system (CNS) [101]. Docking structures have been observed *in vitro* with all leukocytes. *In vivo*, similar structures, so-called domes, have been described for neutrophils [102]. For more details, see the endothelial part below.

3.6. Diapedesis. In order to cross the endothelial monolayer between intercellular junctions, the VE-cadherin/catenin complex needs to be disassembled as it constitutes a physical barrier for transmigrating leukocytes [47]. Real-time imaging of transmigration *in vitro* using VE-cadherin-GFP overexpressing HUVEC revealed that monocytes as well as neutrophils used preexisting and *de novo*-formed VE-cadherin gaps to achieve paracellular transmigration [103]. Interestingly, initial transmigration of monocytes causes downregulation of VE-cadherin and upregulation of PECAM-1 that facilitates subsequent monocyte transmigration [104]. Other molecules of the interendothelial junctions can actually be exploited as counter-receptors by transmigrating leukocytes to facilitate transmigration. For example, monocyte LFA-1 can bind to JAM-A and JAM-A deficiency greatly reduces transmigration of monocytes [105, 106]. In the brain, blocking JAM-A interactions with LFA-1 reduced transmigration of monocytes and neutrophils and ameliorated the overall neurological damage after ischemia/reperfusion injury [107]. Expression of JAM-like protein (JAM-L) is upregulated on

monocytes during inflammation and binds to the endothelial receptor coxsackie and adenovirus receptor (CAR), an interaction that is regulated in *cis* by VLA-4 [108, 109]. Other adhesion molecules within EC contacts that serve as counter-receptor during inflammatory monocyte recruitment are PECAM-1, CD155, and CD99. PECAM-1 and CD99 interact homophilically, whereas endothelial CD155 interacts with CD226 on monocytes. Blockade of all these molecules that are part of the lateral border recycling compartment (LBRC, [52]) by antibodies drastically reduced monocyte TEM without affecting rolling, adhesion, and crawling [110–112]. Interestingly, these molecules act in a sequential manner with PECAM-1 engagement occurring first followed by CD155 and then CD99 [112]. Moreover, occludin in the tight junction controls monocyte transmigration across the blood-brain barrier in response to methamphetamine in an actin-related protein-2/3- (Arp2/3-) dependent fashion [113]. Inhibiting Arp2/3 prevented methamphetamine-induced occludin-internalization and monocyte TEM.

3.7. Postdiapedesis Events. In order to cross the BM and the pericyte layer, both monocytes and neutrophils exploit areas of pericyte gaps and low matrix protein expression within the BM [10, 69]. However, while neutrophils were capable of enlarging these low matrix protein expression regions, monocytes showed increased deformability and rather squeezed through them [69]. $NG2^+$-pericytes also guide and bind transmigrated monocytes and neutrophils by chemokine secretion and ICAM-1 expression, respectively [114]. A common mechanism for all leukocytes after crossing the EC layer is the elongation of uropods and a delayed detachment of the leukocyte from the basal endothelial surface [68]. This process depended on LFA-1 interaction with abluminal ICAM-1 that maintained the connection of the uropod with the endothelium and, on the other hand, on VLA-3 that mediated movement through the BM. Signaling required for tail retraction after diapedesis involves RhoA [115]. Inhibiting RhoA in monocytes renders the cells unable to complete diapedesis and leads to β2-integrin accumulation in the unretracted tail. Vice versa, RhoA activation in EC is also required for efficient monocyte transmigration [116] which involves ROCK-mediated myosin light chain phosphorylation and disruption of VE-cadherin-dependent EC contacts [117]. Monocytes that fully transmigrated express the surface receptor discoidin domain receptor-1α (DDR1α) that can bind collagen and seems to facilitate monocyte migration within collagen-rich extracellular matrices (ECM) in inflamed tissues [118]. Interestingly, monocytes can undergo reverse transmigration when JAM-C-mediated adhesion is disrupted by antibodies *in vivo* [119].

4. Mechanisms Exploited by Lymphocytes to Achieve TEM

T and B lymphocytes (T and B cells) are adaptive immune cells capable of recognizing and distinguishing antigens leading to functional specificity and memory. T and B cells arise from the bone marrow and populate the peripheral lymphoid organs (spleen and lymph nodes), where they complete maturation and become activated in response to specific antigens presented by antigen presenting cells, including dendritic cells (DCs). Trafficking to the lymph nodes occurs via lymphocyte L-selectin-dependent adhesion to its ligands on high endothelial venules (HEV), glycan-bearing cell adhesion molecule-1 (GlyCAM-1), and CD34, followed by integrin-dependent arrest via LFA-1 and VLA-4. The affinity of these integrins is rapidly increased by the chemokines CCL19 and CCL21 that are mainly produced in lymphoid tissues by HEV. These chemokines then bind to CCR7 expressed in both naïve T cells and B cells. Upon antigen presentation, naïve T and B cells become activated and lose L-selectin expression [120–122]. Activated lymphocytes can either become effector cells or memory cells. Effector B cells produce antibodies that contribute to the elimination of extracellular microbes. Effector T cells can be separated into $CD8^+$ cytotoxic T cells, which kill virus-infected cells, and subsets of $CD4^+$ T cells including T helper (Th) cells that help other immune cells carry out their functions, and T regulatory (Treg) cells that suppress the functions of effector T lymphocytes. T and B cell subsets are further divided into well-defined subtypes that play different roles in health and disease [123]. In this section, we summarize the different mechanisms exploited by T and B cells to achieve transendothelial migration. Extravasation of T cell subsets has been studied in more detail *in vitro* and *in vivo*. B cells also infiltrate sites of inflammation in several diseases [124–126] but it is assumed that B cells largely exploit mechanisms similar to those of T cells. Current studies focusing on B cell extravasation are discussed at the end of this section.

4.1. Mechanisms of T Cell Extravasation. T lymphocytes (T cells) are specialized leukocytes that have the ability of recognizing antigens and participate in the adaptive immune response. The process of T cell recruitment to sites of infection or injury is fundamental in the immune response to exogenous antigens, with implications for responses to autoantigens that trigger chronic tissue inflammation when immune tolerance is disrupted in autoimmunity [127]. Within the T cell recruitment cascade, T cell adhesion is perhaps the best-understood process under flow conditions, with several *in vitro* studies involving human $CD3^+$ T cells and T cell-like cell lines characterizing the molecular signals regulating chemokine-induced integrin activation [128]. These *in vitro* studies using human T cells have also demonstrated that the behavior of T cells differs from other leukocytes once contact with the activated vascular endothelium is established. T cells randomly adhere to the apical side of the endothelium and locomote on the surface for some time. This is thought to be required to allow for enough time to induce the chemokine-chemokine receptor crosstalk necessary for optimal T cell integrin activation and subsequent TEM [129]. This contact time also allows the T cell receptor (TCR) to recognize potential antigens being presented by vascular EC, a recently explored pathway that can also lead to TEM [130]. Recent studies on different T cell subsets expanded the classic paradigm of T cell recruitment

by identifying novel critical molecules and pathways leading to T cell extravasation *in vivo*. These involve common but also T cell-specific molecules that participate in the different steps of the T cell recruitment cascade as summarized below.

4.1.1. Rolling. Rolling of T cell subsets has mostly been characterized using $CD4^+$-T cells, with the understanding that many findings may also hold true for $CD8^+$-T cells [131, 132]. As in other leukocytes, T cell rolling occurs under shear conditions by interactions of endothelial selectins with highly glycosylated T cell-expressed selectin ligands. In contrast to other leukocytes such as neutrophils and monocytes, which constitutively express all the glycosyltransferases required for functional selectin ligand biosynthesis, these are normally inducible and regulated in T cells [21]. Therefore, the ability of T cells to bind endothelial selectins is acquired in response to signals that assure the proper glycosylation of selectin ligands. These signals include specific cytokines, and thus T helper type 1 (Th1) cells and T helper type 2 (Th2) cells, which require different cytokines for differentiation and survival [133, 134], differ in the initial rolling step of the T cell recruitment cascade due to the differential expression of active selectin ligands. Th1 cells are major players in immune responses to intracellular microbes and in tissue damage associated with autoimmunity and chronic infections. They express high levels of glycosyltransferases in response to the Th1 cytokine IL-12 and thus have highly glycosylated selectin ligands for rolling on the activated endothelium. Th2 cells contribute to fight against helminth infections and autoimmune atopic diseases but have a rather low extravasation potential compared to Th1 cells [135–137]. The more recently discovered Th17 cells participate in the immune response to extracellular bacteria and fungi and, similarly to Th1 cells, play a role in organ-specific autoimmunity and chronic inflammation [138]. Interestingly, both Th1 and Th17 cells share selectin ligands such as PSGL-1 to roll on the vascular endothelium via P-selectin [139]. More recent data indicate that these subsets, in contrast to the Th2 or naïve cells, can also use the T cell immunoglobulin and mucin domain 1 protein (TIM-1) as a P-selectin ligand to mediate T cell trafficking during inflammation and autoimmunity [140]. Similarities and differences in rolling mechanisms are observed in E-selectin-ligand/E-selectin mediated interactions among T cell subsets, with Th17 cells being more dependent on E-selectin-mediated interactions than Th1 cells [139]. Besides PSGL-1 which functions similarly in Th1 and Th17 cells as a ligand for both E-selectin and P-selectin, other E-selectin ligands have been identified in Th1 cells that are functional only in cooperation with PSGL-1, and these include CD44 [141] and CD43 [142, 143]. Further research will determine whether these can also function in Th17 cells in a subset specific manner.

4.1.2. Adhesion. The emerging novel roles for different T cell subsets in acute and chronic inflammatory processes and the differential expression of chemokine receptors in different T cell subsets have recently been recognized as being critical for integrin-mediated adhesion in response to specific chemokine-chemokine receptor signaling [144]. The integrin associated protein (CD47) has been shown to regulate adhesive functions of the β2-integrins LFA-1 and VLA-4 in T cells *in vitro*. This was proven to be a critical mechanism regulating T cell adhesion to the cremaster microvasculature *in vivo* in studies involving competitive recruitment of CD47 wild type and CD47-deficient Th1 cells [145]. The integrin coactivator Kindlin-3 has also recently been shown to reinforce T cell integrin activation and adhesion. Interestingly, this mechanism is specific for T cell adhesion but did not play a role in T cell diapedesis [146]. Studies using mouse Th1 and Th17 cells generated *in vitro* have also demonstrated that these cells express a different repertoire of chemokine receptors. Depending on the chemokine ligand exposed, these two subsets adhered to immobilized ICAM-1 only in the presence of SDF1α and CCL20, respectively, under shear flow conditions *in vitro*. Other studies using human T cells and HUVEC also demonstrated that CCL20 mediates Th17 adhesion to EC [147]. These studies nicely correlate with *in vivo* studies showing a role for CCR6, the receptor of CCL20, in specific $CCR6^+$-Th17 cell recruitment to the gut [148], to the central nervous system [149], and to the skin [139]. These adhesion mechanisms are therefore chemokine/chemokine receptor-specific. They may also be tissue specific, as Th17 cells in liver endothelium utilize vascular adhesion protein-1 (VAP-1) and the chemokine receptor CXCR3 in addition to CCR6 to mediate adhesion via β1 and β2 integrins [150].

4.1.3. Diapedesis. T cell TEM follows rolling and adhesion and ultimately leads to T cell infiltration into inflamed sites. Compared to other leukocytes such as neutrophils, the percentage of T cells that undergo TEM in *in vitro* assays under flow conditions is much smaller, and it normally requires additional chemokines such as SDF1α in order to facilitate T cell arrest and TEM. Similarities with other leukocytes include ICAM-1-mediated signaling upon T cell adhesion, and VE-cadherin gaps at the site of junctional transmigration [151–153]. Recent work has demonstrated that T cells can trigger the dissociation of the endothelial receptor phosphatase VE-PTP from VE-cadherin as a mechanism leading to VE-cadherin phosphorylation and gap formation to facilitate transmigration [154]. Endothelial CD47 can also promote VE-cadherin phosphorylation and participate in T cell transmigration *in vitro*. Interestingly, CD47 expressed on T cells is also required for T cell TEM *in vitro* and for T cell recruitment at sites of dermal skin inflammation *in vivo* [155]. *In vitro*, VAP-1 can mediate TEM of T cells across liver EC [156]. More recent studies have demonstrated that VAP-1 together with the common lymphatic endothelial and vascular endothelial receptor (CLEVER-1) and ICAM-1 can specifically regulate TEM of Treg cells [157]. VAP-1, CLEVER-1, and ICAM-1 are highly expressed at sites of leukocyte recruitment to the inflamed liver suggesting that they also regulate T cell transmigration into the liver *in vivo*. From all these studies it is clear that chemokines presented by the endothelium are critical for integrin-dependent adhesion and TEM of effector and memory T cells *in vitro*. This has significant implications *in vivo*, where chemokine gradients

are present in the context of infection or injury leading to T cell transmigration. A novel alternative mechanism described for T cell transmigration is the ability of effector T cells to access not only extracellularly deposited chemokines, but also intraendothelial chemokines such as CCL2 stored in vesicles inside the EC to achieve transmigration [158].

As different T cell subsets were identified as principal players in chronic inflammation, a role of the vascular endothelium has been considered as critical for the migratory patterns acquired by antigen-experienced effector T cells that migrate to sites of chronic inflammation. Whether these pathways are T cell subset specific or organ/vascular bed/disease-specific remains to be investigated [164]. Given that EC express major histocompatibility complex molecules (MHC) I and II and therefore can function as antigen presenting cells for both $CD4^+$- and $CD8^+$-T cells, it is recently being recognized that, at the time of contact and antigen presentation, EC can imprint restricted, specific trafficking molecules in T cells. These are thought to be acquired in the organ where the T cells were generated [165], or at the site of infection or inflammation, where these antigenic signals are thought to contribute to the recruitment of these T cell subsets [166, 167]. Thus, the classic paradigm of chemokine-induced T cell arrest and TEM is now being challenged with alternative ways that T cells use to achieve TEM in different inflammatory contexts. *In vitro*, both effector and memory $CD4^+$- and $CD8^+$-T cells dynamically probe the endothelium by extending actin-rich invadosome/podosome like protrusions (ILPs) [168], thought to actively participate in TEM by distorting the actin filaments and breaching the endothelial barrier [9]. *In vivo* mouse studies have shown that antigen specific $CD4^+$-effector T cells use cognate antigen driven signals presented by MHC-II for entry into pancreatic islets in autoimmune diabetes [169, 170]. Apical presentation of cognate antigenic peptides by MHC-I and perivascular dendritic cells are thought to increase integrin adhesiveness and TEM of $CD8^+$-T cells in vascular beds deficient in adhesive and chemotactic activities such as the pancreatic islets in diabetes [167] and vascularized transplants [171]. *In vitro* studies under flow conditions using human effector and memory $CD4^+$-T cells have contributed to gain further insight into the mechanisms taking place in TEM mediated by antigen presentation and TCR signals versus classic chemokine-induced TEM not involving antigenic signals. These studies have demonstrated that T cells can rapidly transmigrate in response to both chemokines and TCR-activating antigenic signals, but these two mechanisms differ in some of the molecular pathways regulating TEM: TCR stimulated TEM was highly dependent on fractalkine (CX3CL1), PECAM-1, CD99, nectin-2, poliovirus receptor (CD155), and ICAM-1, whereas chemokine-stimulated TEM involved ICAM-1 and JAM-A but not any of the other molecules [172]. Furthermore, both of these TEM pathways triggered the activation of the protein ZAP-70 in the transmigrating T cell but differ in the signaling downstream of ZAP-70. Vav-1, Rac-1, and myosin 2A activation occurred only in the T cells that have been in contact with vascular EC in an antigen-TCR dependent way [173]. Phenotypically, this signaling resulted in different T cell cytoskeleton reorganization during transmigration, with the T cell microtubule organizing center (MTOC) being organized in the contact region between the T cell and the EC. Dynein-driven transport of granzyme-containing granules to the contact region between the T cell and the EC was identified as the mechanism regulating the T cell cytoskeleton reorganization during TEM [174]. Thus, these specific molecular signals observed in TCR-driven T cell transmigration closely resemble immune synapse formation and seem to be a novel process that T cells utilize to achieve successful TEM.

Taken together, T cells are uniquely specialized to respond to antigens, proliferate, and differentiate into subsets that acquire migratory phenotypes that allow them to traffic to sites of inflammation previously accessed by neutrophils and monocytes. T cells share some of these recruitment mechanisms with other leukocytes and trigger similar signals on the vascular endothelium to achieve TEM. The specialized T cell response to different antigens and the cytokine milieu results in distinct expression of active selectin ligands and a different repertoire of chemokine receptors involved in rolling and arrest on the vascular endothelium. Once adhered to the endothelium, they can use classic TEM routes and novel antigen-dependent routes. Understanding the mechanisms that regulate the recruitment of effector T cells in different inflammatory settings will shed new light on potential ways these pathways can be exploited for immunotherapeutic purposes.

4.2. Mechanisms of B Cell Extravasation. As mentioned above, B cells utilize in general the same basic mechanisms as naïve T cells to home to secondary lymphoid organs. How activated B cell subsets migrate into specific tissues during inflammation has not been explored in such detail as for T cell subsets, for which each step of the recruitment cascade has been analyzed *in vitro* and *in vivo*. However, some studies have identified some differences in inflammatory B cell extravasation as compared to T cells that will be discussed here. Many mature B cells, named plasma cells, migrate from the lymph nodes to the bone marrow, where they secrete IgG antibodies for long periods of time that are distributed through the body via the blood stream. This B cell subset expresses VLA-4 and CXCR4, which bind to VCAM-1 and CXCL12, respectively, expressed in bone marrow sinusoidal endothelial cells. In contrast, mature B cells that produce IgA antibodies, express $\alpha 4\beta 7$, CCR9, and CCR10 which bind to MadCAM-1, CCL25, and CCL28, respectively, expressed in mucosal endothelial cells to migrate to mucosal tissues such as the gut [175, 176]. These molecules are also thought to mediate IgG and IgA-producing B cell recruitment to sites of chronic inflammation in the synovium [124], the brain [125], and the vessel wall [126]. Moreover, CXCL12 was able to stimulate diapedesis of human B cells across human brain microvascular endothelial cells under flow conditions, and this was blocked by CXCR4 function blocking antibodies [159]. Recently, ADAM28, which is highly expressed in B cells but not in T cells, has been shown to bind to VLA-4 and to increase VLA-4-dependent adhesion of the murine B lymphoma cell line L1-2 to VCAM-1 and subsequent transendothelial migration suggesting that this

metalloprotease affects the efficiency of B cell extravasation [177]. Interactions of ephrin-A4 with its endothelial receptor EphA2 have also been shown to regulate normal as well as leukemic B cell transendothelial migration [178]. B cells are also present within chronically inflamed liver tissue. Using *in vitro* flow adhesion assays and hepatic sinusoidal endothelial cells, human blood-derived B cells were captured via VCAM-1 without requiring a previous rolling phase and remained static before achieving transendothelial migration mediated by ICAM-1, VAP-1, and the chemokine receptors CXCR3 and CXCR4. This mechanism represents a prominent difference in B cell extravasation, since T cells displayed vigorous crawling before transmigration in the same system [160]. Others have observed so-called "intraendothelial canalicular" structures that are especially exploited by B cells to cross the endothelium during homing and inflammation [179]. Whether these structures can also be observed during the extravasation of other leukocyte types needs to be analyzed in the future.

5. The Dynamics of Leukocyte Diapedesis from an Endothelial Point of View

Under quiescent conditions, the endothelium expresses low levels of adhesion molecules, allowing limited immune surveillance. However, upon encounter of an infection or tissue damage, surveilling monocytes and neutrophils are triggered to release inflammatory cytokines, such as TNF-α and IL1β. The importance of this has been recently underscored by the group of Dr. Nourshargh [180]. They showed in an elegant *in vivo* model that neutrophils locally secreted TNF-α which immediately acted on the endothelium and thereby assisted other neutrophils in transmigration. For longer periods of inflammation, EC respond to inflammatory mediators by massive upregulation of adhesion receptors, such as P-selectin, E-selectin, ICAM-1, and VCAM-1, whereas ICAM-2 was found to be decreased [181, 182]. These adhesion molecules attract circulating immune cells to adhere to and transmigrate through the endothelial monolayer (Figure 1). Moreover, inflammatory cytokines induce presentation of chemoattractants on the EC surface, such as CXCL4/5 and IL-8 [14]. During rolling, G-protein-coupled receptors on the leukocytes encounter such chemokines presented by the EC and signal to induce a conformational change of the leukocyte integrins LFA-1 ($\alpha_L\beta_2$) and VLA-4 ($\alpha_4\beta_1$) into a high affinity state enabling these integrins to interact with their endothelial ligands ICAM-1 and VCAM-1, respectively [13, 183]. As a consequence, firm adhesion, crawling, and finally diapedesis occur via interendothelial junctions or through the EC body [184]. Although these processes are believed to occur for any type of leukocyte that crosses the endothelium, some endothelial signals are specifically induced by certain leukocyte subsets that we will highlight in this chapter.

5.1. Adhesion Molecule Upregulation. The inflammatory cytokine TNF-α stimulates EC by binding to its receptor TNFR1 (CD120a) [185, 186]. This binding induces the association of the cytoplasmic adaptor protein TRADD (TNFR1-Associated Death Domain protein) to the intracellular domain of TNFR1. Subsequently, TRADD binds to downstream effectors such as the serine/threonine kinase RIP1 (Receptor Interacting Protein 1), as well as the E3-ubiquitin ligase TRAF2 (TNFR-associated Factor 2). This association in turn triggers a kinase signaling cascade leading to the activation of the mitogen-activated protein kinases (MAPKs) p38, JNK, and ERK [187]. These kinases are able to activate transcription factors, such as activator protein-1 (AP-1). In addition, TRAF2 and RIP1 induce the activation of the transcription factor NF-κB. Under quiescent conditions, NF-κB is retained in the cytosol by inhibitor of κB (IκBα). Upon activation of the IκBα kinase (IKK) complex by TRAF2 and RIP1, IκBα is phosphorylated, which leads to its degradation and the subsequent nuclear translocation of NF-κB [188].

The promoters of the adhesion molecules E-selectin, VCAM-1, and ICAM-1 contain several NF-κB-binding sites, and NF-κB has been shown to be the primary regulator of TNF-α-induced adhesion molecule expression in EC [189–194]. Although the promoters of E-selectin, VCAM-1, and ICAM-1 also contain AP-1-binding motifs, these sites have varying contributions to TNF-α-induced upregulation of these adhesion molecules [192, 193, 195, 196]. In addition, other transcription factors such as Interferon Regulatory Factor-1 (IRF-1), Specificity protein 1 (Sp1), and GATA are also known to become activated via poorly characterized signaling pathways and contribute to TNF-α-induced adhesion molecule upregulation in EC [191, 197–199].

5.2. Signaling by CAMs. The integrin expression patterns differ per leukocyte type. For example, neutrophils primarily express Mac1 and LFA-1 and hardly any VLA-4, whereas monocytes and also T-lymphocytes and dendritic cells express all three integrins, albeit at different levels [200]. This already indicates that different leukocyte types through their integrin repertoire can cluster different ligands on the endothelium resulting in distinctive intracellular signals in the endothelium that differ per leukocyte type. For example, Th17-lymphocytes showed increased adhesion to E-selectin compared to Th1-lymphocytes, most likely because of a better integrin activation on these cells through the CCL20-CCR6 axis [139].

ICAM-1 and VCAM-1 are members of the immunoglobulin (Ig) superfamily of adhesion molecules, whose extracellular domains are characterized by the presence of five and six Ig-like domains, respectively. Compared to their ectodomains, ICAM-1 and VCAM-1 have relatively small carboxyl- (C-) terminal intracellular domains of only 28 and 19 amino acids, respectively. Although the C-terminal domains do not contain any apparent signaling motifs, the intracellular domain of ICAM-1 has been shown to be required for efficient leukocyte TEM [201, 202]. Moreover, ICAM-1 engagement by LFA-1/Mac1 has been linked to F-actin reorganization and to the initiation of signaling events within EC [203]. Several studies have shown that leukocyte adhesion and clustering of ICAM-1 induced an increase in intracellular Ca^{2+} levels [204, 205] leading to activation of

the tyrosine kinase Src by protein kinase C (PKC). In turn, Src induced tyrosine phosphorylation of focal adhesion proteins such as paxillin, cortactin, and FAK [204, 206, 207]. ICAM-1 clustering led to the activation of the small RhoGTPase RhoA which stimulated the formation of F-actin stress fibers and increased endothelial monolayer permeability [204, 208–210] (Figure 2). Moreover, RhoA activity was also demonstrated to be required for efficient ICAM-1 recruitment around adherent monocytes [210] suggesting an upstream role for RhoA within the ICAM-1-induced signaling cascade. Recently, it was shown that ICAM-1 clustering induced tyrosine phosphorylation of VE-cadherin in a Src- and Pyk2-dependent manner, which coincided with increased endothelial permeability [203, 211, 212]. Martinelli and colleagues showed that ICAM-1 clustering induced the phosphorylation of eNOS on S1177 and this was regulated by Src kinase, as well as RhoA, calcium, CaMKK, and AMP kinase, but not PI3 kinase. They additionally showed that this pathway controlled the phosphorylation of VE-cadherin and lymphocyte trafficking [203]. In contrast to ICAM-1, only a few studies have reported signaling events induced upon engagement and clustering of VCAM-1. The leukocyte integrin VLA-4, expressed on monocytes and lymphocytes, showed strong binding preference for VCAM-1 [213]. Clustering of VCAM-1 was shown to promote activation of Rac1 leading to the production of reactive oxygen species (ROS) [214–216]. VCAM-1-dependent ROS production was demonstrated to regulate the activation of matrix metalloproteases, which may contribute to the local breakdown of the endothelial adherens junctions [217]. In addition, VCAM-1 clustering was shown to regulate lymphocyte TEM by activation of the kinase PKCα and the tyrosine phosphatase PTP1B in a ROS-dependent manner [218, 219].

In addition to the classical adhesion molecules on the endothelium (e.g., ICAM-1/2 and VCAM-1), several other molecules are known to play an important role in leukocyte traffic. Several of them belong to ectoenzymes, which are cell surface molecules having catalytically active sites outside the cell. For example, the adhesion molecule Vascular Adhesion Molecule-1 (VAP-1) with amine oxidase enzymatic activity was discovered to be present at the endothelial surface and controls the traffic of lymphocytes [220–222], monocytes [161], and neutrophils [162, 163]. However, if these ectoenzymes transmit intracellular signals into the endothelium that remodel the actin cytoskeleton during leukocyte TEM is not known.

The transmembrane protein CD47 is also an important mediator of leukocyte trafficking [145, 155]. CD47 is expressed on many if not all leukocyte types as well as EC and interacts with SIRPγ that is expressed on lymphocytes [223]. The same group showed that CD47 can phosphorylate VE-cadherin and in this way mediate lymphocyte TEM, again in a Src- and Pyk2-dependent manner [155]. Interestingly, cross-linking of CD47 with antibodies led to formation of stress fibers, similar to what has been observed when cross-linking ICAM-1 [209, 210, 224]. Clearly, changes in the endothelial actin cytoskeleton induced by leukocyte binding control efficient leukocyte TEM.

Tetraspanins form microdomains in the plasma membrane and are involved in intercellular adhesion and migration. For lymphocyte and monocyte TEM, it has been reported that the tetraspanins CD9, CD81, and CD151 distribute to the contact site with transmigrating leukocytes and associate laterally with both ICAM-1 and VCAM-1 [225, 226]. They control the adhesive capacity of the adhesion molecules and thereby control leukocyte binding strength to the endothelium. In addition, Barreiro and coworkers found that tetraspanins can form so-called endothelial adhesive platforms (EAPs) to which leukocytes can bind [95]. These platforms can function as signaling hubs in the plasma membrane and may include lipid rafts as well. Interestingly, ICAM-1 and VCAM-1 can both be present in these platforms, independent of the presence of its receptor.

A summary of the above-described signaling pathways downstream of clustered ICAM-1 and VCAM-1 is shown in Figure 2, where we have color-coded the endothelial proteins that are activated by specific leukocyte subsets.

5.3. CAM Linkage to the F-Actin Cytoskeleton. To support proper adhesion under physiological flow conditions, ICAM-1 and VCAM-1 need to be intracellularly anchored to the cytoskeleton. In the past two decades, several actin adapter proteins have been reported to interact with the intracellular domains of VCAM-1 and ICAM-1. These adapter proteins link these molecules to the F-actin cytoskeleton (Figure 2). The adapter proteins ezrin and moesin from the ERM-family were found to interact with VCAM-1 in a direct manner. Moreover, they colocalized with VCAM-1 around adherent lymphoblasts [94]. Their ability to bind both phospholipids and F-actin allows ERM proteins to organize adhesion molecules into specialized membrane domains [227]. In addition to VCAM-1, ERM proteins were also reported to interact with ICAM-1 in a PIP_2-dependent manner and colocalized with ICAM-1 in microvilli-like structures [94, 210, 228, 229]. However, unlike the binding to VCAM-1, the interaction of ezrin and moesin with ICAM-1 was reported to be indirect [230]. In addition to ERM proteins, the F-actin bundling proteins α-actinin-1 and -4 were also demonstrated to interact with the ICAM-1 C-terminus through a cluster of ICAM-1-C-terminal positively charged amino acids [231, 232]. Interestingly, this same cluster of amino acids was shown to mediate the interaction of ICAM-1 with ezrin [229] suggesting that α-actinin and ERM proteins may compete for binding to ICAM-1. This also indicates the existence of different ICAM-1/actin complexes upon leukocyte-mediated clustering (Figure 2).

The cortical actin-binding protein cortactin was initially shown to become tyrosine phosphorylated upon ICAM-1 clustering [206] and this tyrosine phosphorylation was required for efficient neutrophil TEM [100]. Cortactin is thought to stabilize branched actin networks through interaction with the Arp2/3 complex [233]. It also associated with ICAM-1 upon clustering [234] and was required for ICAM-1 and F-actin recruitment to ring-like structures around adherent leukocytes [99]. Recently, it was shown that cortactin is also required for ICAM-1 clustering around adherent

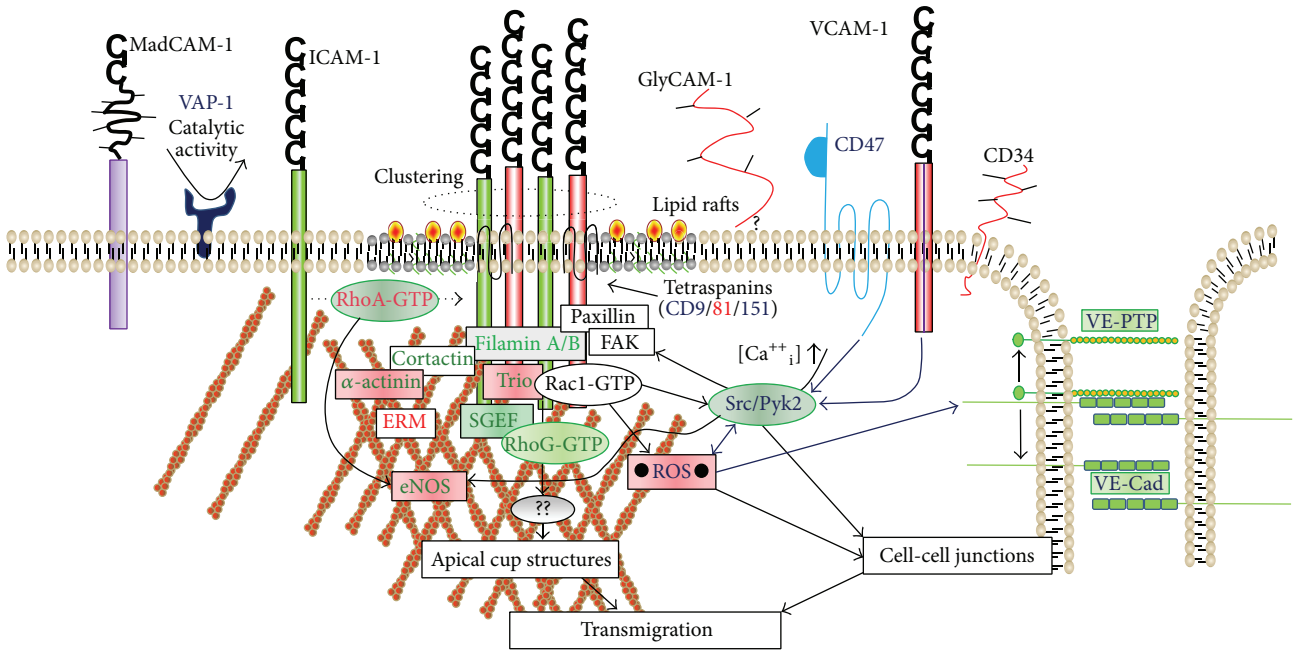

FIGURE 2: Endothelial signaling pathways induced upon clustering of ICAM-1 and VCAM-1 leading to the formation of endothelial F-actin-rich apical cup structures and the dissociation of endothelial adherens junctions. Endothelial signals that are induced by specific leukocyte types are color-coded: neutrophils in green, monocytes in red, T cells in blue, and B cells in purple. In case signaling proteins are identified by studies using different leukocyte types, the background color of the protein name is adapted to the leukocyte type used. In case specific signaling is studied in the absence of leukocytes the color is black. Short stripes indicate glycosylation. Question mark indicates that GlyCAM-1, as a soluble protein, may reassociate to the endothelial membrane.

neutrophils and for efficient neutrophil extravasation *in vivo*, thus highlighting the physiological relevance of the ICAM-1-cortactin interaction [49].

Finally, Kanters and colleagues showed that the F-actin cross-linker protein filamin B interacts with the ICAM-1 C-terminus in a direct manner [235]. Similar to cortactin, filamin B was required for ICAM-1 recruitment to a ring around adherent neutrophils and for neutrophil TEM under physiological flow conditions. In a more recent publication, it was shown that also filamin A interacts with the intracellular tail of ICAM-1 [236]. It has therefore been proposed that these adapter proteins connect ICAM-1 to downstream signaling partners [237, 238]. Silencing of filamin B expression impaired ICAM-1 clustering and leukocyte TEM under physiological flow conditions and since filamin A was still present in filamin B-silenced EC, this suggests that the filamins are not functionally redundant.

Indeed, although filamins A and B share 70% amino acid sequence identity, different effects on ICAM-1 function were observed when either filamin A or filamin B was silenced. Using fluorescence recovery after photobleaching (FRAP) technology, it became clear that silencing of filamin B expression increased the immobile fraction of ICAM-1 in the plasma membrane [235]. In contrast, in EC lacking filamin A, the immobile fraction of ICAM-1 was reduced [239]. Additionally, the clustering-induced ICAM-1-actin association was impaired in filamin A-silenced EC, but not in filamin B-silenced EC (personal observation, JDvB). The effects of filamin A deficiency on ICAM-1 function are similar to those of inhibition of F-actin polymerization. Moreover, deletion of the intracellular domain of ICAM-1 decreased the immobile fraction of ICAM-1 [239]. In addition, filamin A, but not filamin B, also mediated the interaction of ICAM-1 with the lipid raft marker and main constituent of caveolae, caveolin-1 [235]. Since ICAM-1 is recruited to caveolae and caveolin-1 during transcellular lymphocyte TEM [240], filamin A may have a specific role in regulating the transcellular pathway of diapedesis. These findings therefore reveal important roles for the different filamins in controlling ICAM-1 dynamics by regulating the connection with the F-actin cytoskeleton and specific membrane domains.

Recently, it became clear that there is a hierarchy between these actin-binding proteins to bind to ICAM-1 upon clustering. Schaefer and colleagues showed that when ICAM-1 is clustered, α-actinin is the first protein to be recruited to ICAM-1, followed by cortactin and lastly filamin [241]. The recruitment of different adapter proteins to ICAM-1 may result in the composition of a different actin network. For example, α-actinin cross-links actin filaments into actin bundles whereas cortactin cross-links actin into a meshwork and filamin into a "gel-like" structure [233, 237]. The initiation of these different actin networks may generate forces that drive local protrusive activity, that is, docking structures, or create a surface for leukocytes to crawl on. In fact, they showed that the local stiffness of the EC was indeed dependent on α-actinin. Depleting α-actinin resulted in reduced ability of the neutrophils to spread and transmigrate [241]. The group of Dr. Carman showed recently that the cytoskeletal

morphology and as a consequence the local EC stiffness of different vascular beds determined the preferred route for T-lymphocytes to cross the endothelium [9]. In particular, high barrier function was associated with transcellular migration, whereas artificial opening of the junctions resulted in more paracellular migration. They previously showed that T-lymphocytes use invadopodia-like protrusions to probe the endothelial surface, possibly to initiate transcellular migration [242]. It is tempting to speculate that the rate of clustering of adhesion molecules like ICAM-1 or VCAM-1 determines the stiffness of the underlying endothelial surface and that this may be the trigger for, at least, T-lymphocytes to cross. Whether other leukocyte types, for example, neutrophils or monocytes, use the same mechanism to probe the surface is not known.

A recent study by the Woodfin group showed that, next to ICAM-1 and VCAM-1, ICAM-2 plays an important role in immune cell traffic *in vivo* as described above [45]. Whether clustering of ICAM-2 recruits actin adapter proteins and induces similar signals is not clear [202]. However, the role of ICAM-2 seems to be more restricted to certain organs. For example, endothelial ICAM-2 is required for the migration of T-cells across the blood-brain barrier [243, 244].

5.4. Endothelial Docking Structure Formation. Using confocal microscopy, Barreiro and coworkers showed that both ICAM-1 and VCAM-1 were recruited to actin-rich membrane protrusions that surround adherent T-lymphoblasts in cup-like structures that were termed endothelial docking structures [94]. Two subsequent studies by Carman and coworkers demonstrated that the formation of these structures was dependent on F-actin polymerization and correlated strongly with transmigrating leukocytes [96, 97]. They suggested that these structures may function to facilitate and guide leukocyte TEM by forming a cup-like traction structure that is aligned parallel to the direction of transmigration. Interestingly, they showed that the transmigratory cups were essentially equal between monocytes, neutrophils, and lymphocytes. Thus, the cups did not discriminate between the leukocyte types suggesting that these endothelial cups represent a more global mechanism for leukocyte extravasation. In contrast to what Barreiro and coworkers proposed, that is, that the docking structures are required for leukocyte adhesion, Carman and colleagues showed that the cups were highly associated with leukocytes that transmigrated. Disruption of the cups did not alter the capacity of leukocytes to adhere to the endothelium, even under flow conditions. Interestingly, the formation of the cups depended on the intracellular tail of ICAM-1 [98]. In line with the notion that cups are not involved in adhesion, several reports have shown that the intracellular tail of ICAM-1 is needed for proper diapedesis but not for firm adhesion [201, 202, 229]. In addition to the *in vitro* observations, numerous studies have also described the formation of endothelial docking structures *in vivo* [102, 245–249]. Thus, although definite proof is still lacking, the formation of docking structures shows a strong correlation with the diapedesis step.

The initial formation of endothelial docking structures is dependent on the activity of the small GTPase RhoG [238]. RhoG colocalized with ICAM-1 upon ICAM-1 clustering and got activated. Moreover, depletion of SGEF, a GEF for RhoG, or RhoG significantly reduced the formation of docking structures and neutrophil TEM. Using a murine model to study the formation of atherosclerosis, it became clear that SGEF is most likely involved in the recruitment of monocytes to the site of injury since SGEF-deficient animals showed a significant reduction of plaques compared to control animals [250]. This work highlights the importance of docking structures in the development of inflammation-based diseases such as atherosclerosis.

Interestingly, Doulet and colleagues showed that the signaling molecules that are normally responsible for the induction of the apical cup structures can be used by bacteria (e.g., *Neisseria meningitides*) to enter EC [251]. These bacteria titrated the actin adapter protein ezrin and moesin away from sites where leukocytes interacted on the endothelium and thereby prevented the formation of cup structures and leukocyte diapedesis. This study shows the potential clinical relevance of cup structures in leukocyte TEM during inflammation. How these pathogens manage to cross the vessel wall and enter host cells can tell us a lot on the basic principles of the signaling mechanisms during leukocyte TEM.

6. Conclusions

The initial multistep paradigm of leukocyte extravasation largely describes adhesion and diapedesis from a leukocyte point of view and regarded the endothelium merely as just a passive substrate for leukocyte adhesion. However, it is now well appreciated that the endothelium is also an active participant in this process. Clustering of adhesion molecules, such as ICAM-1 and VCAM-1, has been demonstrated to induce signaling leading to significant changes in EC morphology allowing for leukocyte passage. In addition, endothelial cup structures are formed that may capture and guide leukocytes to transmigrate across the endothelium. Thus, the essential role of endothelial adhesion receptors and actin-binding proteins in mediating leukocyte TEM makes them promising candidates for a targeted regulation of leukocyte extravasation. On the other hand, several mechanisms in leukocytes have been identified that activate, for example, integrins for proper interactions with EC and actin dynamics causing the required morphological changes during TEM. Several of such mechanisms have been identified in all leukocyte subsets while others seem to be specific for a given subset. However, whether they are really specific or have just not yet been investigated in other subsets remains to be seen for most of the described mechanisms. It is important to keep in mind that all leukocyte types, besides their potential for tissue destruction, fulfill beneficial functions during many pathophysiological conditions so that pharmacological targeting of leukocyte recruitment will most likely always cause beneficial and detrimental effects. Thus, a lot of work remains to be done until we can fully appreciate whether there are truly unique mechanisms exploited by different leukocyte subsets during TEM that could be targeted pharmacologically in certain pathological conditions that would benefit from

interference with the recruitment of only one given leukocyte type without affecting others.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

All authors contributed equally to this work.

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Review Article

Leukocytes: The Double-Edged Sword in Fibrosis

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Skin tissue scar formation and fibrosis are often characterized by the increased production and deposition of extracellular matrix components, accompanied by the accumulation of a vast number of myofibroblasts. Scaring is strongly associated with inflammation and wound healing to regain tissue integrity in response to skin tissue injury. However, increased and uncontrolled inflammation, repetitive injury, and individual predisposition might lead to fibrosis, a severe disorder resulting in the formation of dense and stiff tissue that loses the physical properties and physiological functions of normal tissue. Fibrosis is an extremely complicated and multistage process in which bone marrow-derived leukocytes act as both pro- and antifibrotic agents, and therefore, few, if any, effective therapies are available for the most severe and lethal forms of fibrosis. Herein, we discuss the current knowledge on the multidimensional impact of leukocytes on the induction of fibrosis, focusing on skin fibrosis.

1. Introduction

Skin tissue integrity is a crucial factor to maintain the homeostasis generated through physical barriers, separating the organism from the environment. Every disruption of dermal integrity triggers a complicated cascade of events, including rapid blood clot formation, inflammatory response, and wound healing, leading to the restoration of the integrity and formation of new tissue. However, repaired structures, known as scars, are nonfunctioning, tight, and tense masses of fibrotic tissue that maintain 70–80% of normal strength, with even less flexibility [1]. Inflammatory responses are necessary for wound healing, preventing multiple infection and contamination and stimulating the proliferation, revascularization, and remodeling of the extracellular matrix [2, 3]. Nevertheless, wound healing might become uncontrolled and, combined with the inflammatory response, results in massive fibrotic tissue formation called fibrosis. In this review, we will focus on the molecular mechanisms underlying skin fibrosis as a post-wound-healing pathological disorder and the impact of bone marrow-derived cells and inflammation on the formation of scars.

2. Fibrosis and Wound Healing: Two Faces of the Same Story

Fibrosis is a pathological process that occurs in many different organs (organ specific fibrosis), such as skin, kidney, heart, lung, and liver [4], which might also take the form of systemic sclerosis (SSc), a global, progressive, and autoimmune disorder, characterized by an extremely poor prognosis and high mortality [5, 6]. According to the United States government, every year, in the USA, around 45% of natural deaths can be associated with different fibrotic disorders [7]. Although the etiology and triggering cascade might differ, fibrosis is characterized by the increased production and deposition of extracellular matrix (ECM) components, including collagen type I, fibronectin, hyaluronan, and elastin, and the accumulation of activated, αSMA-positive, and collagen-secreting fibroblasts, called myofibroblasts [4, 8, 9]. Myofibroblasts exhibit the ultimate fibroblast phenotype. Many authors refer to fibrosis in the context of "uncontrolled" or exceeded wound healing, as an effect of long-term inflammation or mechanical irritation [10].

Wound healing is extremely complex and involves the cooperation of many cell types. This process can be divided into four overlapping phases: coagulation, inflammation, proliferation, and remodeling. Skin injury results, inter alia, in the disruption of endothelial and epithelial cells integrity [10]. Damaged cells release inflammatory mediators that trigger the coagulation cascade, platelet recruitment, and blood clot formation. Degranulated and activated platelets present in the blood clot release multiple chemokines and growth factors (TGF-β1, PDGF), which recruit inflammatory cells. Neutrophils appear first, followed by macrophages and lymphocytes. Platelets also participate in the chemotaxis and recruitment of fibroblasts and endothelial cells [11–13]. The first two phases are often treated as one phase, representing the inflammatory stage. The blood clot comprises cross-linked fibrin and extracellular matrix proteins, such as fibronectin, vitronectin, and thrombospondin. This structure serves as a physical barrier that closes the blood vessel, a reservoir of growth factors, and a matrix on which regenerated tissue is formed [12, 14]. The next phase, proliferation, results from hypoxic conditions and reactive nitrogen species (RNS) production from macrophages [15, 16]. During this phase, angiogenesis occurs, forming new capillaries and facilitating the delivery of nutrients to the wound. In addition to nutrients, collagen-secreting myofibroblasts are recruited to the wound microenvironment [17]. As previously described, myofibroblasts are activated, αSMA-positive, and collagen-secreting fibroblasts that deposit new ECM components, primarily fibronectin and collagen type I, to replace the clot-formed matrix, often forming a scar [10, 18]. In physiological wound healing, remodeling is the final phase. During this phase, myofibroblasts and some vascular cells undergo apoptosis and disappear from the regenerated microenvironment [19]. Moreover, the synthesis of ECM components is reduced but not fully terminated [11], and remodeling is primarily regulated through different matrix metalloproteinases (MMPs) and their inhibitors (tissue inhibitor of metalloproteinases, TIMPs). After the degradation of the overexpressed ECM components, scarring is reduced and an equilibrium between synthesis and catabolism is reached [13, 20]. In fibrosis, the proliferation and remodeling phases have become pathological. Myofibroblasts constantly produce ECM components, disrupting the delicate equilibrium. The increased deposition of collagen type I and fibronectin stiffens and damages the surrounding tissue. In addition, connective tissue cells replace the original cells, creating a scar that in some cases might take an extremely severe form [13, 21, 22]. The wound healing process is shown in Figure 1.

Activated fibroblasts/myofibroblasts accumulate in the fibrotic tissue environment by three different, simultaneous mechanisms. First, these cells are derived from preexisting fibroblasts in the affected tissue through activation due to specific, profibrotic, and proproliferative mediators released from infiltrating inflammatory cells, such as T cells [23–25]. Second, myofibroblasts are recruited through bone marrow-derived fibroblast resembling cells, such as fibrocytes, CD45 and CD34 positive cells [26]. Fibrocytes transmigrate to the fibrotic environment, and in a TGF-β1-controlled process, these cells undergo transdifferentiation into myofibroblasts [27]. Finally, fibroblasts/myofibroblasts accumulate through the transition from endothelial or epithelial cells to mesenchymal fibroblast-like cells [5, 11].

3. The Endothelial and Epithelial to Mesenchymal Transition as a Key Factor in Fibrosis

During the endothelial and/or epithelial to mesenchymal transition (EndMT and EMT, resp.), cells lose their origin markers, polarity, and cell-cell connections and gain promigratory phenotypes and mesenchymal markers [28–30]. Both EndMT and EMT are physiological processes that occur during embryonic organogenesis and wound healing. Epithelial and endothelial cells establish close cell-cell contacts with a certain cell polarity, forming a solid barrier that maintains homeostasis. This barrier is formed through desmosomes and tight and adherent junctions [28, 31]. In contrast, mesenchymal cells are spindle-shaped solitary cells, possessing migratory and ECM remodeling abilities. These cells produce and secrete ECM components, such as collagen type I and fibronectin [13, 32]. During tissue development or regeneration, tightly connected cells cannot undergo migration. Therefore, after undergoing EndMT/EMT, these cells gain the migratory abilities of fibroblasts, facilitating the recruitment of these cells to certain locations. Cells do not typically undergo full transitions, often terminating in intermediate phenotypes between endothelial or epithelial and mesenchymal, and maintaining some cell-cell contacts to perform group migration rather than single cell migration [28, 33]. The endothelial to mesenchymal transition was first observed and described as the leaking and proliferation of endothelial cells during the development of chick and rat endocardial cushions (cardiac mesenchyme) [34]. EndMT and EMT are involved in pathological disorders as well. EndMT is closely associated with dermal, renal, cardiac, pulmonary, intestinal, and cystic fibrosis through the establishment of fibroblasts and myofibroblasts [35, 36]. EMT is reversible, and fibroblasts might regain epithelial phenotypes (mesenchymal to epithelial transition, MET), whereas EndMT reversibility is not well understood. The reversal of EndMT (mesenchymal to endothelial transition) has been recently observed in cardiac fibroblasts that rapidly adopt an endothelial-cell-like phenotype after acute ischemic cardiac injury [37]. However, more additional evidence suggests that EndMT is irreversible, and transformed cells cannot regain endothelial phenotypes, even after the removal of EndMT inducing factors [38]. Therefore, EndMT, which is not terminated at a certain time, could lead to the accumulation of collagen type I secreted from myofibroblasts and the irreversible transformation into fibrotic tissue [39].

Both EndMT and EMT are regulated through the zinc finger transcription factor Snail family (Snail1, Snail2, and Snail3). Snail1 is the first and most crucial transcription factor activated during mesenchymal transition. After activation, on the molecular level, Snail1 stabilizes the quantity of Twist1 transcription factor, and in cooperation, both of these proteins upregulate *ZEB1* gene expression [40, 41]. As

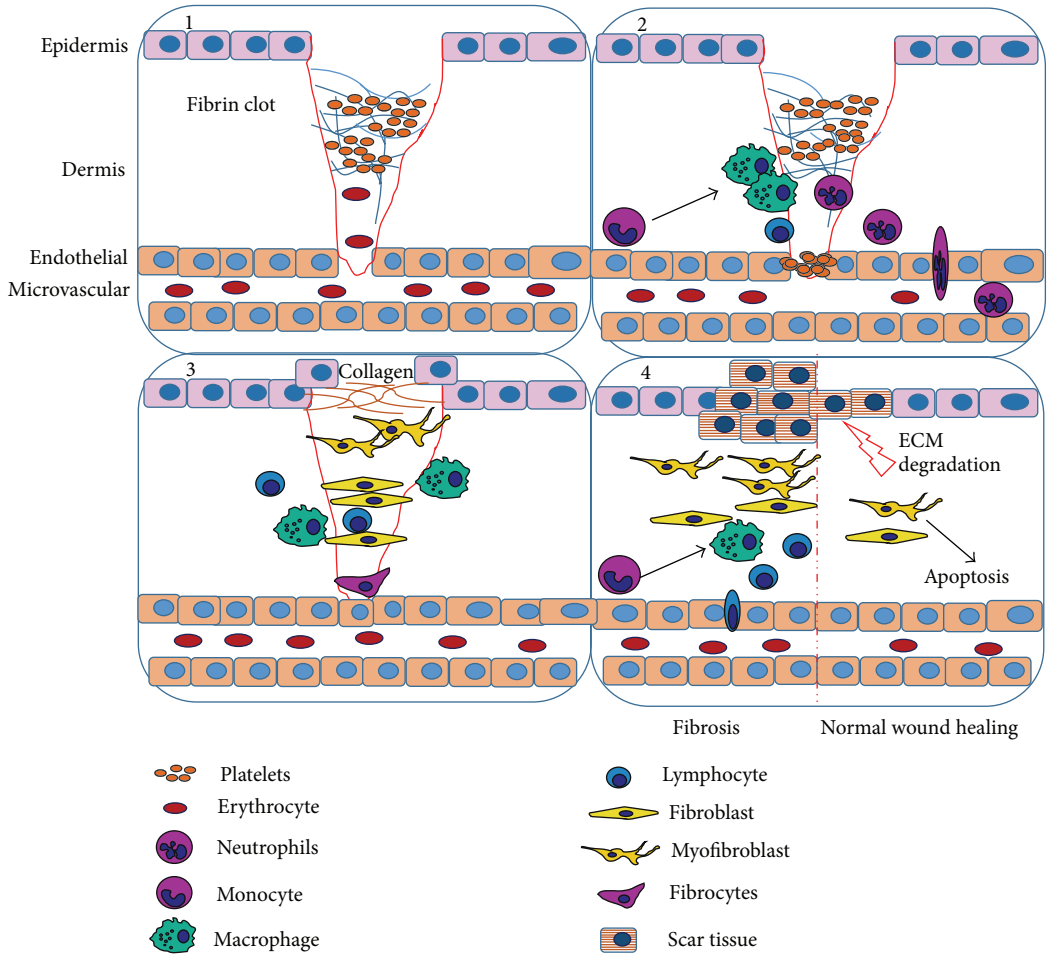

Figure 1: The stages of wound healing. 1, coagulation: after injury, fibrin clot is formed. Trapped platelets degranulate and release inflammatory chemokines. 2, inflammation: leukocytes enter wound site. Neutrophils appear first, followed by macrophages and lymphocytes. Leukocytes clear wound from bacteria and any foreign bodies, respectively, recruiting fibroblasts. 3, proliferation: activated fibroblasts, myofibroblasts, produce and deposit ECM components that serve as skeleton during tissue regeneration process. 4, remodeling, the final stage in normal wound healing: excess amount of ECM is degraded, fibroblasts and myofibroblasts undergo apoptosis, and inflammatory cells leave regenerated tissue. However, during fibrosis, inflammation is prolonged and ECM deposition is highly increased by myofibroblasts.

a repressor, Snail proteins downregulate the expression of genes encoding junction proteins, such as claudin, occludin, E-cadherin (in epithelial cells), VE-cadherin, and PECAM1 (in endothelial cells). It is not clear whether Snail upregulates the genes encoding mesenchymal markers, as observed in the upregulation of myosin Va in some highly metastatic cancer cell lines, such as human lung carcinoma cell lines (A549, PG, and Calu6), human colon cancer cell lines (Lovo and SW480), human breast cancer cell lines (BICR-H1 and MCF7), and prostate cancer cell lines with the same genetic background (PG3M-1E8 and PG3M-2B4) [42], or represses epithelial/endothelial genes and therefore indirectly upregulates mesenchymal markers. Nevertheless, mesenchymal cell proteins, such as vimentin, fibronectin, collagen type I, αSMA, SM22α (transgelin), N-cadherin, calponin, and FSP-1 (fibroblast specific protein 1), are expressed during and after the transition [43–46]. The microRNA profile also changes during mesenchymal transition, revealing the significant upregulation of miR-125, Let-7c, Let7g, miR21, miR30b, and miR195 and downregulation of miR122a, miR127, miR196, and miR375 [47]. A previous study reported that the accumulation of Snail in colorectal cancer cells and in mice utricle sensory epithelia cells, after blocking the degradation of this protein through the glycogen synthase kinase-3 (GSK-3), via lithium chloride treatment or the overexpression of Snail, might trigger the transition into mesenchymal-like cells [43, 48, 49]. However, this transition is typically induced through a variety of proinflammatory cytokines and growth factors secreted from leukocytes, which act synergistically. The most important proinflammatory/profibrotic molecules are transforming growth factors β-1 and β-2 (TGF-β1 and TGF-β2), tumor necrosis factor-α (TNF-α), interleukins IL-1β, IL-6, IL-8, and IL-11, and fibroblast growth factor-2 (FGF-2) [38, 39, 50–54]. It has been suggested that TGF-β receptor is essential for mesenchymal transition signal transduction, and the overexpression of Snail might be an insufficient factor. The inhibition of TGF-β receptor accompanied by simultaneous upregulation of Snail does not lead to EndMT in mouse

embryonic stem cell-derived endothelial cells (MESECs) [44]. However, the upregulation of the transcription factor Snail directly upregulates profibrotic and proinflammatory cytokines, such as IL-8 [55].

The secretion of TGF-β into the fibrotic microenvironment during inflammation is the most important Snail inducer. Snail expression might be triggered through many pathways. The most common pathway is the activation of the Smad2/3 complex. However, studies on skin cancer formation have shown that Smad2 inhibits EndMT, whereas Smad3 acts as an activator [56]. The binding of TGF-β to TGF-β receptor type II (TβRII) triggers heterodimerization through the activation of the TGF-β receptor type I kinase (TβRI), which activates activin-like kinase 5 (ALK5) and transduces a signal through the Smad2/3 complex with Smad4, which activates the expression of Snail [53]. TGF-β2 activates Smad2/3 via ALK2. The inhibition of either ALK5 or ALK2 results in the inhibition of EndMT [29, 57]. TGF-β also activates Snail in a non-Smad pathway, involving Wnt and Noch, via the sequestration of GSK-3 and Akt2, through the transcriptional repression of the miR-200 superfamily and the activation of the inflammatory transcription factor NFκB [30, 46, 50, 58].

4. Leukocytes in Fibrosis: Unanswered Questions

As previously discussed, chronic inflammation is one of the main factors triggering fibrosis, particularly EndMT-based fibrosis, as EndMT is an irreversible process. Constant inflammation leads to the production of a variety of proinflammatory cytokines and growth factors secreted from different leukocytes present in the fibrotic microenvironment. However, fibrosis formation is a multidimensional and multistage process that not only involves EndMT. Leukocyte recruitment triggers many different mechanisms and pathways that might lead to disordered wound healing, myofibroblasts and collagen type 1 accumulation, scarring, and fibrosis.

4.1. Neutrophils. Neutrophils appear first at the site of the wound. The recruitment of these cells is initiated immediately after activated platelets degranulate and release TGF-β1 and PDGF. TNF-α, IL-1, and IL-8 released from endothelial cells also stimulate neutrophil recruitment, leading to selectin-mediated rolling adhesion towards the chemoattractant gradient. In the next phase, tight adhesion to endothelial cells occurs via integrin β2, followed by transmigration through the endothelial tissue. When necessary, neutrophils cross the ECM barrier along fibroblasts and transmigrate through epithelial cells to enter the wound [59]. Neutrophils begin phagocytosing invading bacteria and damaged necrotic cells to clear the wound, preparing it for the regeneration of homeostasis through scar formation. However, fetal wounds heal without scar formation, and fetal neutrophils are physiologically distinct from adult neutrophils, as these cells are less adept than adult cells, producing less cytokines and presenting lower contributions to the inflammatory response [60, 61]. Neutrophil serine protease, elastase, is secreted into the microenvironment, increasing IL-8 expression in the surrounding cells [62]. IL-8 not only is responsible for leukocyte recruitment but also might trigger EndMT and increase the survival and proliferation of endothelial-derived fibroblasts/myofibroblasts, leading to fibrosis [6, 63]. Additionally, elastase is also believed to cleave the IL-8 receptor CXCR1, interfering with neutrophil functions and antibacterial abilities, thereby prolonging inflammation, which in turn increases additional fibrosis-based changes [64]. Prolonged inflammation might also occur through the elastase-mediated degradation of complement, releasing the strong neutrophil chemoattractant, C5a [65]. Moreover, neutrophil derived oxidative burst, leading to the formation of $HOCl^*$ from H_2O_2 catalyzed through myeloperoxidase, induces injury to epithelial cells, thereby implicating the switch to fibrotic tissue deposition [66, 67]. Two different populations of neutrophils have been observed to enter the wound in mice after the induction of acute inflammation: one population has a proinflammatory function, and the second population is responsible for anti-inflammatory responses. These cells differ in size, granularity, and the expression of CD11b and Ly6G [68]. Respectively, the anti-inflammatory neutrophil response is strongly associated with the secretion of the anti-inflammatory cytokine IL-10 [69]. Moreover, a certain population of mature neutrophils, characterized as $CD11c^{bright}/CD62L^{dim}/CD11b^{bright}/CD16^{bright}$, have been reported to suppress T cell proliferation via the expression of the integrin Mac-1 ($\alpha M\beta 2$) [70].

The impact of neutrophils on fibrosis has been observed in pulmonary fibrotic disorders, as these cells transmigrate to pulmonary fluids (such as bronchoalveolar lavage fluid) and recruit other leukocytes [71, 72]. However, only a few clinical studies have successfully established anti-inflammatory strategies in patients with pulmonary fibrosis. Inhibition of neutrophil derived elastase as strategy of downregulation of self-destructive process of neutrophil derived protease activity as well as elastase derived IL-8 expression is currently being elucidated and brings more questions than answers. Clinically useful concepts have only just started to evolve and bring promising, but not yet convincing, answers [59, 73].

4.2. Macrophages. Macrophages appear as the second type of bone marrow-derived cells invading the wound site, and three to five days after injury, they become the dominant leukocyte type [10]. Monocytes, recruited through PDGF, undergo differentiation towards macrophages. Similar to neutrophils, different populations of macrophages have been reported, depending on the activation path through different chemokines and growth factors, as shown in Figure 2. Classical macrophage activation, or M1, is obtained, in particular, through the combination of interferon gamma (IFN-γ) and tumor necrosis factor-α (TNF-α) signaling pathways. Classically activated macrophages produce proinflammatory cytokines, including interleukin-12 (IL-12) [74]. Alternative activation, or M2, is far more complex, leading to the formation of regulatory and wound-healing macrophages. Regulatory macrophages release anti-inflammatory cytokines IL-10 and TGF-β, which downregulate inflammation, and also lead to the endothelial to mesenchymal transition and increase the fibroblast number at the wound site [75]. TGF-β pro- and

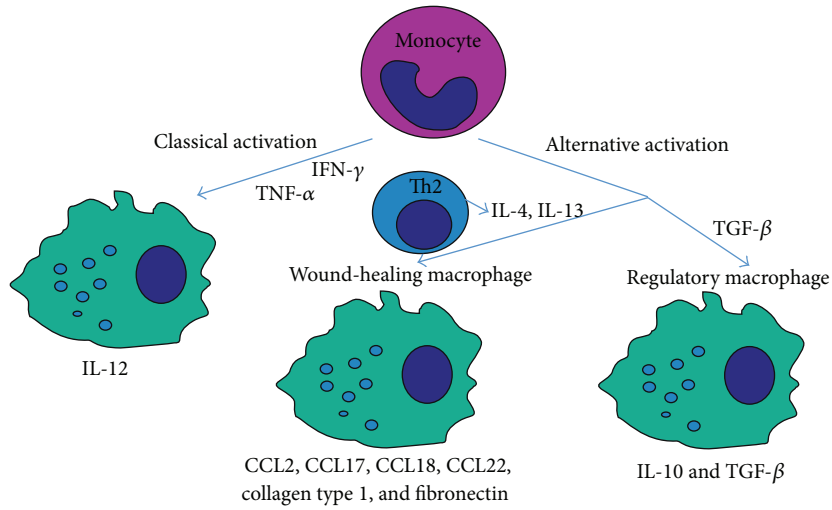

Figure 2: The divergent macrophage activation pathway. Macrophage activation and differentiation from monocyte in the wounded tissue depends on chemokine and growth factors availability. Two macrophage activation pathways might be distinguished, classical activation (M1) depending on interferon gamma (IFN-γ) and tumor necrosis factor-α (TNF-α) and alternative activation pathway (M2). Alternative activation is divided into two separate macrophage populations, IL-4 and IL-13 derived wound-healing macrophage population and TGF-β derived regulatory macrophage population. Despite monocyte origin, different activation pathway results in production and secretion of different chemokines and proteins into wounded tissue.

anti-inflammatory roles are often described as paradox. Its abilities might shift, depending on other cytokines availability and cell type [76, 77]. It was shown that TGF-β administered to animals with infection or inflammation reduces severity of disease and production of proinflammatory IL-1 and TNF [78]. A second group of M2 macrophages, wound-healing macrophages, are derived through IL-4 induction. These cells secrete CC chemokine ligands, including CCL2, CCL17, CCL18, and CCL22 [72]. Wound-healing macrophages are extremely profibrotic, as these cells produce high levels of fibronectin and through CCL18 activation promote collagen production from fibroblasts/myofibroblasts [79]. Moreover, arginase activation in M2 macrophages, stimulated through IL-4, leads to the conversion of arginine to ornithine, a precursor of collagen [74, 80]. Blocking IL-4 with specific antibodies significantly decreases wound-healing, macrophage accumulation, and fibrosis formation [81].

Recent studies showed that overexpression of MMP9 in macrophage might attenuate bleomycin induced pulmonary fibrosis [82]. Respectively, production of MMP13 by Kuppfer cells was shown to be sufficient in preventing pig serum-induced rat liver fibrosis [83]. These data suggest that high level of MMPs might play key role in fibrosis reversibility. Macrophages are the main sources of MMPs that facilitate ECM degradation during remodeling phase in wound healing process; they also phagocytose apoptotic myofibroblasts and cellular debris preventing advance in the fibrotic process. However, some authors suggest strong profibrotic role of macrophage derived MMP13, as they observed that liver fibrosis was suppressed, along with fibrotic markers and inflammatory mediator expression, in MMP13-deficient mice during cholestasis-induced liver fibrosis [84]. Surpassingly, prolonging inflammation and recruitment of activated macrophages might be involved in fibrosis reversing process, as accumulating evidence strongly correlates macrophages and the macrophages derived MMPs (MMP1, MMP2, MMP8, MMP9, and MMP13) with this process [85]. Nevertheless, the role of MMP13 remains unanswered.

4.3. Lymphocytes. Lymphocytes recruited to injured tissue are activated through various antigens. After arrival to the wound site, these cells produce lymphokines, which in turn activate other inflammatory cells, such as macrophages [14]. Among all lymphocyte subpopulations, Th1 and Th2 are most relevant for tissue fibrosis. Th1 and Th2 lymphocytes contribute different responses to wounded tissue. Th1 acts as an antifibrotic, releasing IL-10, and Th2 acts as a profibrotic. Studies using mouse models have shown that the polarized Th2 response leads to massive collagen deposition and increased fibrosis formation. However, the Th1 response activates the genes responsible for apoptosis and acute-phase reactions [86, 87]. Among all cytokines released from Th2, the two most important and most profibrotic cytokines are IL-4 and IL-13. Both IL-4 and IL-13 share functional similarities, as these molecules transduce signal via the IL-4R/Stat6 pathway [88–90]. As previously described, IL-4 activates M2 wound-healing macrophages, resulting in collagen production and deposition. Moreover, IL-4 stimulates in the dose-dependent manner collagen synthesis in fibroblasts and is two times more effective than TGF-β [91]. The scleroderma mouse model (tight-skin mutant mouse Tsk/+) presented extremely increased dermal collagen expression, secretion, and deposition correlated with IL-4. Treatment with an anti-IL-4 antibody resulted in collagen downregulation and provided less fibrosis-based pathological changes [92]. The Th2-mediated secretion of IL-4 and IL-13 enhanced fibrocyte differentiation from CD14-positive precursors, thereby leading to increased fibroblast recruitment and potential fibrosis [93].

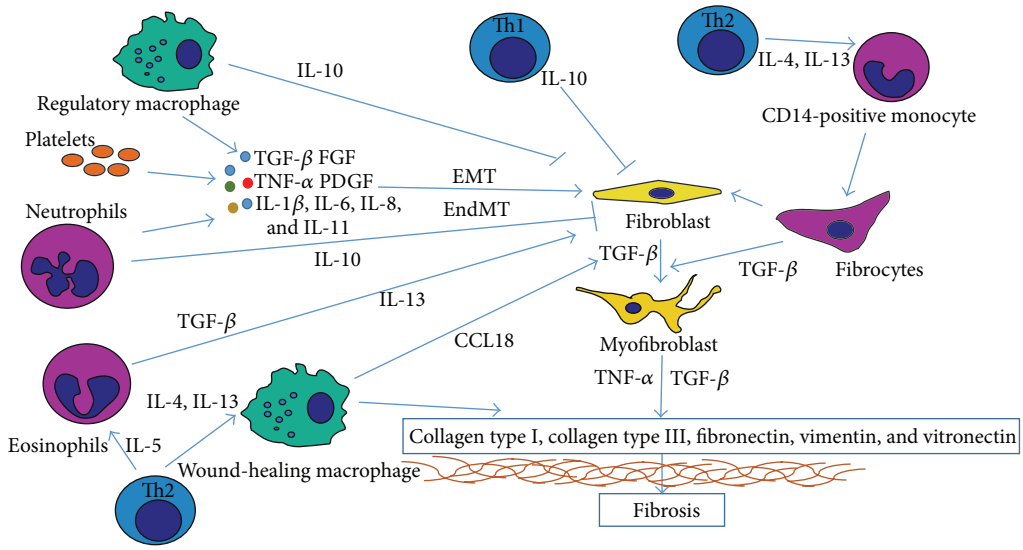

Figure 3: The comprehensive view on complex leukocytes impact on fibrotic tissue formation. The direct impact depends on ECM components production and deposition by leukocytes, such as fibrocytes and wound-healing macrophages. The indirect impact is composed of variety of different chemokines and growth factors interacting with cells, which in turn leads to ECM deposition.

The impact of Th2-derived IL-5 on fibrosis is strongly associated with the recruitment and activation of eosinophils. Activated eosinophils secrete inflammatory factors, such as IL-13 and TGF-β1, into wounded tissue, resulting in fibrosis development, as shown for dermal fibrosis in a mouse model of skin allograft rejection [94]. Both IL-13 and TGF-β1 might induce collagen secretion from fibroblasts present in the wound; however, TGF-β1 is strongly associated with EndMT- and EMT-based fibrosis [95, 96].

As discussed above, Th1 and Th2 lymphocytes affect differently fibrotic tissue. Recently, it was shown that patients with cystic fibrosis and *P. aeruginosa* infection present an age-dependent dysregulation of lymphocyte T response that shifts towards Th2 lymphocyte, resulting in enhanced fibrotic tissue deposition. However, precise regulatory immune mechanism remains poorly understood [97].

4.4. Fibrocytes. Fibrocytes are circulating, bone marrow-derived cells that exhibit mesenchymal phenotypes. As previously described, these cells are both CD45- and CD34-positive cells that transdifferentiate into myofibroblasts. The name fibrocyte represents a combination of shared features of these cells: fibroblast and monocyte [26]. Circulating fibrocytes rapidly enter the wound site. Subsequently, TGF-β1 triggers the transdifferentiation of these cells into αSMA-positive myofibroblasts that express collagen type I, fibronectin, and vimentin and increase the amount and deposition of ECM components [98, 99]. Normally, circulating fibrocytes comprise less than 1% of all leukocyte populations, but during fibrotic changes derived from inflammation, the amount of these cells systematically increases [26]. Fibrocytes can be distinguished in at least 4-day-old skin wounds, and the quantity of these cells raises with time and increasing wound age [100]. For the differentiation of fibrocyte precursors, CD14-positive monocytes are stimulated through lymphocyte Th2-derived IL-4 and IL-13 [93]. The induction of fibrosis through fibrocytes is primarily based on the deposition of ECM components, as discussed above. Nevertheless, we cannot omit a variety of proinflammatory cytokines secreted from fibrocytes into the wound, namely, TNF-α, IL-6, IL-8, IL-10, and macrophage inflammatory protein 1α/β (MIP 1α/β) [27]. Moreover, it has been demonstrated that the fibrocytes in burned patients secrete TGF-β1, which activates myofibroblasts from existing fibroblasts [101] or triggers differentiation toward fibroblasts-like cells from surrounding endothelial or epithelial tissues through EndMT or EMT. However, due to dynamic nature of fibrocytes and constantly changing phenotype and functions of these cells, during their migration, some serious inconsistency appeared on the exact definition and identification of fibrocytes. These discrepancies are related to different methodology used to investigate fibrocytes involvement in variety of fibrotic disorders on variable stages. It has been suggested that one must categorize fibrocytes as functionally different depending on the isolation condition [102]. What is more, it is still unclear whether fibrocytes contribute only to worsening or improving tissue repair, as they possibly represent "the wrong cells in the wrong time" [102].

5. Concluding Remarks

Fibrosis is a complicated and composed process, leading to severe pathological disorders. The scarce formation of fibrotic tissue, comprising excess amounts of collagen type I, fibronectin, and other ECM components, deregulates normal tissue functions. Inflammation and inflammation-associated bone marrow leukocyte recruitment in wounded tissue trigger a cascade of events, leading to wound enclosure, scar formation, and, in case of prolonged inflammation, massive fibrosis. The impact of leukocytes on fibrosis formation might be generally divided into direct and indirect effects as shown in Figure 3. The direct impact is strongly associated with

the production and excess deposition of ECM components. This effect is primarily observed with fibrocytes and alternatively activated, wound-healing macrophages. The indirect impact is far more complicated, as this effect is multistaged and associated with the activation and recruitment (including cells transdifferentiation and EMT/EndMT fibroblasts formation) of collagen-secreting cells, such as macrophages and myofibroblasts, and increased myofibroblast survivability through the downregulation of proapoptotic signals and increased inflammatory response times. All indirect profibrotic events occur through different chemokines or growth factors secreted from leukocytes. The most common, and likely, best-known indirect impact is correlated with TGF-β family proteins, as these molecules trigger both the endothelial and epithelial to mesenchymal transition and activate myofibroblasts from fibroblasts and fibrocytes, thereby increasing the production of ECM components. However, although this mechanism is well known, no antifibrotic therapy, based on TGF-β deactivation, has been implicated without disruption of the physiological function of this molecule. Several drugs for the downregulation of TGF-β transcription or signal transduction have been examined in the last stages of clinical trials [9]. Nevertheless, the impact of IL-6, IL-8, IL-4, IL-13, or TNF-α cannot be neglected. Th2-derived IL-4 and IL-13 are primarily responsible for macrophage collagen deposition and fibrocyte generation, whereas TNF-α, IL-6, and IL-8 are strongly associated with EMT and EndMT and myofibroblast survival and stimulation of collagen production. Thus, leukocyte interactions with wounded tissue cells and other leukocytes are extremely complicated and complex, bringing more questions than answers.

Abbreviations

ALK2: Activin-like kinase 2
ALK5: Activin-like kinase 5
C5a: Complement component 5a
CD: Cluster of differentiation
CXCR1: Interleukin-8 receptor
ECM: Extracellular matrix
EMT: Epithelial to mesenchymal transition
EndMT: Endothelial to mesenchymal transition
FSP-1: Fibroblast specific protein 1
GSK-3: Glycogen synthase kinase 3
IFN-γ: Interferon gamma
IL: Interleukin
Mac-1: Integrin $\alpha M\beta 2$
MESECs: Mouse embryonic stem cell-derived endothelial cells
MIP 1α/β: Macrophage inflammatory protein 1α/β
MMPs: Matrix metalloproteinases
NFκB: Nuclear factor kappa-light-chain-enhancer of activated B cells
PDGF: Platelet-derived growth factor
PECAM1: Platelet-endothelial cell adhesion molecule (cluster of differentiation 31, CD31)
RNS: Reactive nitrogen species
SM22α: Transgelin
SSc: Systemic sclerosis
TGF-β: Transforming growth factor
Th1/2: T helper cell 1/2
TIMPs: Tissue inhibitor of metalloproteinases
TNF-α: Tumor necrosis factor-α
TβRI: TGF-β receptor type I
TβRII: TGF-β receptor type II
αSMA: α smooth muscle actin.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Regulation of Endothelial Adherens Junctions by Tyrosine Phosphorylation

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Endothelial cells form a semipermeable, regulated barrier that limits the passage of fluid, small molecules, and leukocytes between the bloodstream and the surrounding tissues. The adherens junction, a major mechanism of intercellular adhesion, is comprised of transmembrane cadherins forming homotypic interactions between adjacent cells and associated cytoplasmic catenins linking the cadherins to the cytoskeleton. Inflammatory conditions promote the disassembly of the adherens junction and a loss of intercellular adhesion, creating openings or gaps in the endothelium through which small molecules diffuse and leukocytes transmigrate. Tyrosine kinase signaling has emerged as a central regulator of the inflammatory response, partly through direct phosphorylation and dephosphorylation of the adherens junction components. This review discusses the findings that support and those that argue against a direct effect of cadherin and catenin phosphorylation in the disassembly of the adherens junction. Recent findings indicate a complex interaction between kinases, phosphatases, and the adherens junction components that allow a fine regulation of the endothelial permeability to small molecules, leukocyte migration, and barrier resealing.

1. Introduction

Intercellular adhesion is a hallmark of all Metazoa. Complex organisms have evolved sophisticated methods to create adhesive forces that are strong enough to hold the organisms together but, at the same time, flexible enough to allow tissue remodeling and physiological adhesive changes. In particular, the adherens junction (AJ) is a multiprotein structure present in most organisms ranging from insects to mammals [1, 2]; its basic structure comprises transmembrane cadherins and cytosolic catenins linking the cadherin to the cytoskeleton [3]. All classical cadherins are composed of five extracellular domains (EC1–EC5), a single transmembrane domain, and a short cytoplasmic C-terminal tail. Trans-homodimerization occurs by the interaction of two EC1 domains of opposing cadherins [4]. VE-cadherin (cadherin 5) was discovered in the early 1990s [5, 6] and is a major component of endothelial cell-cell contacts. VE-cadherin is critical for endothelial biology and is required for vessel maturation in multiple species ranging from zebrafish [7] to mice [8, 9]. Similar to other classical cadherins, the cytoplasmic tail of VE-cadherin contains the binding regions for p120 catenin at the juxtamembrane domain (JMD) and for β-catenin or γ-catenin at the C-terminal catenin binding domain (CBD). Binding of p120 catenin stabilizes junctional cadherins by preventing cadherin endocytosis (reviewed in [3, 10]), while β-catenin associates with α-catenin, providing the link to the actin cytoskeleton [11, 12].

Endothelial cells play a critical role in the regulation of vasoreactivity, hemostasis, and leukocyte recruitment. Vascular endothelial cells are also critical for maintaining normal tissue function by acting as a selective barrier that regulates the passage of fluid, macromolecules, and leukocytes from the vascular space to the interstitium. The activation of proinflammatory pathways induces a loss of endothelial barrier function through activation of membrane receptors in endothelial cells, triggering several signaling cascades, including the activation of kinase signaling, small GTPase-mediated actin cytoskeleton remodeling, and calcium release (reviewed in [13–16]). Two pathways mediate the passage

across the endothelial barrier. Endothelial cells may allow the transport of proteins and even cells through their cell body in what has been called the transcellular pathway. In this pathway, fluid and proteins are actively transported in a complex system of vesicles from the luminal to the basal side of the cell, where the vesicular content is released [13, 17]. Leukocytes have also been shown to migrate through single endothelial cells both *in vitro* and *in vivo* [18, 19]. In contrast, many proinflammatory mediators promote the disengagement of the AJ-based contacts, allowing the passage of fluids and leukocytes through a paracellular pathway, that is, between two endothelial cells. By regulating the paracellular pathway, VE-cadherin-based cell-cell contacts maintain the strong intercellular adhesion required for the vessel's barrier function, while at the same time allowing for sufficient plasticity when required. This review will focus on the regulation of the paracellular pathway by tyrosine kinase signaling, with special emphasis on discussing the findings that support and those that argue against a direct effect of cadherin and catenin phosphorylation in the disassembly of the adherens junction. Recent findings indicate a complex interaction between kinases, phosphatases, and the adherens junction components that allow a fine regulation of the endothelial permeability to small molecules, leukocyte migration, and barrier resealing.

2. Intercellular Adhesion Is Regulated by Phosphorylation of Cadherins and Catenins

The development of antibodies that recognize phosphotyrosine residues quickly enabled research that demonstrated a critical role for tyrosine phosphorylation in the modulation of intercellular adhesion, in particular through the regulation of AJ-based contacts. Maher et al. were the first to show that cell-cell junctions in epithelial cells (PtK2 and MDCK) and chicken embryo fibroblasts contained proteins phosphorylated on tyrosine [20]. Within the following decade, it became very clear that treatment of cells with pervanadate (a pan-specific tyrosine phosphatase inhibitor) or oncogene-induced transformation in cell culture can induce a dramatic increase in the phosphotyrosine content at the cell junctions by increasing the phosphorylation of VE-, N- and E-cadherin, as well as α-catenin, β-catenin, and γ-catenin [20–32]. A similar observation was made in rats, in which an intravenous injection of sodium orthovanadate increased the junctional staining of an anti-phosphotyrosine antibody in the intestine, heart, and liver [23]. Further, EGF treatment in human epidermoid carcinoma A431 cells induced β-catenin and γ-catenin phosphorylation [33], demonstrating that endogenous kinases could also promote catenin phosphorylation in response to growth factors. At the same time, Reynolds et al. [34] described a 120 kDa protein that was highly phosphorylated on tyrosine in v-Src transformed chicken embryo cells, whose identity was later found to be the catenin family member p120 [35] and to associate with E-cadherin [36]. Src was then shown to be able to phosphorylate multiple tyrosines at the amino terminus of p120 [37]. Early research unambiguously demonstrated that tyrosine phosphorylation can disrupt cadherin-based adhesions. Short-term pervanadate treatment increased phosphotyrosine content at MDBK cell junctions, while long term treatment disrupted cell adhesion [22]. Using v-Src as a model, it was shown that oncogene-driven overactivation of tyrosine kinases promoted a loss of intercellular adhesion in a number of epithelial and fibroblast cells [21, 24–28, 30, 38].

Oncogenic Src mutants may have different substrate specificity than endogenous kinases [39], which may lead to unintended consequences in cells overexpressing v-Src. Indeed, it soon became clear that not all tyrosine kinase activity led to the disruption of the adherens junctions and that phosphorylation in tyrosine could also mediate junctional stability [40–46]. While massive phosphorylation caused by phosphatase inhibition or v-Src overexpression induces dramatic changes in cell adhesion strength, inhibition of protein tyrosine kinase (PTK) signaling can also lead to disrupted cell adhesion (Figure 1). In MCF7 human mammary adenocarcinoma cells, a delicate balance of Src activity was required for maintaining normal adherens junction integrity, since either blocking Src activity (via dominant negative Src constructs or pharmacological inhibition using PP2) or overactivation of Src (by expression of the constitutively active mutant Y530F Src) induced a marked junctional disruption [47]. These results suggest that the effects of Src-mediated signaling in the regulation of the adherens junctions strictly depend on the level of activity and that while high levels of Src signaling disrupt intercellular adhesions, low basal levels of Src are required for normal cellular adhesion. These findings led the authors to propose that the loss of cell-cell adhesion observed in gain-of-function studies using oncogenic v-Src constructs may reflect events of cell transformation and epithelial to mesenchymal transition, while the role of basal endogenous SFKs promoting the strengthening of cell adhesion reflected a physiological role in AJ maintenance [48]. In that regard, it was shown that, in mouse keratinocytes, p120, β-catenin, and γ-catenin (but not E-cadherin) tyrosine phosphorylation was increased after calcium-induced differentiation, which coincided with an increased association of α-catenin and p120 with E-cadherin [40]. Conversely, addition of the kinase inhibitors genistein, tyrphostin, or PP1, or Fyn deficiency, diminished cell-cell adhesion in a dispase-mediated cell release assay and mice deficient for Fyn and Src displayed deficient cell-cell adhesion in skin [40]. Similarly, PP2 or genistein treatments reduced N-cadherin-based adhesion in Rat-2 fibroblasts, an effect that was attributed at least in part to a requirement for cortactin phosphorylation to sustain N-cadherin adhesion [41]. Src activity is also required to maintain junctional stability in *Drosophila*, as a dominant negative mutant of the Src homolog Src42A induced the disorganization of DE-cadherin contacts [42] and *Drosophila* embryos lacking both Src homologs Src42A and Src64 showed diminished DE-cadherin and armadillo staining at cell-cell junctions [43]. Together, these findings demonstrate that tyrosine kinase signaling can lead to AJ formation and stability.

The mechanisms involved in Src-mediated AJ formation are not well understood. Tyrosine kinases may promote AJ assembly through phosphorylation of its components or

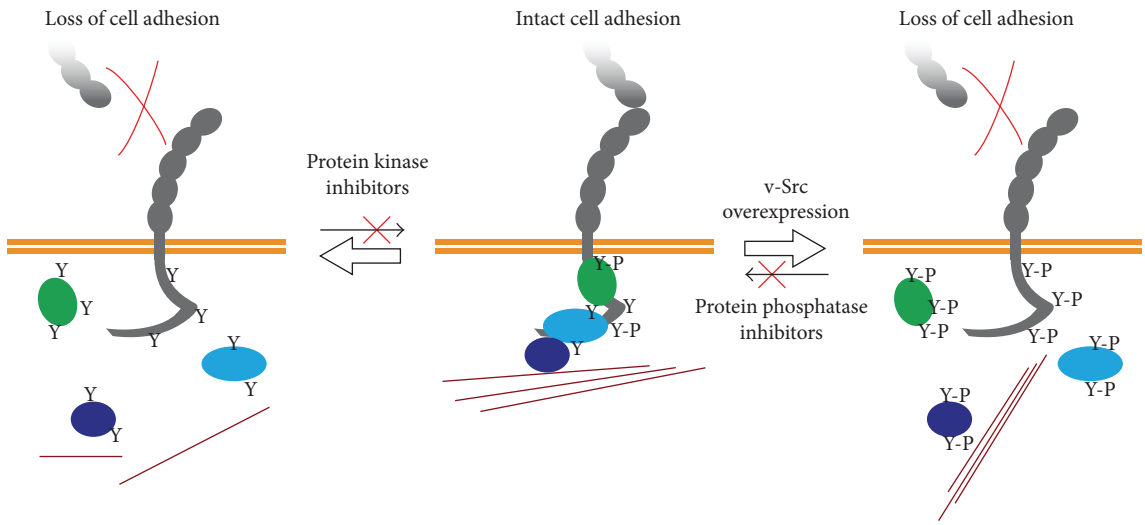

Figure 1: Adherens junction-based cell adhesion requires a tight balance of tyrosine kinases and phosphatases. Oncogenic Src signaling and blockade of phosphatase activity, as well as complete inhibition of kinase activity, can lead to AJ disruption and loss of cell-cell adhesion.

indirectly via the regulation of tyrosine phosphatases as well as small GTPases. For example, Abl kinases can promote AJ formation through the regulation of Rho and Rac signaling [44, 45], while in E8 chicken retina cells, p120-associated Fer is essential to maintain β-catenin binding to N-cadherin, by promoting Y152 phosphorylation in the phosphatase PTP1B, which in turn was responsible for dephosphorylating Y654 in β-catenin [46]. PTK signaling can thus mediate both assembly and disassembly of adherens junctions in a complex interplay. Understanding exactly how and when a phosphorylation event will lead to loss or strengthening of cell adhesions is one of our main challenges ahead.

3. Cadherins Also Can Be Upstream of Tyrosine Kinases and Regulate RTK Signaling

The research discussed so far has placed cadherins and catenins downstream of PTK activity, but E-cadherin engagement can also regulate PTK activity, placing cadherins correspondingly upstream of these kinases (Figure 2). For instance, preventing E-cadherin engagement in MCF7 cells using E-cadherin blocking antibodies reduced the amount of active Src at cell-cell junctions, while beads coated with E-cadherin/Fc chimera promoted a rapid increase in active Src [47]. The mechanism by which E-cadherin activates Src signaling was found to depend on the tyrosine phosphatase RPTPα [49–51], presumably by removing the phosphate at the Src autoinhibitory tyrosine 530 [52]. Cadherins also have been shown to bind and modulate receptor tyrosine kinase (RTK) signaling, both positively and negatively [53]. E-cadherin-mediated cell adhesion inhibited EGFR signaling in MDCK cells [54] but induced ligand-independent EGFR activation leading to increased Erk signaling in HaCat keratinocytes [55] and in mammary epithelial cells [56]. N-cadherin, on the other hand, was not found to be associated with EGFR [54]. However, N-cadherin engagement stimulated neurite outgrowth in cerebellar neurons through the activation of FGFR [57], a pathway that was later found to promote tumor metastasis [58, 59]. Similar interactions between VE-cadherin and VEGFR2 are required for contact inhibition of endothelial cell growth [60] and for the endothelial response to shear stress [61–63]. Further, VEGF-induced Src activation required the dissociation of Csk (a kinase that inhibits SFK activation [64]) from VE-cadherin and the recruitment of SHP2, which then dephosphorylated Src at tyrosine 530, allowing its activation [65]. This mechanism is reminiscent of the E-cadherin/RPTPα-induced Src activation in MCF7 cells [47, 49–51]. Conversely, p120 overexpression in HUVECs blocked neutrophil TEM through preventing ICAM-1-induced VE-cadherin phosphorylation and the association of VE-cadherin with active (pY419) Src [66, 67], placing p120 association with VE-cadherin upstream, rather than downstream, of Src activation at least in the context of neutrophil transendothelial migration. Together, these results show that cadherins can either promote or prevent RTK-mediated signaling.

Although some of the molecular mechanisms are being teased out, it still remains largely unknown how cadherin association can regulate RTK signaling. An important clue comes from the VE-cadherin and VEGFR2-dependent response to shear in endothelial cells [61–63]. Fluid shear force is transmitted by PECAM-1, leading to VE-cadherin-dependent activation of VEGFR2 and Akt signaling [63]. The mechanism involves an increase in PECAM-1 tension, triggering PECAM-1/vimentin association, and a reduction in the levels of VE-cadherin tension [68]. It is possible that tension-mediated changes in cadherins may not be limited to VEGFR2 activation under shear. Cadherins are constantly under tension [12, 68, 69] and it was recently shown that tension at VE-cadherin junctions can regulate cell-cell contacts [70, 71]. It is not known, however, whether changes in tension

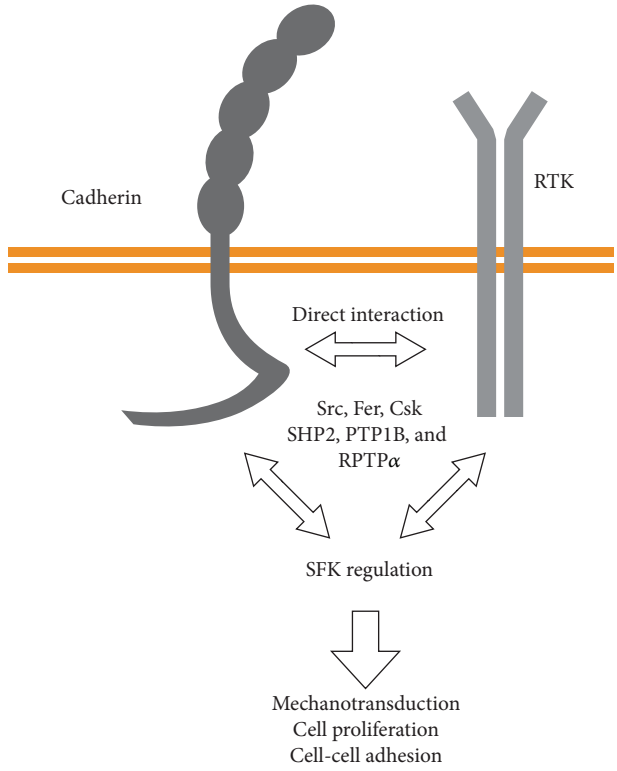

Figure 2: Cadherins can regulate PTK activity. The proposed mechanisms include the direct modulation of kinases and phosphatases as well as interactions with receptor tyrosine kinases. Cadherin-mediated PTK activity has been shown to be involved in mechanosensory transduction, contact inhibition of cell proliferation, and strength of cell adhesion.

at the AJ can modulate other RTK responses or cadherin phosphorylation itself.

4. The Adherens Junction Is Phosphorylated in the Endothelium

Similar to other classical cadherins, the phosphorylation state of VE-cadherin was found to be associated with differences in endothelial function. Lampugnani et al. first showed that a phosphotyrosine antibody labeled endothelial cell-cell contacts [72] by showing that loosely confluent HUVECs displayed strong junctional phosphotyrosine labeling, while this staining was reduced in tightly confluent cultures. Further studies demonstrated that VEGF induced an increase in endothelial permeability that required tyrosine kinases [73] and that VEGF promoted the phosphorylation of tyrosines in VE-cadherin, β-catenin, γ-catenin, and p120 [74]. VEGF also stimulated the dephosphorylation of p120 serine residues [75]. Most importantly, inhibition of SFK activity prevented edema formation in several animal models [76–79], demonstrating a causal role for SFKs in VEGF-induced loss of endothelial barrier function.

The particular phosphorylation sites of VE-cadherin in response to VEGF are a matter of intense investigation. This question became further complicated by the lack of specificity of some commercial antibodies used in previous studies. Wallez et al. [80] showed that VEGF-165 induces VE-cadherin phosphorylation at tyrosine 685 in HUVECs, as measured by phosphopeptide mapping. *In vitro*, Src was able to directly phosphorylate a short peptide containing this residue. Other peptides containing C-terminal tyrosines (from Y645 to Y784) were not a good substrate for this *in vitro* assay, suggesting that either Src is not capable of directly phosphorylating these tyrosines in VE-cadherin or that other docking site(s) in the full length protein are needed for this reaction. No phosphorylation in serine was detected upon VEGF treatment [80]. This is in sharp contrast with the findings by Gavard and Gutkind [81], who showed that VEGF induces VE-cadherin phosphorylation at serine 665 in HUVECs, a key step to promote β-arrestin binding and VE-cadherin endocytosis. VE-cadherin serine 665 phosphorylation was also implicated downstream of R-Ras, a small GTPase required for vascular differentiation that is downregulated in the leaky tumor vasculature [82]. In HUVECs, expression of an active form of R-Ras (R-Ras38V) prevented VEGF-induced phosphorylation at S665 and VE-cadherin endocytosis, without affecting VEGF-induced tyrosine phosphorylation at Y658 or Y731 [83].

VE-cadherin phosphorylation also occurs in response to other stimuli, including TNF-α, LPS, H_2O_2, and high glucose [84–87], albeit at slower kinetics than after VEGF treatment. Addition of TNF-α [84] or LPS [85] to human lung microvascular endothelial cells (HMVEC-L) induced a sustained loss of barrier function, together with VE-cadherin tyrosine phosphorylation. This phosphorylation, however,

was only detectable in cells treated with orthovanadate and phenylarsine oxide, while TNF-α- or LPS-induced increase in permeability did not require phosphatase inhibition, raising the question of whether endogenous phosphatases are sufficient to blunt the tyrosine phosphorylation induced by these agonists, without affecting the increase in permeability. In any case, a requirement of tyrosine kinase activity was demonstrated by the ability of several kinase inhibitors to block the increase in monolayer permeability induced by TNF-α or LPS [84, 85]. Notably, nonspecific inhibitors such as genistein, herbimycin A, and geldanamycin were much more efficient at preventing TNF-α-induced loss of barrier function than SFK-specific inhibitors PP1 and PP2, suggesting that other kinases may be involved in parallel pathways [84].

A wealth of data unmistakably points to a very important role for SFK-mediated tyrosine phosphorylation of endothelial AJ in the regulation of barrier function. However, as in the case of epithelial cells presented above, the link is not straightforward or unidirectional. SFK activity and VE-cadherin phosphorylation can be observed *in vivo* in the absence of any pathological condition. Lambeng et al. [88] showed that VE-cadherin is highly phosphorylated in some tissues of healthy adult mice, particularly lung and uterus, and that VE-cadherin phosphorylation increased upon angiogenic stimuli. More recently, it was shown that VE-cadherin phosphorylation at tyrosine 685 in mouse ovary and uterus varied throughout the estrous cycle [89]. Moreover, Orsenigo et al. [90] showed that venules and capillaries, but not arterioles, in mouse bladder and diaphragm display constitutive VE-cadherin phosphorylation at tyrosines 658 and 685 and that this phosphorylation is dependent on basal SFK activity in venules. In rats, carotids showed much lower VE-cadherin phosphorylation at tyrosine 685 than jugular veins. Interestingly, a jugular bypass to expose the vein to arterial bloodstream drastically reduced VE-cadherin phosphorylation [90]. More recently, Wessel et al. [91] showed that tyrosine 731, but not tyrosine 685, was constitutively phosphorylated in mouse lungs. Substitution of wild-type VE-cadherin for Y685F mutant, but not Y731F mutant, resulted in an attenuation of the dermal vascular leakage after injection of VEGF or histamine. A similar knock-in strategy was used by Sidibé et al., who found that Y685F VE-cadherin mice displayed increased vascular leakage in the uterus and ovary, suggesting that VE-cadherin phosphorylation at this site may have an important role maintaining vessel integrity [92]. In contrast to the observations by Orsenigo et al. [90], Wessel et al. [91] did not find that VE-cadherin was constitutively phosphorylated at tyrosine 685 in the venules of the cremaster vasculature. However, treatment of mice with pervanadate promoted tyrosine 685 phosphorylation in venules, suggesting that differences in basal tyrosine phosphatase activity (either due to differences in the cremaster vasculature or more general mouse strain differences) could explain the difference between these two reports. Strikingly, the pervanadate treatment was unable to promote an increase in VE-cadherin phosphorylation in arterioles, which the authors attributed to a possible lack of active kinases in the vicinity of VE-cadherin [91]. Tyrosine 685 has been proposed to be the binding site for Csk and Y685F VE-cadherin mutant does not associate with Csk [65, 93], raising the possibility that differential Csk association could regulate the access of active kinases to VE-cadherin C-terminal tyrosines. Alternatively, catenin binding may be involved in the regulation of VE-cadherin phosphorylation. For example, p120 overexpression can reduce the association of VE-cadherin to active Src [66, 67], while it may recruit Fer and PTP1B to the AJ as shown in retina cells [46]. A potential role for catenins regulating VE-cadherin phosphorylation in venules remains untested. In all, these findings show not only that VE-cadherin phosphorylation at particular tyrosines is an important step in the loss of endothelial cell-cell adhesion leading to an increase in permeability and TEM, but also that VE-cadherin can be phosphorylated in the absence of vascular leakage, demonstrating *in vivo* that other signals must be activated concurrently.

In vitro, the effect of SFK activation on human dermal microvascular cells depends on the method of activation [94]. Consistent with the findings in epithelial cells, overexpression of a constitutively active form (Y530A) of Src promoted VE-cadherin phosphorylation, monolayer gap formation, and loss of TEER. However, activation of endogenous SFKs by blocking Csk increased VE-cadherin phosphorylation without promoting an increase in monolayer permeability [94], demonstrating that while SFK activity may be required for the hyperpermeability induction by VEGF and other mediators, SFK activation alone is not sufficient to induce a loss of barrier function. Instead, SFK-induced AJ phosphorylation may act as a gatekeeper that allows edemagenic stimuli to promote an increase in permeability. In fact, bradykinin was able to induce vascular leakage on venules that displayed increased Src and VE-cadherin phosphorylation, but not in sites with low basal tyrosine phosphorylation [90]. Interestingly, the phosphorylation at tyrosine 685 in trachea venules quickly disappeared after bradykinin or histamine injections. *In vitro* assays suggested that this dephosphorylation event was due to clathrin-dependent VE-cadherin endocytosis and ubiquitin-mediated degradation, rather than a direct action of a phosphatase [90].

5. Leukocyte Transendothelial Migration Requires Multiple Tyrosine Phosphorylation Steps

Leukocyte infiltration into inflamed tissues is a major aspect of the body's response to damage. To arrive at the required location, leukocytes must travel through the endothelium, in a process called extravasation. This is a multistep process that involves complex interactions between the leukocyte and the endothelial cell. Leukocytes bind to activated endothelium and initiate a cascade of intermolecular contacts that allow them to traverse from the bloodstream into the stroma through the endothelium via either a transcellular route (i.e., through an endothelial cell) or a paracellular route (opening a gap between two adjacent ECs) (for reviews, see [95–97]). The endothelial response to leukocyte adhesion and migration involves the activation of multiple signaling pathways,

notably Ca^{2+} release, Rho activation, actin remodeling, and tyrosine kinase activation, surrounding the leukocyte in what is called the adhesion cup and promoting the cytoskeletal changes to make room for the transmigrating leukocyte. This review will focus on tyrosine kinase signaling, and the reader is referred to recent excellent reviews [16, 98, 99] for a comprehensive discussion of all other known players involved.

Early on, a critical role was recognized for tyrosine phosphorylation in leukocyte transendothelial migration (TEM), at least in part mediated by leukocyte integrins binding to ICAM-1, leading to remodeling of the actin cytoskeleton (Figure 3). ICAM-1 ligation induces the tyrosine phosphorylation of multiple proteins, including focal adhesion kinase (FAK), paxillin, Cas [100], and cortactin [101]. ICAM-1 antibody-coated beads promoted the association of Src and tyrosine phosphorylated cortactin to ICAM-1. Inhibition of SFK activity prevented cortactin phosphorylation but not association with ICAM-1. Consistent with a model in which phosphorylated cortactin is required for ICAM-1 clustering, PP2 treatment significantly reduced the ability of fixed THP-1 monocytes to bind to activated HUVECs and prevented ICAM-1 clustering around the adhered cells [101]. Similarly, cortactin knockdown abolished PMN transmigration through TNF-α-activated HUVECs, which could be rescued by reexpression of wild-type cortactin-GFP, but not by a cortactin mutant in which three tyrosines (Y421, Y466, and Y482) were mutated to phenylalanine (cortactin 3F-GFP) [102]. ICAM-1 cross-linking-induced formation of actin stress fibers in TNF-α-treated HUVECs was also blocked by cortactin knockdown, PP2 treatment, or expression of either tailless ICAM-1-GFP or cortactin 3F-GFP. More importantly, cortactin siRNA blocked the clustering of actin and ICAM-1 around adherent PMN [102]. Altogether, these findings strongly argue for a critical role for SFK-mediated cortactin phosphorylation regulating ICAM-1 clustering and TEM. The definitive proof that cortactin mediates TEM *in vivo* was provided by Schnoor et al. [103] who found that loss of cortactin in mice reduced neutrophil recruitment. Cortactin knockout mice showed increased leukocyte rolling velocities, which was associated with a reduced adhesion to postcapillary venules and diminished ICAM-1 clustering around neutrophils. The mice also showed increased basal vascular leakage, thus mechanistically separating the regulation of barrier function from TEM. *In vitro*, an EPAC-specific cAMP analog rescued the increased permeability, while TEM efficiency was restored by expression of a constitutive form of RhoG [103], a GTPase that is activated downstream of ICAM-1 and Src by the SH3-containing guanine-nucleotide exchange factor (SGEF), a Rho-specific exchange factor [104].

Consistent with a role for cortactin-mediated ICAM-1 clustering, apical ICAM-1 mobility was reduced in HUVECs after ICAM-1 antibody-mediated cross-linking in cells expressing full length ICAM-GFP, but not tailless ICAM-GFP [102]. However, expression of an ICAM-1 deletion mutant lacking the intracellular tail was much more effective at preventing transcellular than paracellular TEM [105]. The implications of this finding are not completely clear, as ICAM-1 clustering via association with the actin cytoskeleton appears to be a critical component of the response to leukocyte binding regulating both paracellular and transcellular migration. ICAM-1 mobility was also reduced at the sites of ICAM beads binding to HeLa cells expressing wild-type ICAM-1-GFP, but not a C-terminal tail deletion [106]. ICAM-1 bead adhesion to HUVECs was prevented by inhibitors of Rac1, actin polymerization, or myosin II. Interestingly, MEFs from Src, Yes, and Fyn (SYF) triple SFK knockout mice reexpressing or not Src displayed similar ICAM-1-GFP FRAP kinetics and bead binding, suggesting that at least in MEFs SFK activity is not a major player in ICAM-1 dynamics [106].

Interestingly, leukocyte receptors can be phosphorylated on tyrosine as well (Figure 3). Src was shown to phosphorylate ICAM-1 *in vitro* on tyrosine 512 [107]. Binding of activated HUVECs to fibrinogen induced the tyrosine phosphorylation of ICAM-1 and promoted ICAM-1/SHP2 interaction through a mechanism that required tyrosine 512 phosphorylation [107]. A possible role for this phosphorylation in ICAM-1 function was proposed because TNF-α-induced ICAM-1 cleavage was abolished by Y512A mutant [108]. In HUVECs, ICAM-1-mediated Src and eNOS activation was dependent on ICAM-1 tyrosine phosphorylation, because expression of a mouse Y518F mutant ICAM-1 construct (corresponding to the human Y512 residue) blocked ICAM-1 cross-linking-induced Src, eNOS, and Akt phosphorylation [109]. Importantly, this Y518F ICAM-1 construct was not as efficient as wild-type ICAM-1 in promoting PMN TEM in HUVECs. PP2 inhibition experiments showed that SFK activity was required for ICAM-1, Akt, and eNOS phosphorylation, while the PI3K inhibitor wortmannin was able to block eNOS, but not ICAM-1 phosphorylation, suggesting that PI3K acted downstream of Src and upstream of eNOS [109]. However, expression of Y512F ICAM-1 was almost as effective at promoting lymphocyte migration as wild-type ICAM-1 in GP8/3.9 immortalized rat brain microvascular endothelial cells [110], suggesting that this Src substrate site was not critical for ICAM-1 function. Further, endogenous ICAM-1 was not phosphorylated after incubation with lymphocytes in these cells [110]. Elucidation of the role of ICAM-1 phosphorylation in leukocyte transmigration will require further research, especially *in vivo*.

PECAM-1 is another leukocyte receptor known to be phosphorylated on at least two tyrosines, Y633 and Y686 [111]. Contrary to most leukocyte-interacting proteins, PECAM resides in a specialized compartment, named the lateral border recycling compartment (LBRC) [96]. Tyrosine phosphorylated PECAM is enriched in the LBRC [112] and this phosphorylation appears to be important for successful TEM, because either PP2 treatment [112] or mutation of tyrosine 633 [111] prevented PECAM recycling to the cell surface and TEM (Figure 3).

To allow TEM via the paracellular route, the endothelial cell must disassemble the adherens and tight junctions that sustain the strong homotypic intracellular contacts in order to create the space for their migration (Figure 3). Early work showed that leukocytes transmigrate through endothelial gaps *in vivo* [113] and *in vitro* [114] and that monocytes and U937 cells induced the reversible loss of junctional

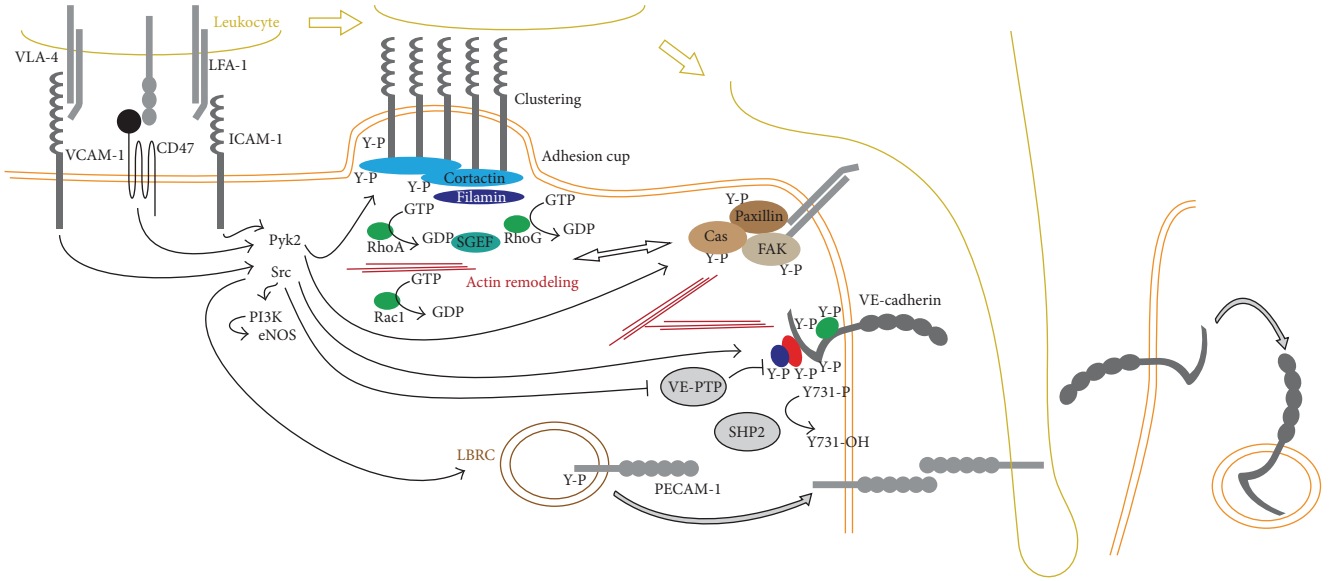

Figure 3: Simplified endothelial signaling cascades in response to leukocyte attachment. Adhesion of leukocytes through multiple transmembrane proteins such as ICAM-1, VCAM-1, and CD47 promotes activation of small GTPases (depicted as Rac1, RhoA, and RhoG) and PTK signaling, such as activation of Src and Pyk2. PTK activity leads to phosphorylation of actin binding proteins (e.g., cortactin) and focal adhesion components (FAK, paxillin, and Cas) that together with filamin promote ICAM-1 clustering and actin remodeling that is required for the formation of the adhesion cup. PTKs also promote the phosphorylation of VE-cadherin, which together with VCAM-1-mediated dissociation of VE-PTP from VE-cadherin leads to junctional hyperphosphorylation. At the same time, SHP2 mediates VE-cadherin dephosphorylation specifically at tyrosine 731. VE-cadherin endocytosis may follow. Src-mediated phosphorylation of PECAM-1 is required from PECAM-1 translocation from the LBRC to the plasma membrane.

VE-cadherin and catenins during TEM [115]. There has been considerable interest in understanding how tyrosine phosphorylation affects the stability of adherens junctions in the context of TEM. Antibody-mediated ICAM-1 ligation promoted VE-cadherin phosphorylation in GPNT rat brain endothelial cells and bEND5 mouse brain endothelioma cells [116] and bead-mediated ICAM-1 cross-linking induced a rapid Src- and Pyk2-dependent phosphorylation of VE-cadherin in TNF-α-treated HUVECs [66, 117]. In particular, ICAM-1 ligation-induced, Src-mediated VE-cadherin phosphorylation can be blocked by p120 overexpression [66]. Similar phosphorylation events were observed after adhesion of monocytic THP-1 cells to TNF-α-treated HUVECs [117] and binding of rat peripheral lymph node lymphocytes to GPNT cells [116]. Tyrosine phosphorylation of catenins, ZO-1, or occludin was not detected after ICAM-1 cross-linking in GPNT cells [116]. Other receptors may activate similar downstream pathways as well, as cross-linking of CD47 in the endothelium induced Src, Pyk2, and VE-cadherin phosphorylation in activated HUVECs [118] and VCAM-1 cross-linking promoted VE-cadherin and VE-PTP dissociation in bEnd5 cells [119, 120]. In HUVECs, active pY419 Src and pY402 Pyk2 labeling showed a similar pattern surrounding the ICAM-1 beads, but neither β-catenin nor VE-cadherin was seen at sites of ICAM-1 engagement [117]. Nevertheless, treatment with the SFK inhibitor PP2 or expression of the Pyk2 dominant negative CRNK reduced the pY658 and pY731 signals and prevented neutrophil TEM. Further confirming a role for these two phosphorylated tyrosines, overexpression of Y658F and Y731F nonphosphorylatable VE-cadherin-GFP mutants strongly reduced paracellular TEM compared to wild-type VE-cadherin-GFP [117]. Surprisingly, VE-cadherin phosphorylation after ICAM-1 cross-linking in GPNT cells was insensitive to PP2 treatment, ruling out SFKs as the main kinases involved in this phosphorylation [116]. The reason for this discrepancy is unknown, but the differential localization of Src and other kinases in response to bead- or IgG-induced ICAM-1 cross-linking could potentially explain this conflicting result. To determine the specific sites of VE-cadherin phosphorylation in response to ICAM-1 ligation, CHO cells were engineered to express ICAM-1 together with either wild-type or mutant VE-cadherin-GFP. Tryptic digestion of ^{32}P-labeled CHO-ICAM-1 cells showed that ICAM-1 cross-linking promoted VE-cadherin phosphorylation at Y731. Interestingly, the authors mentioned that the majority of phosphorylation events in VE-cadherin occur at serine and threonine, rather than tyrosine residues [116], but the effect of these phosphorylated residues remains unknown. To test the causal role of VE-cadherin phosphorylation, a series of Y/F point mutants was expressed in endothelioma cells derived from VE-cadherin null mice. Surprisingly, reexpression of wild-type VE-cadherin or a VE-cadherin-GFP fusion construct increased twofold, rather than decreased, TEM of antigen-specific T cells. Expression of the different mutants in CHO cells suggested that tyrosine 731 is the main phosphorylation site involved in TEM. When compared to wild-type VE-cadherin-GFP, expression of Y731F-VE-cadherin-GFP mutant allowed only 50% of

TEM without affecting T-cell adhesion, while cells expressing Y658F and Y685F mutants allowed similar levels of TEM as wild-type VE-cadherin. A role for Y731 *in vitro* was confirmed in GPNT cells, which express endogenous VE-cadherin. In these cells, expression of Y731F (as well as Y645F and Y733F, but not Y/F mutations in Y658, Y685, Y725, or Y757) significantly reduced T-cell TEM. No mutant affected lymphocyte adhesion to these cells [116]. Critically, mice harboring a Y731F knock-in mutation in VE-cadherin displayed drastically diminished leukocyte infiltration, thus directly demonstrating a crucial role for this tyrosine in leukocyte TEM *in vivo* [91]. While this tyrosine appears to be constitutively phosphorylated, leukocyte attachment induced its dephosphorylation through a mechanism that involved the phosphatase SHP2. It is yet unknown whether tyrosine 658 is also required *in vivo*, as suggested by the data obtained by Allingham et al. [117]. A similar knock-in approach might help answer this question. Thus, *in vitro* data show that leukocyte attachment can promote VE-cadherin phosphorylation, but *in vivo* experiments suggest that the critical VE-cadherin tyrosine is constitutively phosphorylated, suggesting that the main target for SFK-mediated phosphorylation downstream of leukocyte attachment (through at least ICAM-1, VCAM-1, and CD47 engagement) may be other proteins than VE-cadherin, such as cortactin, FAK, or eNOS.

6. The Case for Tyrosine Phosphorylation Regulating Cadherin/Catenin Association

Catenin binding is essential to support cadherin-based adhesions. Accordingly, p120 binding increases E-cadherin lateral clustering and adhesion strength [121, 122] and prevents endocytosis of E-cadherin [123, 124] and VE-cadherin [125–129]. β-catenin also increases VE-cadherin adhesion strength [130] and functions as a bridge to connect cadherins to α-catenin and thus the actin cytoskeleton [11, 12]. A critical role for this latter association was demonstrated by the expression of locked cadherin constructs that are fused directly to α-catenin and are thus independent of β-catenin association and dissociation. Expression of an E-cadherin construct fused to α-catenin promoted strong cell-cell adhesion [131, 132]. Moreover, cells expressing this construct were resistant to dissociation induced by pervanadate treatment [132]. Elegant studies by Schulte et al. [133] using knock-in mice expressing VE-cadherin-α-catenin chimera demonstrated that the dissociation of α-catenin from VE-cadherin is a required step for the induction of vascular permeability by VEGF or histamine and for allowing neutrophil or lymphocyte recruitment into inflamed tissues.

The observation that tyrosine phosphorylation regulates cell-cell adhesion, together with the finding that cadherins and catenins are targets for tyrosine kinases, led to intense research to determine how phosphorylation affected the adherens junction structure, with the notion that the phosphorylation of cadherins and/or catenins may lead to changes in cadherin/catenin association. Consistently, β-catenin phosphorylation may function as a switch to allow or prevent cadherin association with the actin cytoskeleton by affecting its ability to bind E-cadherin and α-catenin. In F9 cells, a phosphomimetic Y142E mutation in β-catenin did not coimmunoprecipitate with α-catenin, while Y654E β-catenin mutation reduced but did not abolish the ability to coimmunoprecipitate with E-cadherin [134], suggesting that these two tyrosines are distinctly involved in β-catenin association with E-cadherin and α-catenin. Accordingly, in IEC18 intestinal epithelial cells, overexpression of K-Ras led to β-catenin phosphorylation at tyrosines 142 and 654, inducing the dissociation from both E-cadherin and α-catenin [135]. Fer and Fyn promoted the phosphorylation of a GST-β-catenin construct at tyrosine 142, leading to the dissociation of β-catenin from α-catenin but not from E-cadherin. Y142F β-catenin mutant was resistant to Fer-induced loss of α-catenin binding. In contrast, Src and Yes were able to phosphorylate β-catenin at sites other than tyrosine 142 [135]. In mouse hearts, VEGF induced FAK activation, binding to VE-cadherin, and phosphorylation of β-catenin at tyrosine Y142, promoting the dissociation of β-catenin from VE-cadherin [136].

Even though phosphorylation-induced loss of catenin binding is an attractive mechanism to explain the reduction of cell-cell adhesion, accumulated evidence clearly shows that AJ phosphorylation cannot be directly linked to AJ disassembly in every case. Instead, the net effect is the overall sum of multiple actions to either increase or decrease AJ protein association, depending on the kinase involved and the specific tyrosines phosphorylated. For example, in 3Y1 fibroblasts [25] and MDCK cells [27] transformed with v-Src, E-cadherin was able to coimmunoprecipitate with α-catenin or β-catenin, respectively, even when v-Src induced a marked increase in E-cadherin phosphorylation. Further, v-Src activation reduced cell adhesion strength in L fibroblasts expressing an E-cadherin-α-catenin fusion construct that did not bind β-catenin, demonstrating that Src can inhibit cell adhesion independently of junction disassembly through β-catenin phosphorylation [30]. Keratinocytes induced to differentiate by culturing in high Ca^{2+} media displayed increased phosphorylation of β-catenin and γ-catenin, which correlated with an increased association of α-catenin and p120 with E-cadherin [40]. Further, p120 and β-catenin may be regulated independently. For example, Ras-induced transformation of MCF10A human mammary epithelial cells promoted the tyrosine phosphorylation of AJ components and a loss of E-cadherin/β-catenin binding concurrently with an increase in E-cadherin/p120 association [29]. Similar observations were made in IEC cells expressing K-Ras [135]. Consistently, *in vitro* phosphorylation and binding assays demonstrated that Src can directly phosphorylate p120 and β-catenin with markedly different outcomes: while β-catenin phosphorylation at Y654 reduced β-catenin affinity for an E-cadherin cytosolic domain, p120 phosphorylation increased E-cadherin binding [137]. In E8 chicken retina cells, p120-associated Fer was essential to maintain β-catenin binding to N-cadherin through phosphorylation and activation of the phosphatase PTP1B, which in turn was responsible for dephosphorylating β-catenin at tyrosine 654 [46]. γ-catenin phosphorylation can also lead to different results, depending

on the kinase involved and the phosphorylated tyrosine. While Src-mediated phosphorylation at tyrosine 683 reduced the association of γ-catenin with E-cadherin and α-catenin, Fer-induced phosphorylation at tyrosine 549 increased γ-catenin binding to α-catenin [138].

Tyrosine phosphorylation has also been linked to AJ dissociation in the endothelium. Tyrosines 658 and 731 in the VE-cadherin tail are required for binding to catenins [139], since phosphomimetic mutations Y658E and Y731E in VE-cadherin constructs expressed in CHO cells prevented the binding to p120 and β-catenin, respectively. Expression of the same mutants prevented the formation of a tight barrier in these cells [139]. Similarly, Y658F VE-cadherin, but not wt or Y658E VE-cadherin, was able to bind to p120 in rat fat pad endothelial cells that lacked endogenous VE-cadherin [140]. Consistently, VEGF treatment in human pulmonary microvascular cells induced the loss of β-catenin and p120 binding to VE-cadherin, which correlated with phosphorylation of both VE-cadherin and β-catenin at tyrosine 654 [141], and expression of an Y658F/Y731F VE-cadherin mutant blocked VEGF-induced permeability and loss of VE-cadherin binding to β-catenin and p120 [141]. Further, overexpression of a catalytically inactive C459S SHP2 mutant in rat lung microvascular endothelial cells resulted in increased phosphorylation of VE-cadherin, p120, and β-catenin and reduced p120 association with VE-cadherin that was associated with a loss of barrier function [142]. VE-cadherin phosphorylation, however, does not always correlate with a decreased association with p120 or β-catenin. Early on, Esser et al. [74] showed that, in HUVECs, VEGF stimulation promoted VE-cadherin and catenin phosphorylation, but this treatment did not affect the level of cadherin/catenin coimmunoprecipitation, clearly dissociating the phosphorylation events from a loss of cadherin association with catenins. Similarly, histamine-induced VE-cadherin phosphorylation in HMEC-1 cells was not followed by a loss of VE-cadherin association with p120, β-catenin, or γ-catenin [143]. Moreover, while bradykinin treatment in HUVECs promoted VE-cadherin phosphorylation at tyrosine 658 and this phosphorylation was required for VE-cadherin endocytosis, internalized VE-cadherin was still associated with p120 [90]. Thus, multiple factors can promote AJ tyrosine phosphorylation without promoting a loss of cadherin binding. To directly assess the ability of Src-induced AJ phosphorylation to disassemble the adherens junction complex, increased tyrosine signaling in human dermal microvascular cells was induced by overexpression of a constitutively active (Y530A) Src construct or by inhibiting Csk activity [94]. Inhibition of Csk was achieved by overexpression of a kinase dead (K222R) mutant Csk that acts as a dominant negative [144]. While cells displayed markedly increased tyrosine phosphorylation, including VE-cadherin, the ability of endogenous VE-cadherin to colocalize and to coimmunoprecipitate with p120, β-catenin, and γ-catenin was not affected [94]. Y530A Src (but not DN-Csk) induced a dramatic loss of barrier function, as measured by monolayer gap formation, TEER, and albumin permeability [94], demonstrating that diminished cadherin/catenin association may not be required for endothelial barrier function loss.

Thrombin, another potent agent that induces a rapid increase in endothelial permeability, promoted the tyrosine phosphorylation of β-catenin without affecting its association with VE-cadherin, as measured by coimmunoprecipitation [145]. In HPAE cells, thrombin-induced monolayer gaps contained thin membrane projections that still connected the two adjacent cells [146]. Interestingly, the authors observed a reduction in the levels of colocalization of cadherin and catenins only in these projections, without a general loss of cadherin/catenins coimmunoprecipitation or colocalization in the rest of the cell body [146], suggesting that AJ disruption may occur only at the sites of adhesion loss. These projections are morphologically identical to the "finger-like" structures observed after TGF-β treatment in bovine pulmonary artery cells [147], the discontinuous junctions induced by TNF-α [148], and the focal adherens junctions shown by Huveneers et al. to contain vinculin molecules linking VE-cadherin to radial actin junctions [149]. In these other studies, VE-cadherin and catenins remained present within these thin structures after the formation of the gap and are probably responsible for maintaining the connection between the two endothelial cells surrounding the gap [147–149]. In fact, at least in the case of TGF-β, it appears that gap formation precedes a loss of catenin staining [147], suggesting the possibility that adherens junction complex disruption is a consequence, and not a cause, of the sustained loss of adhesion. Focal adherens junctions may be involved in junctional formation and remodeling *in vitro* [149, 150] and this remaining connection might be critical *in vivo* to ensure a fast gap closure as proposed by Baluk et al. [151] after detailed description of substance P-induced gap formation in rat trachea venules. Detailed experiments performing multicolor live imaging at high resolution will be required to set this issue.

The regulation of the adherens junction complex may also involve phosphorylation of serine and threonine. In HPAE cells, thrombin effects were associated with a PKC-dependent dephosphorylation of VE-cadherin and β-catenin and p120 phosphorylation [146]. As VE-cadherin phosphorylation was assessed by 2D electrophoresis rather than phosphotyrosine blots, it is possible that many of the observed dephosphorylation events occurred in serine and threonine residues, rather than tyrosines [146]. In fact, PKCα was shown to mediate thrombin- and LPS-induced p120 phosphorylation at serine 879, leading to the dissociation from VE-cadherin and AJ disassembly [152]. E-cadherin serine phosphorylation regulates α-catenin, β-catenin, and γ-catenin binding, and mutation of a cluster of eight serine residues from S838 to S853 prevented E-cadherin binding to the catenins (as measured by E-cadherin IP of ^{35}S-labeled cells) and abolished the ability of E-cadherin to promote cell aggregation, a method to determine cell-cell contact strength [153]. Structural studies demonstrated an increased affinity between phosphorylated E-cadherin and β-catenin [154, 155]. E-cadherin serine phosphorylation may be mediated by casein kinases (CK). CK-II-mediated E-cadherin serine phosphorylation increased β-catenin binding in NIH3T3 expressing exogenous mouse E-cadherin [156, 157]. *In vitro*, CK-II phosphorylated wild-type E-cadherin, while S840A, S853A, and S855A E-cadherin mutants were resistant to CK-II-mediated phosphorylation

[156]. In another study, CK-I phosphorylated E-cadherin at S846 *in vitro* [158], while CK-II was able to phosphorylate S846A E-cadherin mutant, but not an E-cadherin construct in which serines 849, 852, and 855 were mutated to alanine, suggesting that CK-I and CK-II phosphorylate E-cadherin at close but different sites. In GST pull-down assays, S846D phosphomimetic mutant showed decreased binding to β-catenin but did not modify the association with p120 [158]. Serines 846, 849, 852, and 855 in E-cadherin correspond to S742, S745, T748, and S751 in VE-cadherin, but it is unknown whether casein kinases can phosphorylate VE-cadherin. Suggestively, all phosphorylatable residues are conserved in that region, with the only exception of a serine for threonine substitution and two reciprocal substitutions for acidic amino acids ($S_{852}S_{853}E_{854} \rightarrow T_{748}D_{749}S_{750}$), arguing to support a conserved need for negative charges in this domain. CK-I is best known as a component of the β-catenin destruction complex that is part of the Wnt pathway [159], but it was also found to phosphorylate p120 and α-catenin. In response to Wnt3a, CK-I promoted p120 phosphorylation, linking p120 to Wnt-mediated β-catenin transcription [160]. In response to EGF, CK-II phosphorylated α-catenin at serine 641, leading to the release of β-catenin and an increase in β-catenin transactivation [161]. Recently, it was also shown that both CK-I and CK-II can phosphorylate α-catenin *in vitro* on a cluster of serine and threonine residues at the α-catenin flexible linker [162], including S641. Expression of non-phosphorylatable and phosphomimetic α-catenin mutants in MDCK cells in a model of monolayer fragmentation suggested that phosphorylation of α-catenin at this region promotes cell-cell adhesion strength and monolayer integrity. This effect, however, was not due to changes in junctional assembly, as all mutants associated with E-cadherin or β-catenin to the same extent [162].

Overall, the biochemical data suggest that the observation of a particular phosphorylation event may not necessarily imply that large changes in cadherin/catenin association will be detected. While this fact does not negate an important role in junctional phosphorylation in the regulation of the adherens junction assembly and disassembly, several conclusions can be drawn from all the accumulated evidence. First, tyrosine signaling must act in concert with other pathways to promote junctional disassembly and loss of cell adhesion, as direct kinase activation does not always lead to junctional loss. Second, kinase deficient models and small molecule inhibitors demonstrated that a basal level of tyrosine phosphorylation is required for junctional maintenance and that a precise regulation of the levels of activity may be needed in order to ensure normal cell adhesion. Third, fluorescence imaging showed that the loss of cell-cell contacts is reversible and geographically limited, and thus biochemical methods may lack the sensitivity to detect small but important changes. It can be envisioned that junctional disassembly and loss of cadherin/catenin association may be strictly limited to subcellular regions immediately surrounding the formation of a gap or transmigrating leukocyte and temporally restricted to the initiation of such event, reforming quickly to allow junctional recovery.

7. FAK Has Dual Roles

Although most of the efforts to understand tyrosine kinase signaling in the regulation of endothelial contacts have been focused on SFKs, an important role emerged for the focal adhesion kinase family of tyrosine kinases. As its name implies, FAK is best known in the context of integrin signaling (reviewed in [163]). Notably, FAK and Src have complex interactions. FAK is Src substrate, but it can also mediate Src activation, placing FAK both upstream and downstream of Src [163]. FAK has multiple roles regulating epithelial junctions in collective cell migration and metastasis, which are beyond the scope of this review [164, 165]. As with other tyrosine kinase signaling in endothelial cells, FAK has been described to act as a promoter of AJ formation and strengthening as well as an inducer of AJ disassembly. In rat lung microvascular endothelial cells, FAK mediated the increase in TEER induced by hyperosmolarity [166] as well as the recovery of the barrier function after a transient loss stimulated by hydrogen peroxide treatment [167]. Similarly, the recovery of HPAECs from thrombin-induced loss of barrier function was dependent on FAK activity [168]. In that study, expression of FAK related nonkinase (FRNK, which blocks endogenous FAK activity [163]) decreased basal TEER and prevented the recovery after thrombin. FRNK blocked p190 RhoGAP phosphorylation and promoted more sustained Rho activation. Further, cationic liposome-mediated FRNK expression to lungs increased fluid permeability in perfused isolated mouse lungs, demonstrating a role for FAK activity in whole tissues [168]. FAK activation downstream of the PAR1 thrombin receptor was mediated by Gβ1 association with Fyn, leading to the association of activated FAK with p120 [169]. FAK was also recruited to the AJ in cells treated with sphingosine 1-phosphate (S1P), an agonist that promotes endothelial barrier strengthening [170]. In these experiments, FAK coprecipitated with β-catenin after S1P treatment, and knockdown of β-catenin prevented the association between FAK and VE-cadherin. Moreover, Lyn kinase, an SFK, promoted the stabilization of endothelial barrier through phosphorylation of FAK at tyrosines 576/577 and 925 [171] and FAK-deficient mouse endothelial cells displayed increased permeability to FITC-dextran [172]. Thus, there are many strong indications *in vitro* and *ex vivo* that FAK promotes junctional assembly and endothelial barrier function. Nevertheless, published data also argues in favor of another role in promoting barrier disruption. FAK knockdown in immortalized human microvascular cells increased basal TEER [173] and FRNK expression blocked the VEGF-induced increase in permeability in isolated porcine coronary venules and HUVECs [174]. Further, as opposed to the observations made in mouse lungs [168], direct transfection of FRNK into pig coronary venules did not affect basal permeability but prevented neutrophil-induced leakage [175]. Confirming a role for this kinase in VEGF effects, VEGF-induced permeability was abrogated by the FAK inhibitor PF-562271 in HPAECs and by expression of a kinase dead FAK in mouse endothelial cells [136]. Furthermore, FAK can directly phosphorylate *in vitro* VE-cadherin at Y658 [176].

A definitive proof for a requirement of endothelial FAK promoting endothelial barrier function *in vivo* was provided by Schmidt et al. [177], by showing that conditional FAK deletion in the endothelium promoted features of acute lung injury, such as hemorrhage, edema, and neutrophil accumulation. The authors attributed the phenotype to increased activation of RhoA mediated by p115RhoGEF. However, Chen et al. [136] showed that VEGF-induced permeability was abrogated in mice expressing a kinase dead (K545R) FAK in the endothelium. The authors used a mouse model in which one floxed FAK allele was deleted in the endothelium by tamoxifen, while the other allele consisted in either wild-type FAK or a K545R FAK knock-in, thus rendering the mouse ECs with either active or inactive FAK, respectively. Using this model, they demonstrated that FAK is required for VEGF-induced vascular leakage in the dermis. This effect was mimicked by the FAK inhibitor PF-562271 [136]. K545R FAK knock-in mice also showed diminished VEGF-mediated tumor cell extravasation and VE-cadherin Y658 phosphorylation [176]. The data presented above clearly implicates at least two distinct roles for FAK, one as a kinase that is required for normal junctional assembly and another role downstream of the edemagenic effects of VEGF. A possible explanation for these seemingly contradictory roles may lay in FAK's ability to counteract Rho activation (which may be dominant in junctional assembly and in response to thrombin) and to mediate Src activation (a critical step in VEGF signaling). Other Cre-inducible FAK knock-in mice have been developed, including the nonphosphorylatable mutants Y397F and Y861F and the phosphomimetic Y397E [178]. It will important to determine the similarities and differences between the phenotypes of these point mutants versus the FAK null and the kinase dead mutant described above, as they may provide new insight to understand the differential roles of FAK in cell adhesion.

8. The Other Side of the Coin: Tyrosine Phosphatases

Adherens junction proteins can bind to at least 12 distinct tyrosine phosphatases (reviewed in [15, 179]). Of those, several have been shown to affect intercellular adhesion strength, either by directly dephosphorylating adherens junction components or by indirectly affecting their phosphorylation levels through the modulation of RTK signaling and SFK activation. A discussion of the most relevant findings relating to endothelial barrier regulation is provided below.

8.1. SHP2. Src homology-2 (SH2) domain-containing phosphatase 2 (SHP2, also called PTP11, PTP-1D, or PTP-2C) is a ubiquitously expressed phosphatase that is associated with multiple neoplastic malignancies, as well as three closely related inherited developmental disorders [180–182], the Noonan syndrome, the Noonan-like disorder with multiple giant cell lesion syndrome, and the LEOPARD syndrome, that include, among many other defects, lymphatic malformations and bleeding difficulties [183].

A first indication that SHP2 may regulate endothelial cell-cell contacts was provided by Ukropec et al. [184]. In HUVECs, thrombin promoted a transient increase lasting less than 30 minutes of several phosphotyrosine bands in VE-cadherin immunoprecipitates that comigrated with p120, β-catenin, and γ-catenin. This increase correlated with SHP2 phosphorylation and a loss of SHP2 in the VE-cadherin immunoprecipitates. Direct association of SHP2 with β-catenin was likely, since a far-Western blot assay demonstrated that a construct consisting of GST-tandem SHP2 SH2 domains bound to isolated β-catenin, but not VE-cadherin, p120, or γ-catenin from HUVEC lysates [184]. Consistently, Timmerman et al. showed that thrombin promoted transient β-catenin phosphorylation that lasted 15–30 minutes [145]. Thrombin treatment induced Src activation and SHP2 phosphorylation at Y542 with kinetics that correlated with the loss of β-catenin phosphorylation and the recovery of TEER. To prove that SHP2 mediated the recovery, it was shown not only that SHP2 immunoprecipitated from cells treated with thrombin was able to dephosphorylate β-catenin *in vitro*, but also that SHP2 knockdown prolonged β-catenin phosphorylation and thrombin-induced TEER loss [145]. Additionally, SHP2-mediated regulation of cell adhesion may involve Rho GTPases, key mediators of the thrombin response. Early on, it was shown that SHP2 inhibition in fibroblasts promoted Rho activation [185]. In vascular smooth muscle cells, SHP2 mediated the angiotensin II-induced dephosphorylation and inactivation of p190RhoGAP, leading to increased RhoA activation [186]. Proof that this pathway was active in endothelial cells was provided in PAECs [142]. In these cells, inhibition of SHP2 activity by expression of the inactive mutant C459S SHP2 or treatment with the pharmacological SHP2 inhibitor NSC-87877 reduced p190RhoGAP activity and promoted RhoA activation as measured by GST-RBD pull-down assays. SHP2 inhibition reduced basal monolayer resistance in PAECs and promoted an increase in phosphorylated VE-cadherin and β-catenin as measured by IP and phosphotyrosine Western blot [142]. More recently, it was shown that both LPS and thrombin treatment induced a reduction in lung SHP2 activity and association with FAK [187]. Further, liposomal delivery of a constitutively active (D61A) SHP2 mutant reduced pulmonary edema in mice challenged with LPS or *Pseudomonas aeruginosa* [187], suggesting that SHP2 plays an important role in preventing acute lung injury.

SHP2 also regulates the response to other vasoactive mediators. An early observation that VEGFR2 phosphorylation in response to VEGF-165 was much higher in HUVECs grown on vitronectin than in cells grown on collagen I [188] was attributed to differential involvement of SHP2 through direct association with phosphorylated VEGFR2 [189]. SHP2 may indirectly promote Src activation by dephosphorylating the Csk regulator Cbp and inactivating Csk [190]. In fact, SHP2 mediated Src and PI3K activation after VEGF treatment by inducing the dissociation of Csk from VE-cadherin in BAECs [65]. SHP2 interactions with Gab1, an adaptor protein that strongly associates with both SHP2 and PI3K [191, 192], may also explain in part why SHP2 is required for VEGF-induced PI3K activation. In porcine aortic endothelial cells, flow induced the formation of a complex involving

SHP2, Gab1, and PI3K that was required for flow-induced eNOS phosphorylation [193]. Flow also induced SHP2 and Gab1 translocation to the plasma membrane in BAECs [194], as well as increased SHP2 and PECAM-1 association in both BAECs [194] and HUVECs [195]. A complex involving SHP2, Gab1, and Grb2 also mediated PI3K activation downstream of FGFR1 receptors [196, 197]. Importantly, FGF2, a ligand of FGFR1, promoted the formation of tight capillaries in a mouse corneal angiogenesis model [198]. SHP2 mediated regulation of adherens junction stability downstream of FGF appears to involve a different mechanism. In BAECs, inhibition of FGF signaling promoted VE-cadherin internalization and dissociation from p120, an effect with important consequences *in vivo*, since inhibition of FGF signaling in rats using adenoviral delivery of FGF traps destabilized the vasculature integrity and promoted vascular barrier loss [199]. Subsequent research from the same group showed that overexpression of a dominant negative form of FGFR1 (FGFR1DN) reduced VE-cadherin association with SHP2 and p120 [200]. FGFR1DN induced the phosphorylation of VE-cadherin, but not p120, as well as a loss of junctional localization of a VE-cadherin-GFP construct. Phosphorylation of VE-cadherin at Y658 was required for the loss of junctional localization, as Y658F VE-cadherin-GFP construct was resistant to FGFR1DN-induced junctional loss. Confirming a causal role for the loss of SHP2 in this model, overexpression of SHP2 partially rescued FGFR1DN-induced loss of TEER [200].

8.2. Dep1. Dep1 (also called CD148 and PTPη) is a ubiquitously expressed phosphatase that was originally cloned from a HeLa cDNA library [201]. *In vitro*, Dep1 was found to bind directly to Src [202] and to dephosphorylate ZO-1, occludin [203], p120, and β-catenin [204]. Interestingly, Dep1 expression increased with cell density in WI38 and AG1518 fibroblasts [201]. Expression of Dep1 in transformed rat thyroid PCMPSV cells increased Src activity via Y527 dephosphorylation (corresponding to Y530 in human Src) without affecting the level of phosphorylated Y416 (Y419 in human Src). This led to an increase in the tyrosine phosphorylation of FAK and paxillin and overall increased adhesion to the substratum [202]. Experiments using GST pull-downs showed that Dep1 can interact with phosphorylated junctional proteins in endothelial [205] and epithelial [203] cells. A substrate trapping (D/A mutant) Dep1 catalytic domain coprecipitated with p120, β-catenin, and γ-catenin in lysates from HUVECs treated with pervanadate, but not control cells [205]. Similar to observations in HUVECs, GST-C/S Dep1 bound to ZO1, occludin, Src, and p120 in lysates from MCF10A mammary epithelial cells pretreated with pervanadate, but not from control cell lysates [203]. Expression of Dep1 in MDCK-II cells promoted monolayer barrier function, as measured by increased TEER and reduced FITC-dextran permeability after a calcium switch assay [203]. The role of Dep1 was also studied in A431D epidermoid cervical carcinoma cells (that lack endogenous classical cadherins [206]), in which E-cadherin was reexpressed [204]. Coexpression of Dep1 promoted an increase in junctional E-cadherin in these cells, which was dependent on E-cadherin/p120 association, since Dep1 was unable to increase junctional association of 764EED $\rightarrow$ AAA E-cadherin [204], a mutant that is unable to bind p120 [121]. In a calcium switch assay, wild-type but not C/S Dep1 potentiated adhesion-mediated Rac activation without affecting CDC42 or Rho GTP levels. Rac activation by Dep1 was also dependent on the association between p120 and E-cadherin, as it was observed in cells expressing wild-type but not 764AAA E-cadherin [204]. Even though *in vitro* Dep1 was shown to dephosphorylate p120, in A431D cells Dep1 promoted an increase in the phosphorylation level of Y228 p120 after calcium addition. Importantly, the authors found that Dep1 expression also increased junctional VE-cadherin when expressed in HUVECs, although it remains unexplored whether the same p120-dependent mechanism is governing the action in HUVECs [204].

Dep1 was also shown to be expressed in the endothelium *in vivo* and to colocalize with VE-cadherin [207], but its role is not completely understood. Homozygous expression of a mutant Dep1 that lacks the phosphatase domain is embryonically lethal. Embryos die at E11.5 from multiple vascular defects including enlarged vessels and increased endothelial proliferation [208]. Paradoxically, mice completely lacking Dep1 were viable and fertile [209], suggesting that the phenotype of knock-in expressing Dep1 mutant may be due to a dominant function of this construct. However, Dep1 knockout mice, while viable, displayed deficient cerebral arteriogenesis in a model of left common carotid artery occlusion [210], thus confirming a role for wild-type Dep1 in the vasculature *in vivo*. A mechanism for the Dep1-mediated regulation of cell proliferation via the modulation of VEGFR2 signaling was proposed by Lampugnani et al. [60]. Endothelial cell contact inhibition of proliferation correlated with reduced VEGF signaling in confluent cells. The inhibition of VEGF signaling was dependent on Dep1 activity, together with the expression of β-catenin and its interaction with VE-cadherin. Expression of a catalytically inactive C/S Dep1 mutant or siRNA-mediated Dep1 knockdown restored VEGF-induced VEGFR2 phosphorylation and cell proliferation in confluent cells [60], suggesting a negative role of Dep1 in VEGF signaling mediating contact inhibition. This phosphatase, however, may have opposite effects on different VEGFR2 downstream signals. In HUVECs, Dep1 knockdown potentiated VEGF-induced VEGR2 phosphorylation at multiple tyrosine residues, as well as the phosphorylation of PLCγ, eNOS, and Erk1/2, but prevented VEGF-induced Akt activation [211]. Consistent with the previously described role for Dep1 in Src activation [202], this was associated with reduced VEGF-induced Src activation in Dep1 knockdown cells due to increased Src phosphorylation at Y530 and reduced association between Src and Gab1 [211]. In stark contrast, morpholinos directed at either one of the two zebrafish Dep1 genes (Dep1a or Dep1b) promoted vascular defects that could be rescued by PI3K inhibition, suggesting that Dep1 in zebrafish negatively regulates PI3K [212]. Reciprocally, Dep1 can be phosphorylated by Src and Fyn on Y1311 and Y1320, leading to the dephosphorylation of Y530 Src by Dep1 [213]. BAECs expressing Y1311F/Y1320F Dep1 mutant did not display Src dephosphorylation at Y530 after VEGF, while Erk activation was similar in wild-type

and YYFF Dep1-expressing cells. Dep1 knockdown or the YYFF mutant in HUVECs prevented the VEGF-induced VE-cadherin phosphorylation at S665, monolayer gap formation, and increase in FITC-dextran permeability [213].

8.3. VE-PTP. VE-PTP (also called R-PTP-β) is an endothelial-specific transmembrane tyrosine phosphatase that was cloned from a bEnd5 cDNA library [214]. The first demonstration that VE-PTP interacts with VE-cadherin was provided by Nawroth et al. by showing that exogenously expressed VE-PTP and VE-cadherin coimmunoprecipitated from COS-7 lysates [215]. In COS-7 cells expressing VE-PTP, VE-cadherin, and VEGFR2, VE-PTP was able to dephosphorylate VE-cadherin [215]. VE-PTP may have an important role in promoting junctional assembly and in maintaining cell adhesion. VE-PTP relocalization to cell-cell contacts from the endosome recycling compartment and association with VE-cadherin increased with endothelial confluence in bEnd3 cells and HUVECs, suggesting a role in AJ maturation [119]. Further supporting this notion, VE-PTP was able to bind and dephosphorylate γ-catenin [119], a junctional component that also increased binding to VE-cadherin with cell confluence [72]. Similarly, expression of VE-PTP in CHO cells increased VE-cadherin association with γ-catenin, suggesting that this phosphatase can enhance VE-cadherin/γ-catenin binding, but a mutant VE-cadherin in which all C-terminal tyrosine residues were replaced by phenylalanine also bound γ-catenin in the presence of VE-PTP, demonstrating that the effect of VE-PTP in γ-catenin binding was independent of the tyrosine phosphorylation level of VE-cadherin [119]. Conversely, VE-PTP knockdown reduced VE-cadherin-mediated adhesion, increased endothelial permeability to FITC-dextran, and enhanced neutrophil transmigration. Further, neutrophil or T-cell attachment to bEnd5 cells induced the dissociation of VE-PTP from VE-cadherin and promoted VE-cadherin, β-catenin, and γ-catenin phosphorylation [119]. Leukocyte-induced VE-PTP dissociation from VE-cadherin was found to be mediated by VCAM-1 through a pathway that involved Rac1, ROS generation, and Pyk2 activation. Blocking antibodies to VCAM-1 prevented T-cell-induced dissociation, while direct VCAM-1 cross-linking promoted VE-PTP/VE-cadherin dissociation [120]. LPS and VEGF also promoted the dissociation between VE-PTP and VE-cadherin *in vivo* [216]. To determine the role of this dissociation, the authors generated knock-in mice harboring two fusion proteins, VE-cadherin-FKBP and VE-PTP-FRB. When treated with the small molecule rapalog, these VE-cadherin and VE-PTP chimeras were locked in a heterodimeric conformation, thus preventing the dissociation induced by VEGF or leukocyte attachment. Consistent with a role for VE-PTP/VE-cadherin dissociation in TEM, rapalog injections in these knock-in mice prevented leukocyte extravasation in the IL-1β-induced inflammation of the cremaster muscle model, without affecting leukocyte attachment or rolling, and reduced LPS-induced increase of PMN in bronchoalveolar lavage (BAL) fluid. Rapalog injections also diminished VEGF and LPS-induced vascular leakage (measured by a Miles assay and protein content in BAL fluid, resp.) [216]. A role for VE-PTP in vascular permeability was also found in a zebrafish model, suggesting that VE-PTP roles are highly evolutionarily conserved [217]. In this model, injection of morpholinos against zebrafish VE-PTP causes blood cell aggregates, hemorrhage, and hyperpermeability to tetramethylrhodamine-dextran. Electron microscopy demonstrated that, in VE-PTP morphants, 60% of tail vessel ECs did not have junctional complexes, supporting the notion that VE-PTP maintains zebrafish VE-cadherin adhesions [217].

VE-PTP also plays a critical role in vascular development via its regulation of Tie2 and VEGFR2 signaling. VE-PTP null mice [218] or mice expressing a truncated form of VE-PTP [219] are not viable and embryos die at E8.5-10 due to vascular malformations. Allantois explants from VE-PTP-mutant mice displayed enlarged vessels with endothelial cells growing in sheets [219]. Moreover, antibodies against VE-PTP induced vessel enlargement in allantois explants that resemble observations in VE-PTP-mutant mice [220]. These antibodies did not induce vessel enlargement in $Tie2^{-/-}$ allantois, demonstrating that Tie2 mediated this effect. Further supporting VE-PTP-Tie2 axis controlling vessel growth, daily injections of anti-VE-PTP antibodies for 7 days in young mice induced vessel enlargement in the tongue and Tie2 phosphorylation in lung lysates [220]. VE-PTP was shown to associate with Tie2 in bEnd5 cells and to dephosphorylate exogenously expressed Tie2, but not VEGFR2, in COS-7 [214]. VE-PTP and Tie2 may act as a negative regulator of VEGF receptor activation and downstream signaling. VE-PTP knockdown prevented VEGF-induced tube formation in telomerase-immortalized human microvascular endothelial cells grown on a 3D collagen I matrix [221]. This was associated with an increase in VEGF-induced phosphorylation of VEGFR2 and proliferation, without any effect in apoptosis [221]. Further, VE-PTP-deficient embryoid bodies displayed increased angiogenic sprouting and Y1175 VEGFR2 phosphorylation [222]. The same study found that VE-PTP in stalk cells dephosphorylated VEGFR2, in a mechanism that required Tie2. Similarly, in porcine aortic endothelial cells lacking Tie2, VE-PTP did not coprecipitate with VEGFR2, even though in an *in vitro* assay VE-PTP was able to directly dephosphorylate VEGFR2 [222]. The mechanism downstream of VE-PTP may also involve the regulation of VE-cadherin phosphorylation, as angiogenic sprouts in VE-PTP knockout embryonic bodies showed increased pY658 VE-cadherin after VEGF treatment [222]. This effect might be specific to VEGF-induced phosphorylation, since in bEnd5 cells VE-PTP inhibition via blocking antibodies or siRNA-mediated knockdown induced Tie2 phosphorylation without promoting an increase in VE-cadherin phosphorylation [220].

8.4. PTPμ. PTPμ is a transmembrane receptor protein tyrosine phosphatase that was isolated using degenerated PCR primers from mouse brain cDNA based on its homology to other tyrosine phosphatases [223]. It contains an immunoglobulin domain and four fibronectin type III repeats and can mediate homophilic interactions through

its extracellular domains [224]. PTPμ localizes to cell-cell junctions in MvLu mink lung epithelial cells and coprecipitates with E-cadherin, β-catenin, and α-catenin [225]. *In vitro*, PTPμ was able to bind directly to the intracellular domain of E-cadherin but not to α-catenin or β-catenin. Pervanadate treatment did not prevent the coprecipitation of PTPμ with E-cadherin, suggesting that PTPμ can also associate with hyperphosphorylated cadherins [225]. However, a temperature-sensitive v-Src construct promoted E-cadherin phosphorylation and dissociation of PTPμ in WC5 neonatal rat cerebellar cells [226], suggesting that tyrosine phosphorylation events can regulate E-cadherin/PTPμ association. The PTPμ binding site in E-cadherin was located to the C-terminal 38 amino acids, close to the β-catenin binding site [226]. Interestingly, PTPμ can sustain E-cadherin adhesion in LNCaP prostate carcinoma cells through a mechanism that involves scaffolding, but not catalytic activity [227]. AJ/PTPμ association is not restricted to E-cadherin, as PTPμ coprecipitated with E-cadherin, N-cadherin, and R-cadherin in rat lung extracts [226] and promoted neurite outgrowth of chicken retinal ganglion cells grown on N-cadherin-coated surfaces [228]. In human lung microvascular ECs, PTPμ coprecipitated with VE-cadherin, and *in vitro* GST pull-downs demonstrated a direct interaction between PTPμ and VE-cadherin [229]. This interaction may be important in regulating endothelial barrier, because PTPμ knockdown or expression of catalytically inactive constructs increased permeability to albumin. This effect may be due to regulation of VE-cadherin phosphorylation, as PTPμ overexpression in immortalized HMEC-1 cells reduced basal VE-cadherin tyrosine phosphorylation [229]. Expression of PTPμ in endothelial cells appears to be variable. PTPμ expression increased severalfold with increasing monolayer confluence in HUVECs [230] and bovine aortic endothelial cells [231]. *In vivo*, its expression may be restricted to arteries. Immunofluorescence studies showed that PTPμ expression was higher in arterioles and arteries than in veins of multiple rat tissues [231], and PTPμ-LacZ knock-in mice displayed β-galactosidase activity in arterioles and capillaries, but not in veins or in fenestrated endothelium [232]. This observation could explain at least in part why venules display increased Src activation and VE-cadherin phosphorylation [90]. However, the fact that pervanadate (which blocks the activity of multiple phosphatases, including PTPμ [233]) increased VE-cadherin phosphorylation only in venules in the cremaster vasculature [91] argues against PTPμ differential expression as the sole explanation.

8.5. PTP1B. PTP1B is a ubiquitously expressed nonreceptor tyrosine phosphatase that holds the record of being the first tyrosine phosphatase purified and characterized [234–237]. PTP1B can dephosphorylate multiple phosphotyrosine-containing proteins, including several receptor and receptor-associated tyrosine kinases [238], and as such it plays a critical role in heart disease, insulin resistance, and leptin regulation, as well as in multiple neoplastic disorders [238]. The ability of PTP1B to associate with the adherens junctions was first shown by Balsamo et al. [239, 240] in chicken retina cells. In those cells, N-cadherin copurified with tyrosine phosphorylated PTP1B and with nonphosphorylated β-catenin. N-cadherin binding to PTP1B requires PTP1B tyrosine 152 phosphorylation [240, 241]. In turn, PTP1B leads to β-catenin dephosphorylation [46, 239]. Similar to PTPμ [226], PTP1B/N-cadherin association is mediated by a region near the C-terminus of N-cadherin, close to the β-catenin binding site, although β-catenin and PTP1B do not appear to compete for N-cadherin binding [242]. Besides a possible direct role in dephosphorylating β-catenin, it is possible that PTP1B may affect junctional stability through the direct modulation of Src activity [243–246]. For example, fibrinogen binding to $\alpha_{\mathrm{IIb}}\beta_3$-integrin in platelets triggers PTP1B recruitment to a complex involving Src, Csk, and integrins, which leads to Csk dissociation and Src activation through dephosphorylation of tyrosine 530 [244]. PTP1B is recruited in Src-dependent fashion, because pretreatment with PP2 blocks PTP1B association with β_3 integrin. Thus, not only can PTP1B be activated by tyrosine kinase signaling, but it can also promote tyrosine kinase activation. However, little is known about whether a PTP1B-Src axis is important in the endothelium. PTP1B was shown to bind and dephosphorylate VEGFR2 *in vitro*, and expression of wild-type PTP1B, but not a C/S catalytically inactive mutant, prevented VEGF-induced VEGFR2 phosphorylation and Erk, but not p38, in HUVECs [247]. Conversely, PTP1B knockdown increased VEGF-induced VEGFR2 phosphorylation and Erk activation, without increasing basal VEGFR2 signaling. Consistent with an increase in VEGFR2 signaling, blocking PTP1B activity by expression of PTP1B C/S mutant or by PTP1B knockdown induced an increase in VE-cadherin tyrosine phosphorylation and a reduction in TEER [247]. PTP1B can coprecipitate with p120, β-catenin, and VE-cadherin in rat lung microvascular endothelial cells as well as mouse lungs [248]. LPS treatment in mice reduced the association between PTP1B and β-catenin. More importantly, expression of an oxidation-resistant PTP1B mutant reduced LPS-induced lung edema [248].

9. Concluding Remarks and Perspectives

This year marks the 30th anniversary of the first detection of phosphotyrosine at the cell-cell junctions [20]. Since that initial discovery, intense research was aimed at understanding the mechanisms by which the adherens junction phosphorylation is regulated and at determining the functional effect of such phosphorylation events. It is now known beyond doubt that cadherin and catenin phosphorylation is a common event that occurs at multiple tyrosine residues, as a result of a complex balance of the multiple tyrosine kinases and phosphatases that interact with junctional proteins (Figure 4). While massive kinase activation or phosphatase inhibition leads to a dramatic loss of cell adhesion, there is an incomplete understanding of the details of the adherens junction regulation in cells with limited, regulated tyrosine kinase activation.

The ability of SFKs to phosphorylate VE-cadherin and the requirement for SFK activation downstream of multiple receptors, including VEGFR2 and ICAM-1, have been

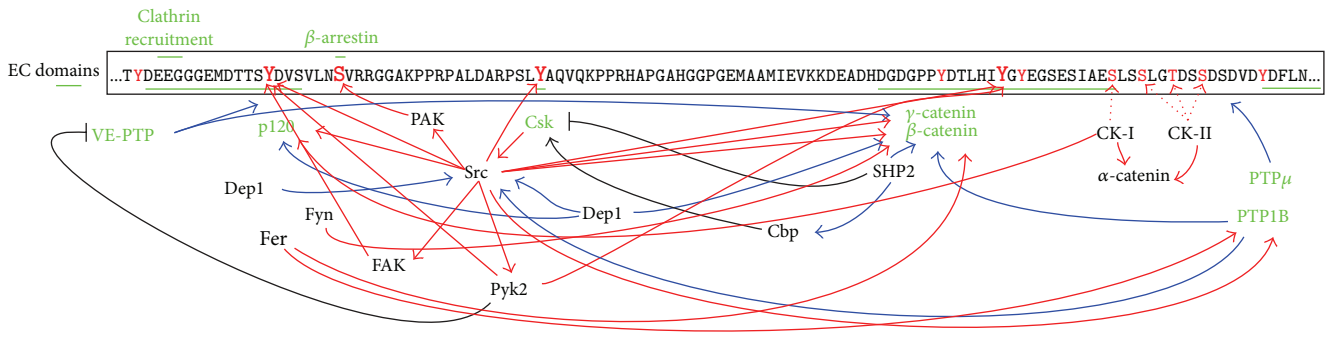

Figure 4: The net effect on cadherin tail phosphorylation depends on the action of multiple kinases and phosphatases. The VE-cadherin cytoplasmic region contains multiple phosphorylatable residues (in red) located in or near the JMD and CBD domains responsible for catenin binding (marked in green). Larger font highlights tyrosines 658, 685, and 731, together with serine 665, which have been more intensely studied. The overall phosphorylation status is the effect of a network of kinase (red arrows) and phosphatase (blue arrows) activities. These kinases and phosphatases can modify the cadherin tail and/or associated catenins directly or indirectly via the regulation of other associated kinases and phosphatases. Dotted arrowheads: CK-I and CK-II activity was shown to phosphorylate homologous residues in E-cadherin tail.

demonstrated. Nevertheless, it became clear that simply the observation of SFK activation cannot predict a loss of endothelial barrier function [94]. The findings that in mice SFKs may be active in the venular endothelium [90] and that VE-cadherin is phosphorylated in the absence of proinflammatory stimuli [88–91] prompt revisiting the role of VE-cadherin tyrosine phosphorylation in barrier function. One possibility is that SFK-mediated VE-cadherin phosphorylation may act as a gatekeeper to allow specific vascular beds to respond to proinflammatory agents that may trigger the loss of cell adhesion through the activation of other parallel signaling pathways. However, other mechanism(s) by which SFK signaling may crosstalk with other pathways cannot be excluded, such as the direct regulation of Rho GTPases as observed downstream of several tyrosine kinases and phosphatases [133, 142, 168, 177, 185, 204]. Moreover, loss of junctional VE-cadherin coincided *in vivo* with cadherin dephosphorylation of specific tyrosines [90, 91] and the inability to phosphorylate Y685 led to increased leakage in angiogenic tissues [92]. These observations raise the possibility that junctional components may require cycles of phosphorylation and dephosphorylation to enable the relocalization to different membrane compartments, as it is the case in focal adhesion turnover [249, 250]. In that scenario, dynamic changes in cadherin phosphorylation may allow transient binding to adaptor molecules. Endothelial gap formation can coincide with the generation of the so-called focal adherens junctions, which recruit vinculin to link cadherins and catenins to radial actin fibers [149], a process that may not be required for the formation of the gap itself, but for enabling the recovery of cell adhesion. However, it is not known whether vinculin association or any other scaffolding protein with the adherens junctions in this context requires a change in VE-cadherin and/or catenin phosphorylation.

A potential mechanism that stands out for its simplicity and logic is the regulation of the association of VE-cadherin to catenins by differential phosphorylation events. The well-known role of p120 in preventing VE-cadherin endocytosis [3, 125, 126, 128, 129] and the inability of a phosphomimetic Y658E VE-cadherin mutant to bind p120 [139, 140] support a model in which VE-cadherin phosphorylation drives the loss of p120 binding and thus endocytosis, leading to disruption of cell adhesion. However, most of the available biochemical data do not validate this model and strongly suggest that even with dramatic changes in VE-cadherin phosphorylation and/or loss of barrier function at least the majority of VE-cadherin remains bound to p120 [74, 90, 94, 143, 145, 146]. Should we disregard then this potential mechanism? We probably should not. The formation of endothelial gaps may require the loss of only a small subset of junctional VE-cadherin/p120 complexes, which would render the biochemical approaches not sensitive enough to detect small but important changes in junctional protein association. Alternatively, some stimuli may require loss of p120 binding and VE-cadherin endocytosis, while others may act via the dissociation of the bridge between β-catenin, α-catenin, and the actin cytoskeleton. Additionally, it is possible that some phosphorylation events in VE-cadherin may be a consequence, and not a cause, of catenin dissociation. The adherens junctions associate with multiple tyrosine kinases [46, 65, 66, 93, 135, 169, 172] and phosphatases [119, 145, 184, 201, 204, 205, 213], and both p120 and β-catenin can bind and recruit them to the junction or, alternatively, compete for a binding site and displace kinases and phosphatases from binding VE-cadherin, with the overall effect of modulating the levels of junctional phosphotyrosine (Figure 4). The exact temporal relationship in the set of events leading to the disruption of cell adhesion is still under study. Lastly, changes in tension at the junction due to catenin-regulated activity of Rho GTPases [251] not only may affect the ability of VE-cadherin to transduce mechanosensory stimuli [68] but could also potentially regulate RTK signaling and the levels of VE-cadherin phosphorylation and cell-cell adhesion. In fact, pharmacological inhibition of MLCK [147], ROCK [148, 149], or myosin II [149] prevented the formation of focal adherens junctions, demonstrating that actomyosin-mediated tension is critical for junctional remodeling in endothelial cells. The use

of new microscopy techniques including the use of novel FRET-based tension sensors [68, 252, 253] as well as kinase and GTPase biosensors [254–257] will undoubtedly allow us to assess the extent of localized and temporally limited changes in junctional association in the context of a forming gap at the specific locations where intercellular adhesion is being affected. Combining these assays with the newly developed phosphospecific antibodies with much improved epitope specificity [81, 89–91] will enable us to correlate in time and space the level of VE-cadherin phosphorylation with its association with catenins in the endothelial cell response to edemagenic stimuli and to leukocyte diapedesis.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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Research Article

Early Detection of Junctional Adhesion Molecule-1 (JAM-1) in the Circulation after Experimental and Clinical Polytrauma

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Severe tissue trauma-induced systemic inflammation is often accompanied by evident or occult blood-organ barrier dysfunctions, frequently leading to multiple organ dysfunction. However, it is unknown whether specific barrier molecules are shed into the circulation early after trauma as potential indicators of an initial barrier dysfunction. The release of the barrier molecule junctional adhesion molecule-1 (JAM-1) was investigated in plasma of C57BL/6 mice 2 h after experimental mono- and polytrauma as well as in polytrauma patients (ISS ≥ 18) during a 10-day period. Correlation analyses were performed to indicate a linkage between JAM-1 plasma concentrations and organ failure. JAM-1 was systemically detected after experimental trauma in mice with blunt chest trauma as a driving force. Accordingly, JAM-1 was reduced in lung tissue after pulmonary contusion and JAM-1 plasma levels significantly correlated with increased protein levels in the bronchoalveolar lavage as a sign for alveolocapillary barrier dysfunction. Furthermore, JAM-1 was markedly released into the plasma of polytrauma patients as early as 4 h after the trauma insult and significantly correlated with severity of disease and organ dysfunction (APACHE II and SOFA score). The data support an early injury- and time-dependent appearance of the barrier molecule JAM-1 in the circulation indicative of a commencing trauma-induced barrier dysfunction.

1. Introduction

Polytrauma with subsequent complications, such as systemic inflammation and multiple organ dysfunction and failure, remains a leading cause of mortality among people aged 45 and younger [1]. Immediately after the trauma impact, the injured patient is exposed to multiple danger- and pathogen-associated molecular patterns, leading to molecular, cellular, and organ dysfunction [2]. A main pathophysiological driver for multiorgan failure is the dysfunction and breakdown of external (skin) and internal barriers, including the brain-, air-, and gut-blood barrier (BBB, ABB, and GBB, resp.) resulting in an uncontrolled "flooding" of tissues and microbial invasion. Physiologically, highly effective paracellular barriers are guaranteed by tight junctions, formed by various integral membrane molecules, such as occludin, claudin, and junctional adhesion molecule-1 (JAM-1/JAM-A/CD321/F11R) [3, 4]. In addition to the sealing of cell-cell contacts of endothelial and epithelial cells, JAM-1 is expressed on circulating cells, including leukocytes regulating transendothelial migration [5], or on hematopoietic stem cells facilitating long-term repopulation and reendothelialization of injured vascular walls [6, 7]. On a molecular level, JAM-1 can dimerize and bind homophilically via its extracellular

domain, whereas its intracellular domain can interact with submembranous structures and signaling proteins [8]. JAM-1 is also present on platelets, where it can dimerize or cross-link with FcγRII, resulting in enhanced platelet adhesiveness and hyperaggregability [9]. Other reports suggest that JAM-1 in resting platelets inhibits premature platelet activation [10]. During inflammatory conditions, including murine autoimmune encephalomyelitis, a significant reduction in tight junction molecules, particularly JAM-1, was found to be associated with BBB dysfunction [11]. Similarly, ABB dysfunction, as modelled by lipopolysaccharide- (LPS-) induced acute lung injury, revealed partially reduced JAM-1 and claudin expression within the lungs [12]. In accordance with this, JAM-1-deficient mice exhibited classical signs of enhanced pulmonary permeability and susceptibility to a remote LPS challenge, suggesting JAM-1 as a key regulator of lung barrier function [13]. Under trauma conditions, ischemia and reperfusion (I/R) injury is a central pathophysiological driver of the inflammatory response. After induction of experimental liver I/R injury, JAM-1 deficiency did not alter hepatocellular necrosis but increased apoptotic rates and attenuated neutrophil infiltration [14]. Release of hemoglobin from extravasated erythrocytes (e.g., after brain injury), mimicked by stereotactic hemoglobin injection into the rat brain, led to a BBB breakdown with significantly reduced JAM-1 expression levels and enhanced nitric oxide production [15]. Furthermore, during chronic inflammatory diseases, including atherosclerosis, JAM-1 can be released from tight junctions into the circulation [16].

However, no data is currently available on a possible JAM-1 release in the context of severe tissue trauma. Therefore, we hypothesized that JAM-1 may be detected in the circulation after polytrauma, reflecting the degree of tissue injury. For the first time, we describe the detection of circulating JAM-1 in a murine polytrauma model and in patients after polytrauma which appears to be injury-pattern- and time-dependent and may therefore represent a useful future clinical tool to assess posttraumatic barrier dysfunction.

2. Materials and Methods

2.1. Experimental Murine Polytrauma. The study protocol for the experimental murine polytrauma was approved by the University Animal Care Committee and the Federal Authorities for animal research, Tuebingen, Germany (number 1016), and all experiments were performed in adherence to the National Institutes of Health Guidelines for the use of laboratory animals. C57BL/6 mice aged 8-9 weeks (Jackson Laboratories, Bar Harbour, USA) with a mean body weight of 25 g (±2.5 g) were used. Mice were anesthetized using a mixture of 2.5% sevoflurane (Sevorane, Abbott, Wiesbaden, Germany) and 97.5% oxygen and were exposed to blunt chest trauma or sham procedure. Following trauma, anesthesia was continued intraperitoneally using Ketavet (Pfizer Pharma, Karlsruhe, Germany) and Xylazine (Bayer Health Care, Monheim, Germany). Mice were randomly divided into the following treatment groups with $n = 6$–12 animals in each: control (sham), bilateral blunt chest trauma (TxT), closed traumatic brain injury (TBI), proximal femoral fracture including contralateral soft tissue injury (Fx + STI) or a combination of the traumata: TxT + TBI, TxT + Fx + STI, and TBI + Fx + STI, or polytrauma (PT = TxT + TBI + Fx + STI) as previously described [17]. Sham animals underwent identical procedures, but without trauma application. Mice were maintained anesthetized until sacrifice. Blood was withdrawn 2 h after trauma by cardiac puncture; plasma was obtained by centrifugation and stored at −80°C until further analysis. Lungs were either used for bronchoalveolar lavage (BAL) or homogenized for ELISA analysis.

2.2. Bronchoalveolar Lavage (BAL) Protein. BAL was collected ($n = 6$ mice per group) to assess the integrity of the alveolocapillary barrier. After sacrifice, the mouse trachea was exposed and cannulated, and the lung was flushed three times using 500 μL ice-cold phosphate-buffered saline (Gibco, Eggenstein, Germany), including 10 μL 1 : 1,000 broad-spectrum protease inhibitor (Sigma-Aldrich, St. Louis, MO). Subsequently, the BAL fluids were centrifuged at 380 ×g for 10 min at 4°C, and the supernatant was stored at −80°C until analysis. Protein concentrations in BAL fluids were determined using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA) as recommended by the manufacturer using a microplate reader (Tecan GmbH, Grödig, Austria).

2.3. Measurement of JAM-1. Concentrations of tight junction protein JAM-1 were determined using a sandwich-enzyme-linked immunosorbent assay technique according to the manufacturer's recommendations. JAM-1 was determined in human (human JAM-1 ELISA, Cloud-Clone Corp., Houston, TX, USA) and murine plasma (mouse JAM-1 DuoSet ELISA, R&D, Minneapolis, MN, USA) as well as in lung tissue homogenates of mice after experimental trauma or sham procedure.

2.4. Polytrauma Patients. We conducted a prospective clinical study including patients with multiple trauma (Injury Severity Score (ISS) ≥ 18) who were admitted to the University Hospital Ulm between 2010 and 2011. The study was approved by the Independent Local Ethics Committee of the University of Ulm, number 69/08. Ten healthy volunteers served as a control group. Written informed consent was obtained from all the patients and volunteers. Exclusion criteria were age <18 years, pregnancy, infection with the human immunodeficiency virus, cardiogenic shock as the primary underlying disease, underlying hematologic disease, cytotoxic therapy given within the previous 6 months, and the presence of a rapidly progressive underlying disease anticipating mortality within the following 24 hours. In the trauma cohort, 8 multiple-injured patients (6 men, 2 women) with a mean age of 51 years (±24 years, standard deviation (SD)) and a mean ISS of 30 points (±4 points, SD) were enrolled to determine the JAM-1 plasma concentration. Blood samples were drawn on admission at the emergency room as well as 4, 12, 24, 48, 120, and 240 hours afterwards, and plasma aliquots were stored at −80°C until analysis.

2.5. Statistical Analysis. All results are given as the mean ± standard deviation (SD). Normal distribution of data was

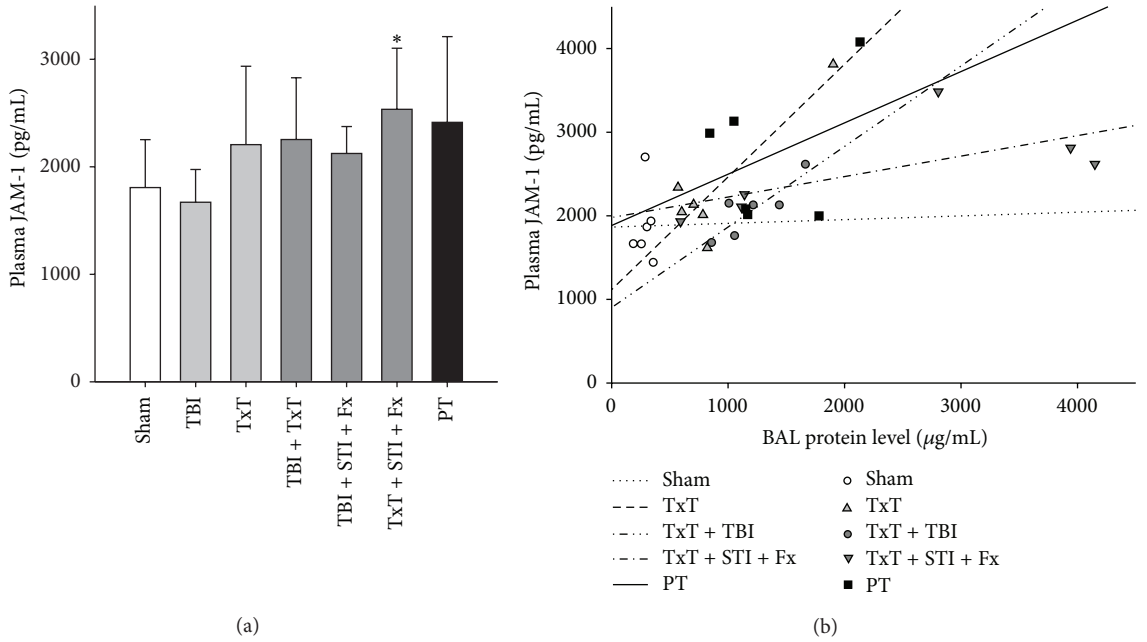

Figure 1: (a) JAM-1 levels in murine plasma 2 h after experimental blunt chest trauma (TxT), closed traumatic brain injury (TBI), femur fracture including contralateral soft tissue injury (STI + Fx), and combination of injuries (PT) (n = 6–9 per group) compared to sham procedure (n = 12). Results are presented as means ± SD; Kruskal-Wallis ANOVA on ranks followed by Dunn's method; $^{*}P \leq 0.05$ versus sham. (b) Correlation analysis was performed between murine JAM-1 plasma levels and protein concentration in bronchoalveolar lavage (BAL) fluids to assess the impairment of the barrier function 2 h after blunt chest trauma (TxT), TxT in combination with traumatic brain injury (TxT + TBI), TxT combined with femur fracture and contralateral soft tissue injury (TxT + STI + Fx), polytrauma (PT), and sham procedure. Pearson correlation coefficient (r) of plasma JAM-1 versus BAL protein: $r = 0.01$ with $P = 0.99$ (sham); $r = 0.89$ with $P = 0.02$ (TxT); $r = 0.86$ with $P = 0.03$ (TxT + TBI); $r = 0.67$ with $P = 0.10$ (TxT + STI + Fx); and $r = 0.36$ with $P = 0.48$ (PT). Symbols show values for individual animals; lines represent linear regression for the indicated groups.

verified using the Kolmogorov-Smirnov test. Data sets were then analyzed using one-way analysis of variance (ANOVA) followed by the Dunnett method as a post hoc test for multiple comparisons. In case of nonparametric distribution, Kruskal-Wallis one-way analysis of variance on ranks followed by Dunn's method was performed. The Pearson method was used to analyze correlations between values. The results were considered as significant with $P \leq 0.05$.

3. Results

3.1. Detection of JAM-1 in Plasma after Experimental Polytrauma: Association with BAL Protein Leakage as Air-Blood-Barrier (ABB) Dysfunction Marker. To determine whether JAM-1 as a key barrier molecule can be detected in the circulation early after tissue trauma, the JAM-1 plasma concentration was investigated in the mice 2 h after different standardized trauma insults. Indeed, there was a slight increase of circulating JAM-1 after thorax trauma alone (TxT) which was markedly increased in combination with an additional soft tissue and fracture trauma (TxT + STI + Fx) or polytrauma (Figure 1(a)). The amount of BAL protein leakage as indicator of an ABB dysfunction was significantly associated with JAM-1 plasma levels among all the animals (Pearson correlation coefficient $r = 0.57$; $P < 0.01$, data not shown). Herein, the blunt chest trauma insult (TxT) alone ($r = 0.89$; $P = 0.02$) or in combination with traumatic brain injury (TxT + TBI) ($r = 0.86$; $P = 0.03$) displayed a significant correlation with JAM-1 release (Figure 1(b)), suggesting that a trauma-induced dysfunction of the alveolocapillary barrier might to some extent be monitored from the plasma JAM-1.

3.2. Local Reduction of JAM-1 in Lung Tissue after Experimental Trauma. Because JAM-1 was increased in plasma mainly after experimental traumata that affected the lung (TxT; TxT + STI + Fx; and PT), we investigated the JAM-1 level in lung tissue homogenates. A slight decrease of JAM-1 in lung homogenates was found 2 h after blunt chest trauma alone (TxT), after an additional fracture and soft tissue injury (TxT + Fx + STI), and after polytrauma compared to sham animals (Figure 2). The combination of thorax trauma with traumatic brain injury (TxT + TBI) led to a significant decrease of JAM-1 in lung homogenates, suggesting that tissue injury leads to signs of ABB disruption as assessed by reduced JAM-1 tight junction protein in traumatized lungs.

3.3. Systemic Detection of JAM-1 in Human Polytrauma. To investigate whether early trauma-induced barrier dysfunction can be monitored *in realiter*, JAM-1 plasma concentrations were determined in patients after severe multiple injuries and compared to healthy controls. There was a significant increase in plasma JAM-1 as early as 4 h after

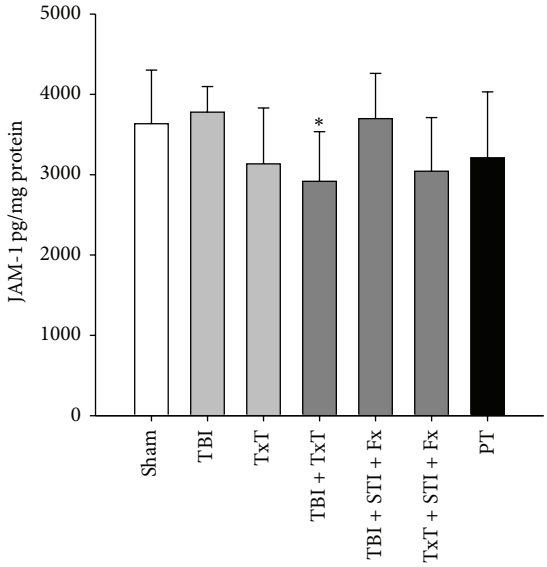

Figure 2: JAM-1 concentration of murine lung tissue homogenates per mg protein 2 h after experimental blunt chest trauma (TxT), closed traumatic brain injury (TBI), femur fracture including contralateral soft tissue injury (STI + Fx), and a combination of injuries (PT). Results are presented as means ± SD with $n = 7$-8 per group; one-way ANOVA/Dunnett's test; $^{*}P \leq 0.05$ versus sham.

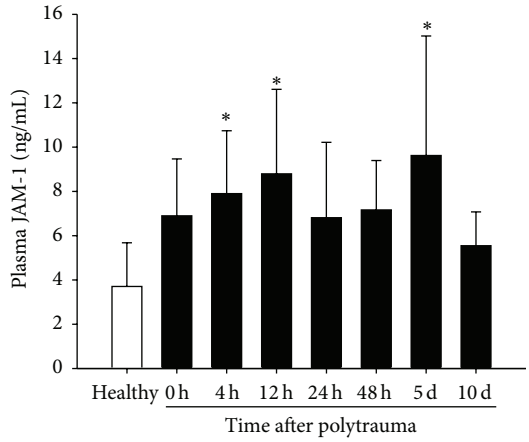

Figure 3: JAM-1 levels in plasma of polytrauma patients at the indicated time points after trauma. Data are presented as means ± SD with $n = 6$–10 per group; one-way ANOVA/Dunnett's test; $^{*}P \leq 0.05$ versus healthy volunteers.

the trauma insult following a biphasic posttraumatic time course with maximal JAM-1 levels at 12 h and 120 h after injury (Figure 3). At 10 d after hospital admission, the JAM-1 concentrations of polytrauma patients had almost returned to control levels.

3.4. Association of Plasma JAM-1 with the Clinical Course Assessed by APACHE II and SOFA Scoring. To determine whether JAM-1 release is associated with the severity of disease and degree of organ dysfunction, correlation analyses between JAM-1 plasma levels of polytrauma patients and established corresponding clinical scores were performed. JAM-1 plasma levels of the patients determined at 0, 24, 48, 120, and 240 h after trauma positively correlated with the "acute physiology and chronic health evaluation II" (APACHE II) score [18] (Figure 4(a)) and with the "sequential organ failure assessment" (SOFA) score [19] (Figure 4(b)). These results indicate that JAM-1 plasma concentration might, to some extent, mirror the severity of organ damage and dysfunction by following a temporal pattern after severe trauma.

4. Discussion

The present study is to our knowledge the first analysis demonstrating a trauma-induced release of tight junction protein JAM-1 into the circulation. We found that plasma JAM-1 increased in response to experimental murine polytrauma and in human polytrauma. In mice, JAM-1 levels were positively associated with BAL protein levels as an early sign of alveolocapillary barrier dysfunction. Furthermore, in patients, plasma JAM-1 correlated with severity of the disease and organ dysfunction as assessed by the APACHE II and SOFA scores, respectively, suggesting that JAM-1 might represent a marker for early trauma-induced barrier dysfunction.

Severe tissue injury causes direct cell and organ injury with disruption of vessels and tissue barriers, leading to transit problems for air, blood, liquor, urine, and feces. Additionally, remote barrier failure with end-organ dysfunction may develop as a result of the systemic danger response after severe tissue trauma. In regard to trauma-induced ABB dysfunction, some clinical data are available. In patients with severe polytrauma (ISS > 40), sequential measurements of BAL fluids revealed signs of an increased alveolocapillary permeability within 6 h after trauma, although none of them suffered a severe lung contusion. The ABB dysfunction was significantly aggravated in those patients who later developed acute respiratory distress syndrome [20, 21]. In our murine trauma model, multiple trauma led to a significant increase of lung myeloperoxidase as a marker for airway inflammation, even in the group that received no chest trauma [17]. Regarding trauma-induced breakdown of the GBB, intestinal-epithelial-cell damage was found to correlate with both the abdominal injury severity (Abbreviated Injury Scale (AIS)) and the general injury severity (determined by ISS), indicative of direct and remote intestinal damage after severe tissue trauma [22]. In a porcine multiple firearm-injury model, multiple trauma caused remote gastrointestinal ischemia and GBB failure with detection of gut-derived endotoxin and diamine oxidase in the portal vein within 6 h even in the absence of a hemorrhagic shock [23].

In the regulation of endogenous blood-organ barrier function, intercellular junctions and particularly tight junctions play a central role [24]. Disruption of the tight-junction structure is a common feature of many evident or hidden inflammatory diseases [24–27], including sepsis [28], shock, and trauma [29, 30]. Several studies suggested that JAM-1 represents a key player in tight junction assembly and regular

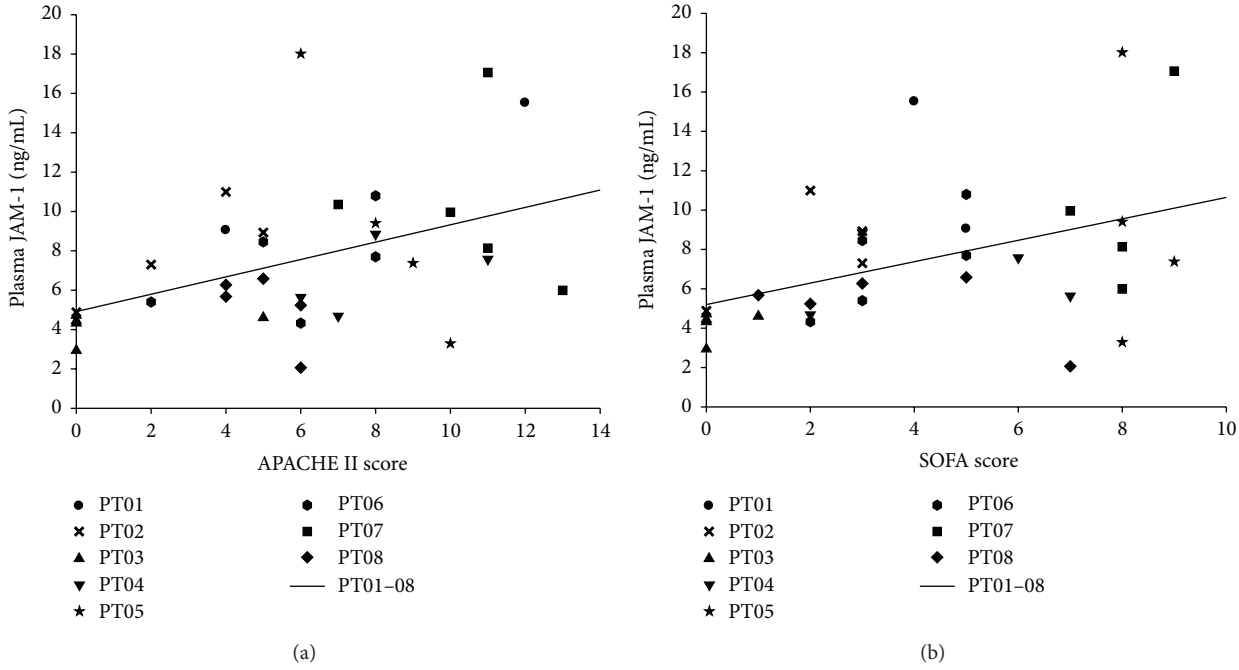

Figure 4: Correlation analysis between JAM-1 plasma levels of polytrauma patients (up to 10 d after trauma) and (a) the APACHE II score (Pearson correlation coefficient $r = 0.50$, $P = 0.01$) and (b) SOFA score (Pearson correlation coefficient $r = 0.37$, $P < 0.04$).

barrier function. In SK-CO15 intestinal cells, downregulation of JAM-1 resulted in increased epithelial permeability [31]. *In vivo*, JAM-1-knockout mice displayed enhanced mucosal permeability as shown by increased dextran passage and decreased transepithelial resistance. Yeung et al. investigated the role of JAM-1 in BBB integrity under inflammatory conditions. In a rat model of cortical cold injury, dual labeling of JAM-1 and fibronectin revealed that only lesioned vessels with BBB breakdown displayed a loss of brain endothelial JAM-1 immune staining [32]. Reduced endothelial JAM-1 immunostaining was also demonstrated in active brain lesions of patients with multiple sclerosis [33]. In primary alveolar epithelial cells, depletion of JAM-1 resulted in increased epithelial permeability as indicated by decreased transepithelial resistance, decreased expression of scaffold tight junction protein zonula occludens 1, and disorder of the structural transmembrane protein claudin 18 [13]. *In vivo*, LPS intraperitoneal injection led to increased susceptibility to pulmonary edema and the reduction or disorder of associated tight-junction proteins in JAM-1-knockout mice, suggesting that JAM-1 is essential for the regulation of tight junction interaction and lung-barrier function [13]. Accordingly, in the present polytrauma model, we found that lung contusion led to an early breakdown of the alveolar barrier as assessed by increased BAL protein, which was associated with a reduction of JAM-1 in lung homogenates and an increase of JAM-1 in the plasma of mice. In the polytrauma patient cohort, 7 out of 8 patients suffered from injuries to the thorax (mean AIS = 3), suggesting that severe organ damage might cause disruption of paracellular tight junctions and the subsequent release of barrier molecules, including JAM-1, into the circulation. In addition to mechanical barrier breakdown, inflammation is considered a pivotal trigger of JAM-1 release. Upon proinflammatory stimulation of human umbilical vein endothelial cells (HUVECs), JAM-1 was shed from the cell surface by a disintegrin and metalloproteinases 10 and 17 [34]. Additionally, JAM-1 shedding was increased in HUVECs 2 h after incubation with stimulated neutrophils. In mice, injection of IFN-γ and TNF-α (both factors are elevated after polytrauma in humans) induced a systemic inflammation, which was associated with increased levels of soluble JAM-1 in the circulation within 2.5 h. Although severe tissue trauma is a well-known progressor of the inflammatory response [2, 35, 36], trauma-induced shedding of JAM-1 is relatively unexplored. In the present mouse model, polytrauma led to a systemic increase of IL-6 [17] and JAM-1, indicating that trauma-induced inflammation might represent a trigger for tight junction release and subsequent barrier dysfunction. Accordingly, in patients, CRP as a clinical inflammatory monitoring marker was significantly elevated starting 12 h after polytrauma (data not shown) as a sign for trauma-induced systemic inflammation. However, whether CRP or IL-6 causally contribute to JAM-1 release after trauma remains to be clarified. Furthermore, JAM-1 plasma levels were associated with disease severity (APACHE II) and organ dysfunction (SOFA), indicating a relationship between the breakdown of paracellular junctions and organ damage.

As a limitation of the study, the present multiple-injured patient cohort was too small for powerful subgroup analysis

to satisfactorily answer further clinical questions, including a theoretical association of JAM-1 concentrations with distinct injury patterns and the development of subsequent complications (e.g., hemorrhagic shock, disseminated intravascular coagulopathy, and septic shock). It has to be clarified whether JAM-1 is shed from the endothelium, leukocytes, platelets, or hematopoietic stem cells. Furthermore, we did not determine whether the released JAM-1 protein represents the full-length molecule capable of redimerizing homophilically or whether we merely detected a cleavage product. Consideration should be given to the fact that JAM-1 shedding might additionally serve as a self-defense mechanism. In this regard, Koenen et al. demonstrated that the shed cleavage form of JAM-1 was able to reduce transendothelial migration of neutrophils *in vitro* and *in vivo*, suggesting that soluble JAM-1 might regulate vascular permeability [34]. Regarding physiological consequences, increased circulating plasma concentrations of shed JAM-1 may reduce migration of leukocytes [34], repopulation of hematopoietic stem cells [7], reendothelialization of injured vessel walls [6], and adhesion of platelets [10]. Thus, elevated JAM-1 plasma levels may stage-dependently reflect beneficial or harmful functional effects during the course of traumatized patients.

For the clinical application, a bedside barrier molecule monitoring would be desirable especially since JAM-1 seems to be different from other markers used in the clinic as it is not considered an inflammatory molecule monitoring the patient's inflammatory state; given its function as a central barrier molecule, blood levels might therefore provide information on the state of the patient's blood-organ barriers and predict their breakdown even before appearance of clinically evident signs of leakage syndrome, such as edema.

The current finding that the barrier molecule JAM-1 is present in plasma early after experimental and clinical polytrauma may help to detect and possibly monitor early barrier and organ dysfunction and, furthermore, the inflammatory response, particularly because it remains unclear whether the barrier dysfunction represents the cause or the consequence of the systemic inflammatory response. The identification of barrier dysfunction markers may aid in the recognition of patients at risk of posttraumatic leakage syndrome and, thereby, improve the clinical management of severely injured patients.

Conflict of Interests

The authors declare that they have no competing interests that might be perceived to influence the results and discussion presented in this paper.

Acknowledgments

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Review Article

Endothelial-Leukocyte Interaction in Severe Malaria: Beyond the Brain

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Malaria is the most important parasitic disease worldwide, accounting for 1 million deaths each year. Severe malaria is a systemic illness characterized by dysfunction of brain tissue and of one or more peripheral organs as lungs and kidney. The most severe and most studied form of malaria is associated with cerebral complications due to capillary congestion and the adhesion of infected erythrocytes, platelets, and leukocytes to brain vasculature. Thus, leukocyte rolling and adhesion in the brain vascular bed during severe malaria is singular and distinct from other models of inflammation. The leukocyte/endothelium interaction and neutrophil accumulation are also observed in the lungs. However, lung interactions differ from brain interactions, likely due to differences in the blood-brain barrier and blood-air barrier tight junction composition of the brain and lung endothelium. Here, we review the importance of endothelial dysfunction and the mechanism of leukocyte/endothelium interaction during severe malaria. Furthermore, we hypothesize a possible use of adjunctive therapies to antimalarial drugs that target the interaction between the leukocytes and the endothelium.

1. Introduction

Malaria is the most important parasitic disease worldwide. It is present in more than 100 countries, putting 1.2 billion people at risk and accounting for more than 800 thousand deaths each year [1, 2]. Cerebral malaria (CM) is the most severe form of malaria and is usually found in children under five years old [3]. Clinically, CM is defined by the identification of *P. falciparum* in peripheral blood, convulsions, and coma, after ruling out any other cause of coma such as meningitis [4, 5]. Pathological findings such as capillary congestion, production of proinflammatory cytokines, and adhesion of infected red blood cells (iRBC) to brain vasculature are responsible for cerebral complications associated with CM [6]. In some patients, a systemic illness called severe malaria (SM) is observed which is characterized by one or more peripheral organ dysfunctions as acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) [7] and acute kidney injury [8, 9] and can be combined with cerebral malaria signals [10]. Some authors suggest that SM is due to pathological events such as parasitized erythrocytes, leukocyte adhesion to the organ microvasculature, systemic production of cytokines, and cytotoxic lymphocyte activation [11, 12]. Despite systemic activation, the leukocyte/endothelial cell interaction differs depending on the studied organ. Here, we discuss endothelial dysfunction during severe malaria and the mechanisms by which leukocytes adhere to the endothelium in distinct organs during this pathology.

2. Leukocyte-Endothelium Interaction during Cerebral Malaria

A main characteristic of brain physiology is the immune privilege conferred by the BBB to brain tissue [13]. However, the BBB composition, especially in the postcapillary venule, allows leukocyte diapedesis during nonmalarial brain injury [14, 15].

During human cerebral malaria, the importance of infected red blood cells adhesion to brain microvasculature is well established [5]. Necropsy of fatal cases of severe malaria shows the adhesion of iRBC in the venules and capillaries, causing congestion [6, 16, 17]. The mechanism of iRBC adhesion to brain microvasculature is well described and depends on expression of membrane proteins such as *P. falciparum* erythrocyte membrane proteins (PfEMP1) [18]. However, the leukocyte-endothelium interaction during human cerebral malaria is not completely clarified [12, 16, 19, 20]. Indeed, it is well established that both endothelium [21] and leukocyte [22, 23] are activated in patients diagnosed with CM; however, how they orchestrate the brain injury to develop CM is still not well understood.

Endothelium activation markers have been used in clinical studies to predict malaria severity [24, 25]. During CM, the endothelium can be activated by different mechanisms as the binding of soluble proteins present in host serum [24], direct contact with iRBC [6], and activation induced by parasite-derived molecules as hemozoin [26] and GPI [27]. Necropsy performed in fatal cases of CM showed increased expression of adhesion molecules on brain microvasculature [28] supporting the idea that the endothelium is able to promote leukocyte adhesion. Some studies show the presence of leukocyte in brain vasculature lumen [16] or in perivascular space [29], although there are no lines of evidence of the importance of leukocyte adhesion to brain vasculature in development of human CM. However, it cannot be ruled out considering the lack of knowledge in this issue [16, 20].

The interaction between leukocytes and endothelial cells during human CM could not depend on cell-cell contact. Instead, leukocytes and lymphocytes produce inflammatory mediators as TNF-α which activate endothelial cells [28, 30]. Endothelial activation induced by TNF-α accounts for many factors involved in development of CM [31] as increased iRBC adhesion [30], expression of leukocyte chemotactic factors [32] and, costimulated by iRBC, increases ICAM-1 expression that improve iRBC adhesion [30].

On the other hand, the adhesion of leukocytes to brain vasculature is often observed during experimental cerebral malaria [33, 34]. A recent report revealed that the majority of leucocytes accumulated in the brain during experimental severe malaria are monocytes. These cells are responsible for the recruitment of $CD4^+$ and $CD8^+$ T cells to the CNS vasculature [35]. However, in the absence of monocytes, T cells are still recruited to the brain to initiate experimental cerebral malaria [35]. Observation of the microvessels within the brains of live animals demonstrated the marginalization of leukocytes and platelets aggregates in postcapillary brain venules but not in capillaries of *P. berghei*-infected mice, showing that leukocytes do not accumulate in brains tissue but induce endothelium dysfunction, leading to vascular leakage, neurological signs, and coma [35, 36]. The role of adhesion molecules, especially ICAM-1, in the leukocyte/endothelium interaction to promote cerebral dysfunction during experimental severe malaria is controversial. The impairment of the ICAM-1/β2-integrin complex abolishes the development of cerebral dysfunction associated with *P. berghei* infection [36–38]. However, Ramos and colleagues deleted ICAM-1 in different cells and showed that only ICAM-1 expressed in leukocytes accounts for experimental severe malaria [39]. The authors speculated that because endothelial cells do not express ICAM-1 counter receptor, leukocytes, platelets, and iRBC aggregates occlude brain microvessels and promote cerebral malaria [39].

A new approach of leukocyte and endothelium interaction in brain during CM has been proposed through interaction between MHC class I molecules and $CD8^+$ T lymphocytes. Recent studies regarding experimental CM show that the membranes of endothelial cells and iRBC fuse by trogocytosis, resulting in the expression of *Plasmodium* antigens [40]. Endothelial cells preferentially phagocytize merozoites and, via proteasome digestion, present plasmodial antigens by MHC class I molecules to $CD8^+$ T lymphocytes, thus contributing to the adaptive immune response to *P. berghei* infection [41]. It is noteworthy that the same results were observed within *P. falciparum* phagocytosis by human endothelial cells [41]. However, *P. falciparum* phagocytosis by endothelial cells *in vivo* and its clinical relevance remain to be elucidated.

Overall, microvascular congestion observed in both human and experimental CM leads to severe cerebral endothelial damage, resulting in the breakdown of the BBB mainly at the level of postcapillary venules [16, 29, 31, 42]. The postcapillary venule BBB (Figure 1) is functionally distinct from other BBB areas and is in direct contact with the perivascular space [42]. In light of the new findings concerning brain anatomy in which the authors described the presence of lymphatic vessels in direct contact with the perivascular space in the central nervous system, in the next few years, the dynamics of the interaction between leukocytes and the endothelium during cerebral malaria will likely be unveiled [43].

3. Leukocyte-Endothelium Interaction in the Lung during Malaria

The brain is not the only organ affected during severe malaria. Twenty percent of patients diagnosed with severe malaria develop acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) [7, 9]. ALI/ARDS is a syndrome derived from pathological conditions such as sepsis and traumatic brain injury. ALI/ARDS diagnosis includes the identification of respiratory failure, diffuse alveolar damage, and inflammatory infiltration in lung tissue [44]. Necropsy in fatal cases of severe malaria revealed that patients present classical symptoms of ALI, including pulmonary edema, pulmonary capillary congestion, thickened alveolar septa, marked inflammatory response in lung tissue, and macrophages in the lumen of the pulmonary capillaries [11]. Murine experimental models of severe malaria also present pulmonary pathology such as edema, cell infiltration, tissue damage, and lung mechanical impairment [45–48]. Furthermore, the lung appears to be a large reservoir of metabolically active parasites, as described in an elegant study by Lovegrove et al. who evaluated the transcriptional responses to *Plasmodium* in different organs [49].

The lung vasculature in malaria infection is essential to initiate the *Plasmodium* cycle within the host. When merozoites leave the liver, they are located inside host-derived

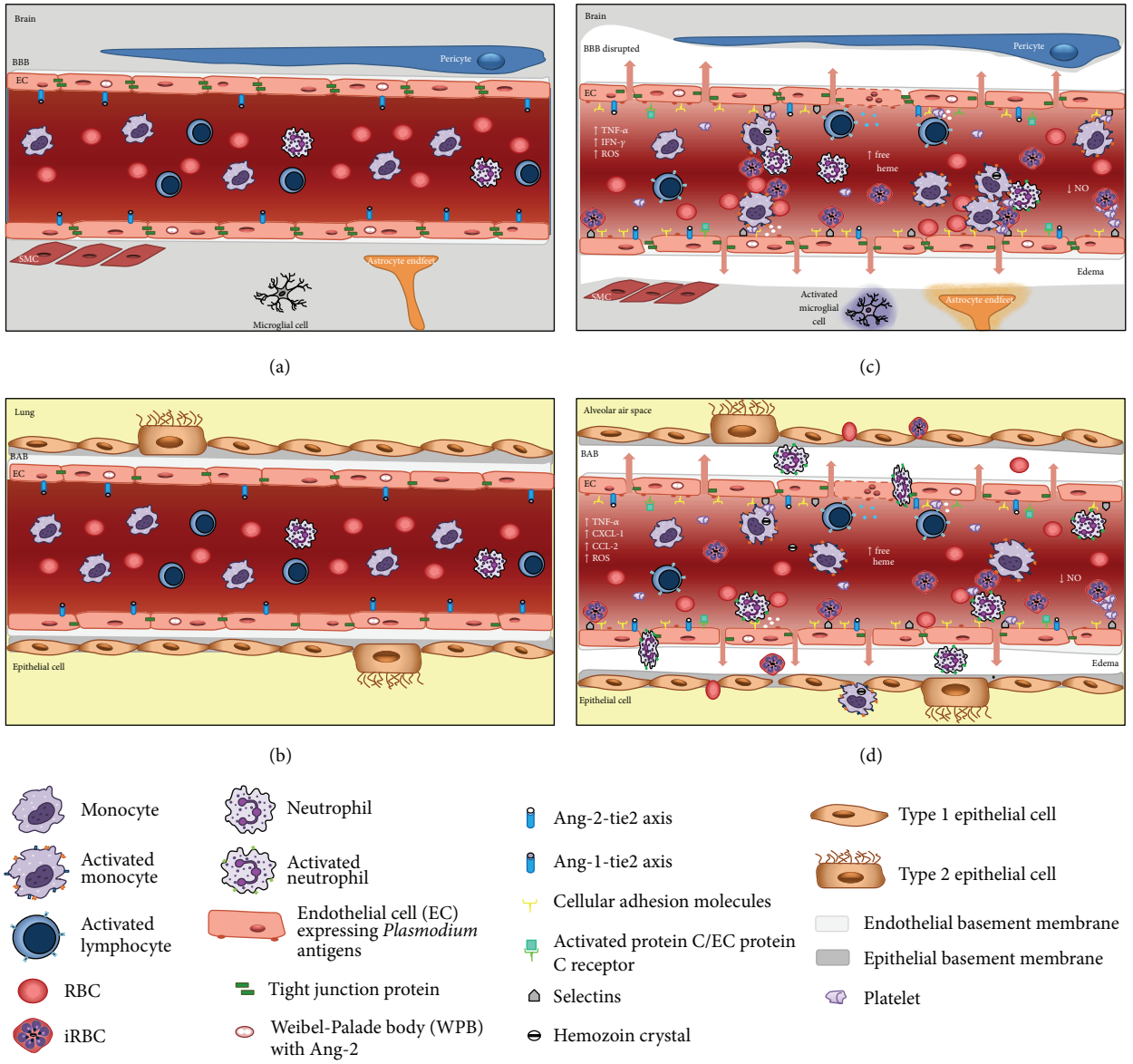

Figure 1: Blood barrier differences between brain and lung during malaria. (a) Cerebral microvasculature and (b) lung microvasculature without leucocytes attached in postcapillary venules and EC expressing Ang-1, under physiological conditions. (c) During severe malaria, we observe production of proinflammatory cytokines, increase of cellular adhesion molecules expression, release of Ang-2, decrease of NO, and adhesion of iRBC and leukocytes (mainly mononuclear cells) to brain vasculature leading to capillary congestion, BBB dysfunction, and edema. Such events activate the subjacent tissue (microglial cells and astrocytes). (d) Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) associated with malaria. The augment of inflammatory cytokines and chemokines, release of Ang-2, and decrease of NO are responsible for activation of EC that increases the expression of cellular adhesion molecules allowing the margination and infiltration of iRBC, leucocytes, and platelets into blood vessels, interstitial tissue, and consequently alveolar air space. BBB: blood-brain barrier; BAB: blood-air barrier; EC: endothelial cell; ROS: reactive oxygen species; SMC: smooth muscle cell.

buds named merosomes, whose membranes are disrupted within the pulmonary capillary beds to allow merozoites to reach the erythrocytes [50, 51]. The close contact between infected erythrocytes and pulmonary endothelial cells triggers a remarkable inflammatory response 24 h after infection, characterized by intense inflammatory cell infiltration as well as the production of proinflammatory cytokines and chemokines in lung tissue that persists for at least five days after infection [45–47]. The quantity of parasites in lung tissue defines the extent of chemokine production in lung tissue [52]. Chemokines such as CCL2, CXCL1, and CCL5 are produced in lungs during experimental malaria and are correlated with macrophage and neutrophil accumulation in pulmonary tissue [45, 53, 54]. Intravital studies in lungs of *Plasmodium*-infected mice reveled edema formation and the migration of monocytes and neutrophils to lung tissue [37]. However, due to the technical limitations in studying leukocyte mobility within the lung [55], until now, there have

been no available data on the dynamics of leukocytes and lung endothelium during malaria-triggered ALI [37]. Indeed, lung endothelial cells are activated during malaria infection and express adhesion molecules. P-selectin, in addition to L- and E-selectin, is part of a family of calcium-dependent (C-type) lectins whose activation induces the expression of β2-integrins and consequent leukocyte arrest in the vasculature [56, 57]. P-selectin is expressed in both lung and brain endothelium during experimental malaria. This molecule mediates leukocyte rolling in brain microvessels of *P. berghei*-infected mice; however, it is not essential for development of experimental cerebral malaria signals [58]. On the other hand, the monocyte/macrophage accumulation in lungs of *P. berghei*-infected mice depends on the expression of ICAM-1 [52, 59], while ICAM-1 expression in the brains of infected mice does not account for leukocyte adhesion [60]. It is interesting to note that while inflammatory cell infiltration in cerebral tissue was not observed in the brain, neutrophil and macrophage infiltration is frequently observed in pulmonary interstitial lung tissue during malaria [45]. Indeed, differences in the blood-brain barrier and blood-air barrier tight junction constitution of the brain and lung are responsible for this phenomenon (Figure 1).

The morphological and biochemical differences between lung and brain endothelial cells account for the distinct inflammatory responses in both organs. Despite both endothelial cell types containing nonfenestrated endothelium, brain endothelial cells present fewer caveolae and are richer in tight junctions than lung endothelium [61, 62]. The lung endothelial bed is rich in adherens junctions and P-selectins and allows leukocyte transmigration by paracellular and transcellular pathways [61, 62]. Endothelial cells from lung tissue can be activated by VEGF [4], TNF-α [63], LPS [64], and *P. falciparum* infected erythrocytes, resulting in the reorganization of their junctional proteins [63]. In addition to inflammatory mediators and pathogen-associated molecular pattern (PAMP), the leukocyte contact also contributes to endothelial cell reorganization, triggering a dephosphorylation cascade followed by the endocytosis of VE-cadherins, which support leukocyte transmigration through lung endothelial cells [65]. Of note, in most organs, leukocyte transmigration happens almost exclusively in post-capillary venules. However, in the lung leukocyte transmigration occurs in capillaries of the blood-air barrier which are surrounded by epithelium forming alveoli [61].

In addition to the direct interaction between leukocytes and the endothelium described earlier, leukocytes can bind platelets and then adhere to the endothelium. Piguet and colleagues showed that platelet and mononuclear cell trapping occurs in the lungs of *P. berghei*-infected mice [66]. In addition, the authors observed that the impairment of platelet activation decreased leukocyte adhesion to the lung vasculature of *P. berghei*-infected mice [66]. The stimulation of the receptor $P2Y_1$ but not $P2Y_{12}$ on platelets induces the downstream activation of the RhoA pathway, resulting in platelet/leukocyte aggregation and migration to the lung [67]. In addition, platelets also contribute to the leukocyte/endothelium interaction by releasing microparticles. Neutrophils stimulated with platelet-derived microparticles increased the expression of αM integrin and adhered to pulmonary endothelial cells via ICAM-1 [68].

The study of leukocyte/endothelium interactions within the lung during malaria is limited but extremely important. Mice depleted of neutrophils showed reduced malaria associated ALI and delayed mortality [38], suggesting that further studies are necessary to show the mechanism of the leukocyte/endothelial interaction in the lung during severe malaria.

4. Leukocyte/Endothelium Interaction during Malaria as a Target for Treatment

In accordance with the findings presented above both in human and animals, the leukocyte/endothelium interaction plays a role in the development of pathogenesis of severe malaria particularly in malaria-induced ALI [9, 12, 45, 47]. In fact, lung dysfunction triggered in both human and experimental malaria shares similarities with lung mechanics impairment, pulmonary edema, production of inflammatory cytokines, and inflammatory cells infiltration in lung tissue [9, 45]. Furthermore, the inflammatory response persists even after the host is cured of infection [10, 69, 70] (unpublished data), which suggests that modulation of inflammatory response in addition to antimalarial therapy would be helpful to patient outcome [71]. The leukocyte-endothelium interaction is not the most important factor regarding development of human cerebral malaria pathogenesis; however, it should not be neglected as actor in severe malaria-induced organ dysfunction.

Recently, Frosch and John suggested that an adjunctive therapy that impaired the inflammatory response induced during malaria should be combined with antimalarial drugs [72]. Several approaches have already aimed at the modulation of the malaria-induced inflammatory response. Figure 2 illustrates several potential targets described in the literature. Patients diagnosed with severe malaria have been treated with modulators of TNF-α production [73], CD36 expression [74, 75], NO precursors [70, 76], or adhesion of iRBC to vasculature [77] and presented decreased inflammation scores when compared to a placebo treated group. Despite evidence suggesting that the modulation of leukocyte and endothelial activation supports the outcome of severe malaria, it is not clear whether an adjunctive therapy targeting the leukocyte/endothelium interaction would predict patient outcome. It is worth noting that the most important class of antimalarial drug to treat severe malaria is artemisinin and its derivatives [78], which also have immunomodulatory activities in pathologies such as microbial infections, tumor growth, and inflammatory diseases [79–82]. Our group demonstrated that, in addition to its antimalarial properties, artesunate exerted a protective effect against severe malaria via its immunomodulatory properties by inhibiting endothelial cell activation, NF-κB nuclear translocation, and the subsequent expression of ICAM-1 [83].

Srinivas and colleagues studied the effect of treatment with activated protein C on a patient with severe malaria coinfected with leptospirosis and observed a rapid outcome

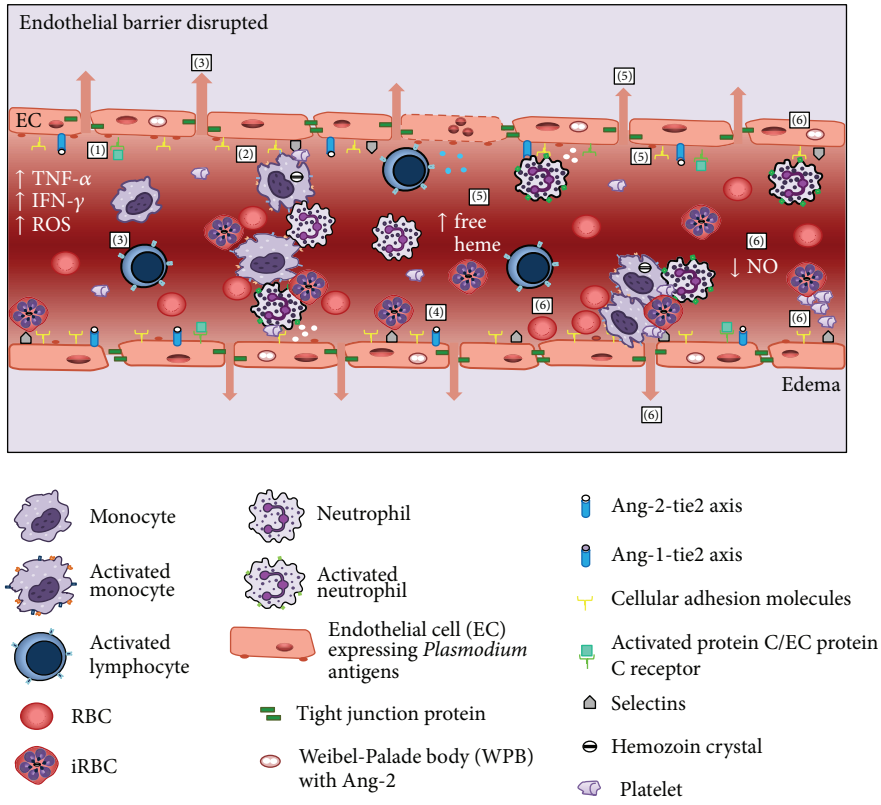

FIGURE 2: Targets of adjuvant therapies during malaria. Scheme showing several approaches that have been investigated aiming at modulation of malaria-induced inflammatory response. EC: endothelial cell; ROS: reactive oxygen species; SMC: smooth muscle cell. (1) *Activated protein C* binds to protein C receptor in activated EC cells decreasing the expression of adhesion molecules. (2) *Statins* decrease the production of chemokine and diminished the adhesion of leukocytes to brain microvasculature. (3) *Sphingosine-1-phosphate (S1P)* decreases the numbers of lymphocytes in brain vasculature and stabilizes the tight junction protein ZO-1 in brains. (4) *Neuregulin-1* and *bone marrow mesenchymal stromal cells* induce Ang-1, which promotes stabilization of EC tight junctions, EC desensitization to TNF-α, and downregulation of ICAM-1 and VCAM-1. (5) *Lipoxin* A_4 decreases production of proinflammatory cytokines, impairs EC activation, and inhibits the expression of cellular adhesion molecules involved in leukocyte adhesion by stimulating the activity of HO-1, which catabolizes free heme. (6) *L-Arginine* or *inhaled NO (iNO)* reduces pulmonary edema and, in addition, decreases cytoadherence of iRBC, hemorrhagic foci, and leukocyte and platelets adherence to brain vasculature by inhibiting of WPB exocytosis that impairs the release of Ang-2 and inhibiting TNF-α production and procoagulant activity of endothelial cells.

[84]. The binding of activated protein C to endothelial cell protein C receptor in activated endothelial cells avoided NF-κB p65 phosphorylation and induced AKT signaling, which decreased the expression of adhesion molecules on the endothelial cell surface [18, 85]. Thus, activated protein C in malaria would increase endothelial barrier integrity, induce antiapoptotic pathways, and decrease adhesion molecule expression [86]. Other modulators of endothelial functions have been used to evaluate malaria outcome in humans and experimental models. Studies in which *P. berghei*-infected mice were treated with statins, a class of drugs that inhibit the rate-limiting step in cholesterol synthesis and that show pleiotropic effects, demonstrated that statins decreased the production of chemokines [87] and decreased the adhesion of leukocytes to the brain microvasculature [88] probably by inhibiting the binding site of LFA-1 on leukocytes [89]. Accordingly, *in vitro* treatment of human endothelial cells with statins followed by stimulation with *P. falciparum*-infected erythrocytes decreased the expression of adhesion molecules, suggesting that statins could exert an antiadhesive role in the treatment of severe malaria [90]. Statins have not been tested in clinical trials for malaria adjunctive treatment. However, statins diminished the risk of sepsis-related mortality in patients, probably by decreasing the inflammatory response triggered during sepsis [91].

The endothelial barrier stabilizer sphingosine-1-phosphate (S1P) also rescued mice from severe malaria by decreasing the numbers of $CD8^+$, $CD4^+$, and $CD45^+$ cells in the brain vasculature of *P. berghei*-infected mice, likely decreasing ICAM-1 expression and stabilizing the tight junction protein ZO-1 in brains [36, 92]. Transfection of bone marrow mesenchymal stromal cells and administration of S1P and other endothelial barrier stabilizers such as neuregulin-1 induce the endogenous Ang-1 anti-inflammatory pathway, which promotes decreased vascular permeability by stabilizing endothelial cell tight junctions, endothelial cell desensitization to TNF-α, and downregulating ICAM-1 and VCAM-1. These Ang-1 actions result in decreased leukocyte/endothelial interaction and, consequently, host outcome [4, 93–97].

Another family of lipids has been studied for its anti-inflammatory activity during severe malaria. Lipoxins (LX) are products of arachidonic acid metabolism and are produced through sequential lipoxygenase activity following cell-cell interactions in the inflammatory milieu (reviewed by [98]). The interaction of LXA_4 and its receptor ALX has anti-inflammatory and proresolving activity in inflammatory models such as allergic airway inflammation [99] and autoimmune diseases [100] by reducing leukocyte adhesion to endothelial cells [101]. The administration of LXA_4 improved survival in *P. berghei*-infected mice by decreasing the production of proinflammatory cytokines but not the accumulation of $CD8^+$/IFN-γ^+ cells in brain tissue [102]. In addition to LXA_4 impairment of leukocyte activation, the mechanism of action of LXA_4 on endothelium during severe malaria was recently disclosed by intravital studies of the microvasculature of *P. berghei*-infected mice. The authors showed that treatment with LXA_4 did not modulate leukocyte adhesion to the brain vasculature or decrease the expression of β2-integrin in leukocytes (unpublished data). On the other hand, treatment with LXA_4 impaired endothelial activation during severe malaria and restored the blood flow in brains of *P. berghei*-infected mice [33]. The authors also showed that LXA_4 exerted its effects by stimulating the activity of heme oxygenase 1 (HO-1), an isoenzyme that catabolizes free heme released under pathological conditions, especially in pathologies such as malaria which are associated with intravascular hemolysis [33]. HO-1 upregulation helps maintain BBB integrity under pathological conditions [103]. During the inflammatory response, HO-1 inhibits the expression of several adhesion molecules involved in leukocyte adhesion to endothelial cells [104, 105]. During experimental severe malaria, HO-1 is differentially regulated in certain tissues at different stages of *Plasmodium* life cycle [106, 107]. Furthermore, HO-1 production in brain tissue is associated with mouse survival, decreased cerebral edema, and decreased leukocyte adhesion to brain vasculature [106].

In hemolytic disorders such as malaria, low bioavailability of NO is observed, as free hemoglobin is a potent scavenger of this gaseous molecule [108]. Therefore, the administration of L-arginine or inhaled NO (iNO) has also been tested as adjunctive therapy in the treatment of severe malaria [4, 76]. Yeo and collaborators showed that impaired endothelial NO production occurred in severe malaria in both children and adults, supporting the idea that further trials of drugs that led to increased endothelial NO bioavailability could attenuate severe malaria symptoms [109]. Studies in which severe malaria patients were treated with inhaled nitric oxide demonstrated that NO reduced pulmonary edema in patients with malaria-derived ALI and decreased pulmonary capillary pressure through selective vasodilatory effects on postcapillary venules [110]. Thus, in severe malaria, nitric oxide is hypothesized to promote vascular quiescence, decrease cytoadherence of parasitized erythrocytes to the microvascular endothelium as a critical mediator of VEGF and Ang-1, and dampen inflammatory responses and thrombosis [4]. Nitric oxide (NO) is a short-lived free radical formed from L-arginine conversion that is involved in many important biological functions including neurotransmission, immune system, cytokine modulation platelet inhibition, vascular homeostasis, and regulation of hematopoiesis [111]. Its production occurs through three different NO synthase (NOS) enzyme isoforms: neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2), and endothelial NOS (eNOS or NOS3) [111]. The constitutive isoforms (neuronal and endothelial) are calcium/calmodulin dependent and permanently active, generating low concentrations of NO. The inducible isoform (iNOS) is only expressed when its transcription is activated by a variety of cytokines, growth factors, and inflammatory stimuli on target cells, leading to the release of high levels of NO [112]. In experimental severe malaria, treatment with exogenous NO (NO donor dipropylenetriamine NONOate, DPTA-NO) showed improved pial blood flow, diminished hemorrhagic foci, and reduced leukocyte and platelet adherence to the brain vasculature [113, 114]. The authors hypothesize that NO attenuates malaria symptoms by (a) inhibition of Weibel-Palade body exocytosis and the consequent release of Ang-2 and increase in Ang-1 expression; (b) decreasing the endothelial expression of ICAM-1 and VCAM-1; (c) inhibiting TNF-α production; (d) inhibiting the procoagulant activity of endothelial cells; and (e) decreasing intravascular platelet aggregation [108, 110, 111].

Indeed, pathophysiological phenomena experienced during experimental severe malaria are not fully translated to human severe malaria [115–118]. Therefore, further studies should be performed before initiating clinical studies of immunomodulatory drugs as adjunctive therapy for severe malaria.

Disclosure

Tatiana Pádua is a student of the Graduate Program in Cellular and Molecular Biology from Oswaldo Cruz Institute, Fiocruz, Rio de Janeiro, Brazil.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Cardiac-Restricted IGF-1Ea Overexpression Reduces the Early Accumulation of Inflammatory Myeloid Cells and Mediates Expression of Extracellular Matrix Remodelling Genes after Myocardial Infarction

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Strategies to limit damage and improve repair after myocardial infarct remain a major therapeutic goal in cardiology. Our previous studies have shown that constitutive expression of a locally acting insulin-like growth factor-1 Ea (IGF-1Ea) propeptide promotes functional restoration after cardiac injury associated with decreased scar formation. In the current study, we investigated the underlying molecular and cellular mechanisms behind the enhanced functional recovery. We observed improved cardiac function in mice overexpressing cardiac-specific IGF-1Ea as early as day 7 after myocardial infarction. Analysis of gene transcription revealed that supplemental IGF-1Ea regulated expression of key metalloproteinases (MMP-2 and MMP-9), their inhibitors (TIMP-1 and TIMP-2), and collagen types (Col 1α1 and Col 1α3) in the first week after injury. Infiltration of inflammatory cells, which direct the remodelling process, was also altered; in particular there was a notable reduction in inflammatory Ly6C+ monocytes at day 3 and an increase in anti-inflammatory CD206+ macrophages at day 7. Taken together, these results indicate that the IGF-1Ea transgene shifts the balance of innate immune cell populations early after infarction, favouring a reduction in inflammatory myeloid cells. This correlates with reduced extracellular matrix remodelling and changes in collagen composition that may confer enhanced scar elasticity and improved cardiac function.

1. Introduction

Cardiovascular diseases (CVD) are the major cause of death globally, with myocardial infarction (MI) being one of the main causes of mortality [1]. After MI, the damaged myocardium releases inflammatory signals that trigger a cascade of cellular processes in order to repair damaged tissue, leading to the formation of scar tissue and left ventricular (LV) dysfunction [2, 3].

Our laboratory has characterised the therapeutic properties of the insulin-like growth factor-1 Ea (IGF-1Ea) propeptide during wound healing/regeneration and pathological inflammation. The *IGF-1* gene is encoded in 70 kb of genomic DNA distributed over six exons and five introns [4, 5]. Use of alternative start codons generates proteins with N-terminal variability while different exon use at the 3′ end generates multiple C-terminal extension-peptides, termed E-peptides. The most predominant is a 35-amino-acid-long E-peptide, termed Ea, alternating with a far less abundant E-peptide termed Eb or mechanogrowth factor (MGF) [6, 7]. The E-peptides control local IGF-1 bioavailability by adhering strongly to the extracellular matrix (ECM), retaining the propeptides locally and preventing their release into the circulation [8]. Expressed as a cardiomyocyte-specific transgene

or delivered locally to the mouse heart, IGF-1Ea improves functional recovery after cardiac injury [9, 10]; however the underlying mechanisms are not fully understood.

Tissue restructuring after infarction involves the breakdown of the ECM by proteolytic enzymes, mainly the matrix metalloproteinases (MMP) MMP-2 and MMP-9, balanced by interaction with tissue inhibitors of metalloproteinases (TIMPs) [11]. Initially a temporary matrix is formed, primarily composed of collagen type III (Col Iα3), providing a scaffold for replacement cells and structural integrity to the heart, thereby reducing the risk of LV dilation and rupture [12, 13]. This is later replaced by collagen type I (Col Iα1) which will constitute the permanent ECM [14]. Col Iα1 confers tensile strength and resistance to stretch and deformation, while Col Iα3 confers compliance. Their balance determines cardiac tissue stiffness with increased Col Iα3 to Col Iα1 ratio generating a more resilient left ventricle [13, 14].

Innate immune cells recruited to the injured myocardium from the blood include neutrophils, monocytes, macrophages, and dendritic cells [15–17]. They play a prominent role in remodelling, producing the MMPs that break down the matrix, synthesising new ECM components, and activating fibroblasts to myofibroblasts which will later in the inflammatory process be the main producers of matrix proteins [14]. The tissue microenvironment at a given time after MI influences the recruitment of immune cells as well as their phenotypic and functional properties. This is especially relevant for the macrophage population which undergoes a time-dependent shift between inflammatory and reparative functions [3, 18]. At early time points after injury, the majority of macrophages produce inflammatory cytokines and reactive oxygen species including interleukin- (IL-) 1β, IL-12, MMP-9, and nitric oxide. These are termed inflammatory or M1-polarised macrophages, which express high levels of Ly6C and function to recruit more inflammatory cells and phagocytose cellular debris and produce growth factors [12]. As the inflammation progresses, reparative macrophages accumulate in the infarct area. In contrast to the inflammatory macrophages, these cells, many of which express CD206, are involved in the suppression of inflammation due to high production of IL-10 and TGF-β [3, 19] and assist in the progression from inflammation to repair.

They also perform reparative roles promoting cell growth, angiogenesis, and remodelling of the ECM. Additionally, different monocyte populations can be distinguished by Ly6C in the mouse and may preferentially give rise to inflammatory versus reparative macrophages.

We have previously shown that IGF-1Ea and its mature circulating form IGF-1 can modulate immune responses and suppress pathological inflammation by inducing regulatory cytokines and immune cell types [20, 21]. In the heart, IGF-1Ea increased expression of IL-10 after cardiotoxin injury and decreased levels of IL-1β suggesting that a shift in immune cell populations may also occur in the heart [9]. In the present study, we investigated whether there was a difference in the immune cell dynamics after MI in transgenic IGF-1Ea hearts and whether this had a carry-on effect on tissue remodelling.

2. Materials and Methods

2.1. Cardiac Injury Model. Myocardial infarction by permanent left coronary artery occlusion was induced in wild-type (WT) and αMHC.IGF-1Ea male mice which were 8 to 12 weeks old. Surgeries were performed under mechanical ventilation with 1–2.5% isofluorane. The chest cavity was opened through the left fourth intercostal space. The heart was exposed and the left coronary artery was ligated using an 8.0 mm non-absorbable suture (Ethicon-Johnson & Johnson, USA) below the left atrium to produce an ischemic region of 20–30% of the left ventricle area. The chest cavity and skin were sutured with 6.0 mm silk sutures (Ethicon-Johnson & Johnson, USA). Analgesic treatment with buprenorphine (0.3 mg/kg, s.c.) was provided before and after surgery. They were housed in individually ventilated cages in temperature-controlled facilities on a 12-hour light/dark cycle on standard diet. All mouse procedures were approved by the Imperial College London Ethical Committee and were in accordance with national and international regulations (UK Home Office Project license 70/7589).

2.2. Echocardiography. Echocardiographic measurements were taken using a high-frequency ultrasound system Vevo 770 (VisualSonics, Inc., Canada) with a 30 MHz linear transducer and recorded images were analyzed by using the Vevo 770 workstation software. Mice were anesthetised with 1-2% isofluorane, and the anesthetic flow rate was adjusted to maintain heart rate of approximately 450 ± 50 beats per minute. Furthermore, warmed ultrasound gel and a heating platform were used to maintain body temperature at 37 ± 0.5°C to minimise variation between mice. This analysis was performed at basal level, 1, 3, 4, 7, and 28 days after MI to evaluate left ventricle cardiac function, chamber dimensions, and infarct size.

2.3. Masson's Trichrome Staining. Samples were fixed in paraformaldehyde (4% in PBS) for 48 h, washed in PBS, dehydrated, and embedded in paraffin wax. Five-micron-thick sections were stained with Celestine Blue for 5 minutes, washed in tap water, and then incubated in haematoxylin for 5 minutes. Slides were then incubated with Acid Fuchsin for 5 minutes, rinsed in distilled water, incubated in phosphomolybdic acid (1%), and then rinsed in distilled water before staining with Methyl Blue for 2-3 minutes. Slides were dehydrated in ascending concentrations of ethanol, cleared in xylene, and mounted in DPX (VWR, UK).

2.4. Cell Isolation. To analyse neutrophils, monocytes, macrophages, and dendritic cells, a single cell suspension was prepared from hearts before or at various time points after MI (days 1, 3, 5, 7, and 28 after operation). The hearts were mechanically dissociated using surgical scissors and subsequently treated with a 1x Hank's Balanced Salt Solution (HBSS) (Invitrogen, USA), enzymatic dissociation buffer containing 0.1 mg/mL Liberase TH Research Grade (Roche Diagnostics, UK), 50 μg/mL of DNaseI (Roche Diagnostics, UK), 10 mM HEPES (Invitrogen, USA), and 30 mM Taurine (Sigma, UK) for 4 cycles of 10 min at 37°C. After each 10 min

incubation cycle, the cells were collected and filtered using a 70 μm cell strainer (BD Pharmingen, USA) and an equal volume of ice cold 1x HBSS containing 10 mM HEPES, 30 mM Taurine (Sigma, UK), and 20% Fetal Bovine Serum (FBS) (GE Healthcare, USA) was added to the enzyme dissociation buffer. The cells were pelleted at 320 g for 7 min at 4°C and washed with the 1x HBSS media solution containing 20% FBS as described above. Under these isolation conditions, adult cardiomyocytes are predominately lysed, as the enzymatic dissociation buffer is toxic to these large, fragile cells [22]. An aliquot of the cell suspension was used to quantify the cell concentration/mL using a Beckman Coulter Vi-Cell XR cell counter (Beckman Coulter, High Wycombe, UK).

2.5. Flow Cytometry and Cell Sorting. Isolated cells were incubated in a 1x Dulbecco's Modified Eagle Medium (DMEM) solution (Gibco, Life Technologies, UK) containing 2% FBS and 10 mM HEPES for 30 min, on ice, in the dark, with the following primary antibodies: CD11b-PE (BD Biosciences; catalogue 553311), F4-80-Biotin (eBioscience; catalogue 13-4801-85), CD45-APC-Cy7 (BioLegend; catalogue 103116), CD206-PerCP-Cy5.5 (BioLegend; catalogue 141716), Ly6C-APC (eBioscience; catalogue 17-5932-82), and Ly6G-AlexaFluor700 (BioLegend; catalogue 127622). Samples stained with biotin-labelled primary antibody were incubated with a streptavidin-PE-Cy7 (eBioscience; catalogue 25-4317-82) secondary antibody for 30 min, on ice, in the dark, with the 1x DMEM media solution as mentioned above. The samples were washed and resuspended in fresh 1x DMEM media solution with 1.5 μM Sytox Blue (Invitrogen. USA) dead cell stain and refiltered using 5 mL, 35 μm filter cap tubes (BD Falcon) just prior to sample acquisition. Flow cytometric cell sorting was performed using a BD FACSAriaI cell sorter (BD Biosciences, Oxford, UK) equipped with a 355 nm UV laser, a 405 nm Violet laser, a 488 nm Blue laser, a 561 nm Yellow-Green laser, and a 640 nm Red laser. The antibody cocktail fluorescence minus one (FMO) controls were used as gating controls for analyses to distinguish positive from negative fluorescence signal (see Supplementary 4–6 in Supplementary Material available online at http://dx.doi.org/10.1155/2015/484357). Total leukocytes (CD45+), neutrophils (CD45+, CD11b+, F4/80−, CD11c−, and Ly6G+), and monocytes (CD45+, CD11b+, CD11c−, Ly6G−, and F4/80−) were analysed. Monocytes were further classified as $Ly6C^{high}$ and $Ly6C^{low}$ monocytes. Macrophages were defined as CD45+, CD11b+, CD11c−, Ly6G−, and F4/80+ and further characterised on the basis of Ly6C and CD206 expression (i.e., $Ly6C^{high}/CD206^{low}$ (inflammatory macrophages) and $Ly6C^{low}/CD206^{high}$ (reparative macrophages)). Dendritic cells were defined as CD45+, CD11b+, F4/80−, CD11c+, and Ly6G−. Flow Jo software (version 9. 8.5) (Tree Star, Ashland, OR) was used for analysis.

2.6. RNA Isolation and cDNA Generation. Mouse heart tissue was harvested and flushed with cold PBS. Samples from the infarct and remote (non-infarct) myocardium were placed in a 1.5 mL tube and homogenised in TRIzol (Invitrogen, USA) reagent using a rotor-stator homogeniser (Polytron PT 2500 E). Total RNA was isolated according to the TRIzol manufacturer's instructions. RNA was pelleted, air-dried, and resuspended in DNase/RNase free water and the yield quantified using Nanodrop (Thermo Scientific, USA) at 260 nm. One microgram of RNA was reverse-transcribed into cDNA using the Quantitect Reverse Transcription Kit (QIAGEN, Crawley, UK).

2.7. Quantitative Real-Time PCR. Following cDNA synthesis, quantitative RT-PCR using TaqMan probes (Invitrogen. USA) was performed on an ABI 7900HT Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA). The probes used were IGF-1Ea (Mm00710307_m1), IL-10 (Mm00439614_m1), IL-1β (Mm00434228_m1), CCL2 (Mm00441242_m1), CCL5 (Mm01302427_m1), TGFβ (Mm03024053_m1), collagen Iα1 (Mm00801666_g1), collagen Iα3 (Mm01254476_m1), Lox (Mm00495386_m1), MMP2 (Mm00439498_m1), MMP9 (Mm00442991_m1), TIMP1 (Mm00441818_m1), TIMP2 (Mm004418225_m1), actin, alpha 1, skeletal muscle, ACTA, (Mm00808218_g1), Atrial Natriuretic Peptide, and ANP (Mm01255747_g1). Gene expression was determined as fold induction over uninjured hearts after normalising to the reference gene, GAPDH.

2.8. Statistics. Data are presented as mean ± SEM. Two-tailed Student's t-test was performed to compare WT and αMHC.IGF-1Ea mice at selected time points after MI. Data were analysed with GraphPad-Prism 5.0 (Graphpad Software, Inc., www.graphpad.com), and differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. IGF-1Ea Improves Cardiac Function after Myocardial Infarction. Endogenous IGF-1Ea expression in WT hearts was measured 1, 3, 5, 7, and 28 days after MI in both ischemic and remote (nonischemic) regions. IGF-1Ea levels increased in both the ischemic and remote regions (Figure 1(a)) with a substantially stronger induction in the ischemic area (8-fold over uninjured levels). These results indicate that, similar to other organs, endogenous IGF1-Ea expression is upregulated after cardiac tissue damage [23, 24]. In αMHC.IGF-1Ea hearts, the baseline expression of transgenic IGF-1Ea was much higher than the expression of endogenous IGF-1Ea in WT hearts (average 286-fold) at all experimental time points (Supplementary 1A and 1B). Although far exceeding the expression of endogenous IGF-1Ea, the αMHC.IGF-1Ea transgenic mice provide a suitable model of IGF-1Ea at supraphysiological concentrations of possible therapeutic relevance.

Previously our group showed an improvement in cardiac function in αMHC.IGF-1Ea compared to WT mice one month after MI [25]. To pinpoint the start of functional improvement we extended this analysis and performed echocardiography before and 1, 3, 5, 7, and 28 days after MI (Supplementary Table 1). One day following MI, both groups displayed a reduction in ejection fraction (EF); however, αMHC.IGF-1Ea hearts showed significant improvement in

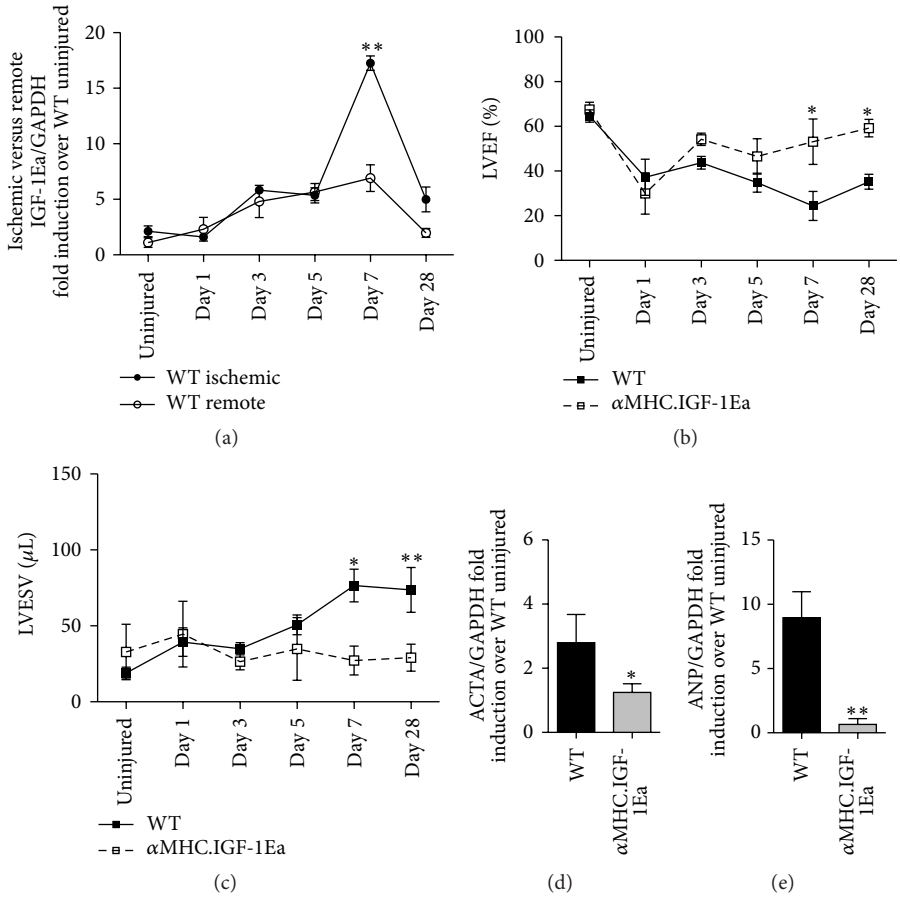

Figure 1: αMHC.IGF-1Ea improves cardiac function and reduces dilation as early as 7 days after myocardial infarction. (a) Levels of IGF-1Ea in the ischemic and in the remote area of WT hearts at 1, 3, 5, 7, and 28 days following MI. (b) Left ventricular ejection fraction (LVEF) and (c) left ventricular end systolic volume (LVESV) after MI. Solid lines represent WT mice. Dashed lines represent αMHC.IGF-1Ea mice. Levels of (d) actin-alpha 1 skeletal muscle (ACTA 1) and (e) atrial natriuretic peptide (ANP) mRNA expression 28 days after MI. Results are expressed as mean fold induction ± SEM over the values of uninjured hearts. $n = 4$–6 per group. Two-tailed Student's t-test was performed to compare WT versus αMHC.IGF-1Ea at selected time points after MI. $^{*}P < 0.05$, $^{**}P < 0.005$.

left ventricular EF by day 7 after MI (Figure 1(b) and Supplementary Table 1). Left ventricular end systolic/diastolic volumes significantly increased after MI in WT hearts, indicating left ventricular dilation, while the αMHC.IGF-1Ea hearts did not display any such signs (Figure 1(c) and Supplementary Table 1). In support of the functional data, the expression of molecular markers for cardiac damage such as actin-alpha 1 skeletal muscle (ACTA) and atrial natriuretic peptide (ANP) was significantly reduced in αMHC.IGF-1Ea compared to WT mice 28 days after MI (Figures 1(d) and 1(e)). The peak of endogenous IGF-1Ea in the ischemic region of WT mice by day 7, along with the improvement in cardiac function in αMHC.IGF-1Ea as early as day 7, indicates that IGF-1Ea signalling at early time points is key for cardiac repair.

3.2. Altered Tissue Remodelling in αMHC.IGF-1Ea after Injury. Three days after MI, WT and αMHC.IGF-1Ea hearts displayed infarcts of equal size, quantified by Masson's trichrome staining (Figure 2(a)). However, by 28 days after infarction, αMHC.IGF-1Ea hearts exhibited smaller scar areas compared to WT (Figures 2(b), 2(c) and 2(d)), as previously reported [25]. This was observed as a reduction in scar length but increased scar thickness, consistent with reduced infarct expansion. As a molecular measurement of fibrosis, we quantified TGF-β mRNA levels which were significantly lower in αMHC.IGF-1Ea than in WT mice after MI (Figure 2(e)). We therefore measured expression of genes involved in ECM turnover and synthesis, MMP-2, MMP-9, TIMP-1, TIMP-2, Col Iα1, Col Iα3, and lysyl oxidase (Lox) at 1, 3, 5, 7, and 28 days after MI. In WT hearts, MMP-9 was upregulated 3 days after MI, followed by MMP-2 at day 7 (Figures 2(f) and 2(g)). Interestingly, neither MMP-2 nor MMP-9 was significantly upregulated in αMHC.IGF-1Ea hearts at any time point. Inhibitors of matrix degradation, TIMP-1 and TIMP-2, had similar expression in WT and αMHC.IGF-1Ea hearts at all time points except for day 7 when TIMP-2 was significantly upregulated in αMHC.IGF-1Ea hearts compared to WT (Figures 2(h) and 2(i)). Taken together, this supports the idea of a net overall reduction in matrix breakdown, which may contribute to the reduced fibrosis observed in αMHC.IGF-1Ea hearts.

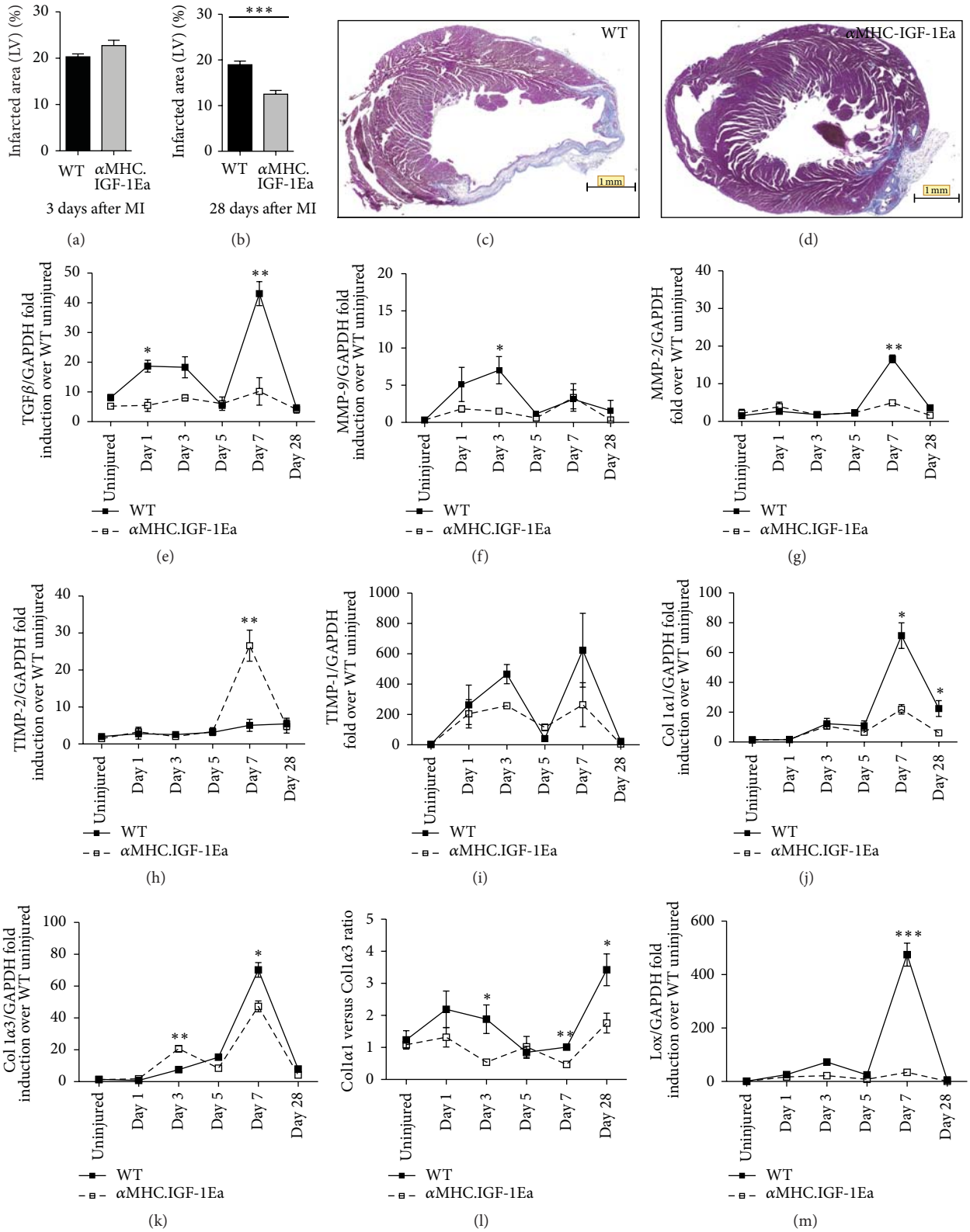

Figure 2: Positive remodeling in the αMHC.IGF-1Ea infarcted region after myocardial infarction. Quantification of the infarcted area at (a) 3 days and (b) 28 days after MI. $n = 3$. Representative histological sections of (c) WT and (d) αMHC.IGF-1Ea hearts stained with Masson's trichrome 28 days after MI. Scale bar: 1 mm. ((e)–(l)) mRNA relative expression of (e) transforming growth factor beta (TGF-β), (f) matrix metalloproteinase 9 (MMP-9), (g) matrix metalloproteinase 2 (MMP-2), (h) tissue inhibitor of metalloproteinase (TIMP) 2, (i) TIMP-1, (j) collagen (Col) Iα1, (k) Col Iα3, (l) ColIα1/ColIα3 ratio, (m) lysyl oxidase in the infarct area. Results are expressed as mean fold induction ± SEM. Solid lines represent WT mice. Dashed lines represent αMHC.IGF-1Ea mice. $n = 3$ per group. Two-tailed Student's t-test was performed to compare WT versus αMHC.IGF-1Ea at selected time points after MI. $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$.

We next analysed the composition of the newly synthesised matrix by measuring mRNA expression of the most abundant cardiac ECM collagen, Col Iα1 and Col Iα3, as well as the collagen cross-linker, lox, which increases matrix stiffness. Upregulation of both collagen types was detected by day 3, peaking at day 7 (Figures 2(j) and 2(k)). At this time point, WT hearts expressed significantly more of both collagen types than αMHC.IGF-1Ea hearts. We also noted a difference in the ratio of the two collagen types; αMHC.IGF-1Ea hearts had a reduced Col Iα1/Col Iα3 ratio compared to WT, which was significant at days 3, 7, and 28 (Figure 2(l)). At the peak of collagen upregulation, lox was also upregulated in WT hearts, yet this was not observed in αMHC.IGF-1Ea hearts (Figure 2(m)). These results indicate that IGF-1Ea overexpression reduces ECM turnover after MI and alters the composition of the matrix, with preferential expression of Col Iα3 over Col Iα1 and less cross-linking, which likely alters the mechanical properties of the fibrotic area.

3.3. Distinct Chemokine and Cytokine Production in αMHC-IGF-1Ea Hearts after Myocardial Infarct. Tissue remodelling after injury is closely tied to the inflammatory process. We therefore compared the inflammatory status of the αMHC.IGF-1Ea and WT hearts, monitoring the production of key immune genes IL-1β, IL-10, MCP-1, and CCL5. Expression of the inflammatory cytokine IL-1β was rapidly induced upon injury in WT hearts (66-fold over uninjured) yet was not upregulated as strongly in αMHC.IGF-1Ea hearts (19-fold over uninjured; Figure 3(a)). Similarly the strong upregulation of MCP-1 in WT hearts was not observed in αMHC.IGF-1Ea hearts (Figure 3(b)). IL-10 was also rapidly upregulated, peaking 1 day after MI. In contrast to IL-1β and MCP-1, this immunosuppressive cytokine was upregulated 3-fold higher in αMHC.IGF-1Ea hearts compared to WT controls. Although a second peak of IL-10 mRNA at 7 days was comparable in both WT and αMHC.IGF-1Ea hearts 7 days after MI (Figure 3(c)). CCL5, which is involved in the recruitment of Ly6C^{low} monocytes [26, 27], was upregulated in αMHC.IGF-1Ea hearts 7 days after MI (Figure 3(d)). These data suggest an early bias towards a less inflammatory environment potentially modulating the recruitment of monocytes in αMHC.IGF-1Ea hearts after myocardial infraction.

3.4. IGF-1Ea Modulates Myeloid Cell Recruitment after Myocardial Infarction. To document accumulation of the main innate immune cell populations involved in cardiac inflammation after infarct, single cell suspensions were prepared from whole mouse hearts and analysed by flow cytometry. All cell populations described in this work were identified using the gating strategy shown in Supplementary Figures 3–6.

In WT and αMHC.IGF-1Ea hearts, the total number of infiltrating leukocytes (CD45+) gradually increased after MI, both reaching comparable peak numbers at day 5 (Figure 4(a)). However at the earlier 3-day time point, αMHC.IGF-1Ea hearts contained 49% less leukocytes per milligram of tissue than WT hearts. In examining specific immune cell populations, this difference was partly explained by a 75% reduction in neutrophils (CD45+, CD11b+, F4/80−, CD11c−, and Ly6G+; 186 versus 46 cells/mg of tissue, Figure 4(b)) and a 67% reduction in monocytes (CD45+, CD11b+, CD11c−, Ly6G−, and F4/80−; 191 versus 62 cells/mg of heart, Figure 4(c)); however it was mostly due to reduced presence of macrophages (CD45+, CD11b+, CD11c−, Ly6G−, and F4/80+; 58%, 2276 versus 949 cells/mg of heart, Figure 4(f)). These data agree with the reduced MCP-1 expression observed in αMHC.IGF-1Ea hearts (Figure 3(b)), as it is the principal chemokine involved in monocyte recruitment [26, 28].

The Ly6C surface marker distinguishes two different subsets of monocytes [2, 3]. Analysis of the Ly6C^{high} (CD45+, CD11b+, CD11c−, Ly6G−, F4/80−, and Ly6C^{high}) and Ly6C^{low} (CD45+, CD11b+, CD11c−, Ly6G−, F4/80−, and Ly6C^{low}) populations revealed that the reduction of total monocyte numbers at day 3 was attributable to a 20% decrease of the Ly6C^{high} population (102 versus 32 cells/mg of heart), whereas the Ly6C^{low} population was not significantly different in WT compared to αMHC.IGF-1Ea (Figures 4(d) and 4(e)) hearts.

To differentiate between inflammatory and reparative macrophage populations, we used the markers Ly6C and CD206. For this work, only cells that were either Ly6C+/CD206− or Ly6C−/CD206+ were analysed, although we noted a double positive cell population (i.e., CD206+, Ly6C+). However no changes were observed over time between WT and αMHC.IGF-1Ea for the double positive population (Supplementary 2). Both Ly6C+ inflammatory macrophage (CD45+, CD11b+, CD11c−, Ly6G−, F4/80+, and Ly6C+/CD206−) and CD206+ reparative macrophage (CD45+, CD11b+, CD11c−, Ly6G−, F4/80+, and Ly6C−/CD206+) normalised cell numbers were reduced by 71% and 48%, respectively, in αMHC.IGF-1Ea hearts at the day 3 time point (522 versus 153 and 596 versus 310 cells/mg of tissue of heart, resp.; Figures 4(g) and 4(h)); however this was significant only for the Ly6C+ population. By day 7 after MI, macrophage dynamics changed and we observed a greater number of total macrophages in αMHC.IGF-1Ea hearts compared to WT, which was mainly due to a 155% preferential increase in the CD206+ population (575 versus 1468 cells/mg heart, Figure 4(h)). Dendritic cells (CD45+, CD11b+, F4/80−, CD11c+, and Ly6G−) were increased by 48% in αMHC.IGF-1Ea hearts compared to WT 5 days after MI (168 versus 248 cells/mg heart; Figure 4(i)).

In summary, cardiac-restricted expression of an IGF-1Ea transgene limited the early accumulation of innate immune cells at day 3 after MI, with a bias towards the reduction of inflammatory myeloid populations rather than regenerative populations. This reduction corresponds with the lower expression of myeloid chemokines and the less inflammatory milieu observed in the αMHC.IGF-1Ea hearts.

4. Discussion

Previous work in our lab showed that local expression of IGF-1Ea promoted functional restoration after MI and observed reduced infarct expansion, thinning, and dilation of the left ventricular wall [25]. We now demonstrate transcriptional modulation of key ECM remodelling genes in the IGF-1Ea hearts associated with tempering of the inflammatory myeloid cell response.

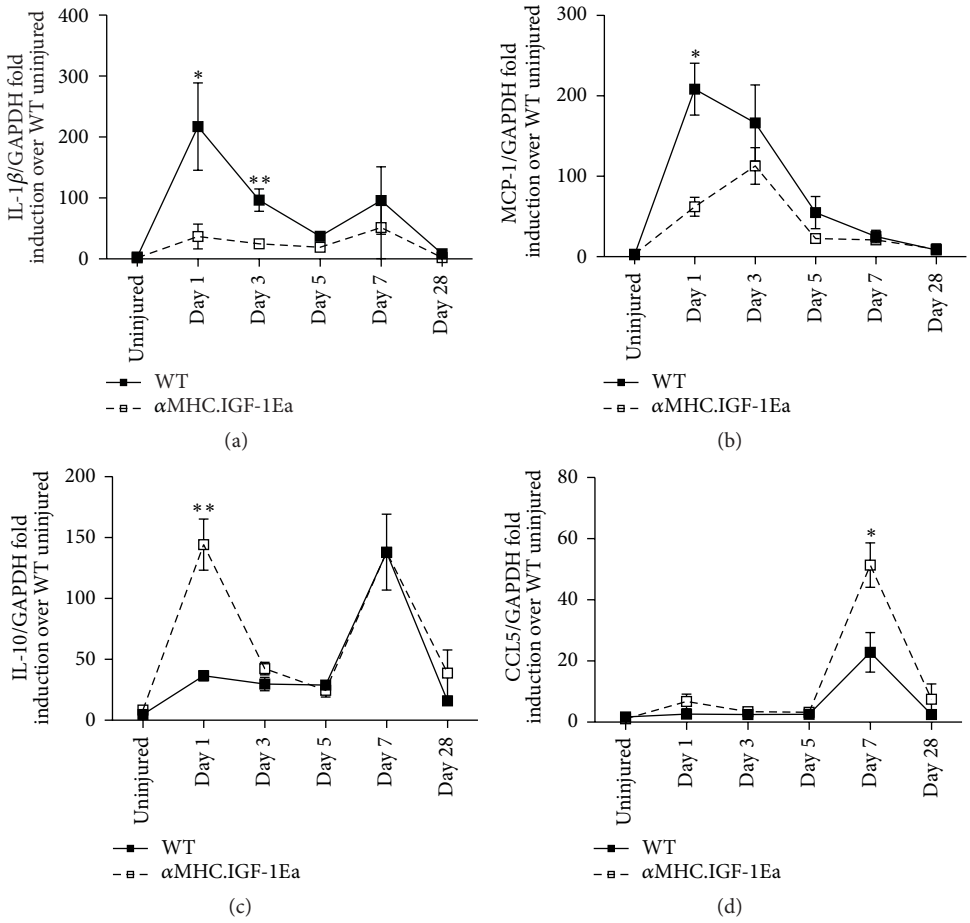

Figure 3: Cytokine dynamics in the infarcted αMHC.IGF-1Ea hearts. (a) Interleukin- (IL-) 1β, (b) MCP-1, (c) IL-10, (d) CCL5 at several time points after permanent ligation. Solid lines represent WT mice. Dashed lines represent αMHC.IGF-1Ea mice. Results are expressed as mean fold induction ± SEM over the values of uninjured hearts. $n = 3$ per group. Two-tailed Student's t-test was performed to compare WT versus αMHC.IGF-1Ea at selected time points after MI. $^{*}P < 0.05$, $^{**}P < 0.005$.

Increased MMP expression after injury has been implicated in contributing to scar destabilisation, as transgenic animal model knockouts of either MMP-2 or MMP-9 have been shown to attenuate LV dilation, rupture, and impairment of cardiac function [29, 30]. While MMP-2 and MMP-9 mRNA expression levels were upregulated in injured WT hearts, this increase was not observed in αMHC.IGF-1Ea hearts. In line with reduced matrix breakdown, mRNA expression of the MMP inhibitor, TIMP-2, was significantly increased in αMHC.IGF-1Ea hearts at day 7 after MI. Thus IGF-1Ea may prevent adverse cardiac remodelling, in part, by modulating transcription of MMPs/TIMPs.

The production of new matrix components was also altered by the presence of the IGF-1Ea transgene with an overall reduction in collagen synthesis, confirmed in histological stains, and a bias towards expression of Col Iα3 over Col Iα1. In MI patients, turnover of cardiac extracellular matrix can be assessed by using circulating collagen peptides as blood biomarkers [31] and high type I collagen is associated with adverse clinical outcome [32]. A prospective multicentre study further showed that a low type III/type I collagen ratio especially at 1 month after MI is predictive of detrimental left ventricular remodelling as well as cardiovascular deaths and hospitalisation cases for heart failure [33]. Changes in collagen ratios are known to affect the strength and tensile properties of the ECM. It would be interesting to determine whether these properties are modified in the αMHC.IGF-1Ea hearts.

IGF-1Ea could promote the changes in collagen deposition by directly acting on fibroblasts as it promotes both their proliferation and activation to myofibroblasts [34, 35]. Alternatively, IGF-1Ea may influence accumulation and activation of immune cells present at the infarct which in turn regulate myofibroblast activation. Indeed, we observed modulation of the inflammatory process in αMHC.IGF-1Ea hearts, with less monocyte ($Ly6C^{high}$) infiltration into the injured myocardium. This effect is associated with reduced expression of the monocyte chemoattractant MCP-1, although no significant changes were observed in the $Ly6C^{low}$ monocyte population in infarcted hearts, possibly due to CCL5 upregulation.

It is interesting that while complete depletion of monocytes at any stage of repair leads to poor recovery [36], a more subtle modulation of the monocyte population in

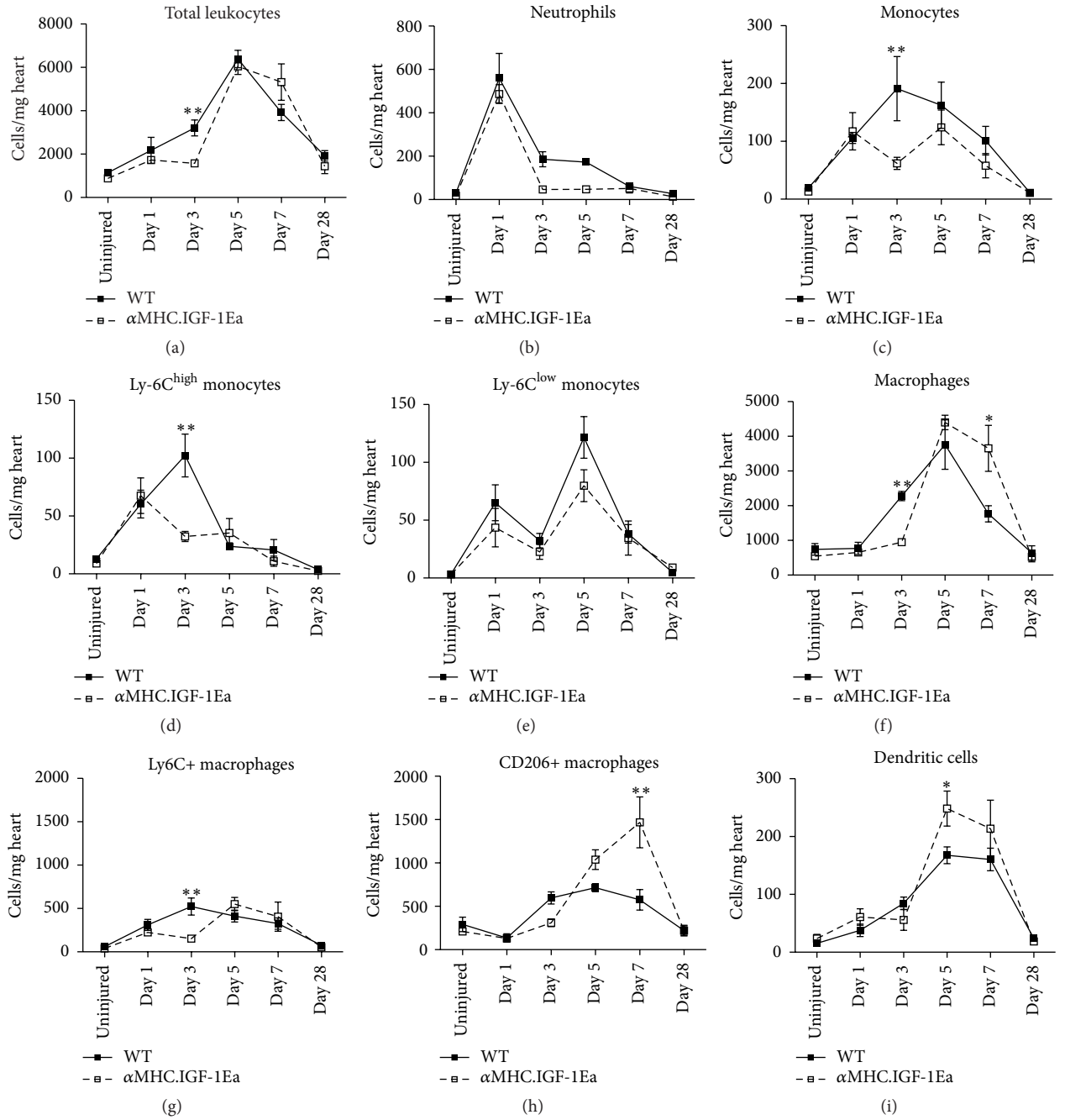

Figure 4: Characterisation and temporal dynamics of immune cell populations in αMHC.IGF-1Ea hearts after MI. Quantification of (a) total leukocytes (CD45+), (b) neutrophils (CD45+, CD11b+, F4/80−, CD11c−, and Ly6G+), and (c) monocytes (CD45+, CD11b+, CD11c−, Ly6G−, and F4/80−) which were further classified as (d) Ly6C^{high} and (e) Ly6C^{low} monocytes. (f) Macrophages (CD45+, CD11b+, CD11c−, Ly6G−, and F4/80+) were further characterised on the basis of Ly6C and CD206 expression as (g) Ly6C^{high}/CD206^{low} (inflammatory macrophages) and (h) Ly6C^{low}/CD206^{high} (reparative macrophages). For this work, only cells that were either Ly6C^{high}/CD206− or Ly6C−/CD206+ were analysed, although we noted a double positive cell population (i.e., CD206+, Ly6C+). (i) Dendritic cells were defined as CD45+, CD11b+, F4/80−, CD11c+, and Ly6G−. Data is presented as the total number of cells per mg of heart. n = 4–6 per group. Two-tailed Student's t-test was performed to compare WT versus αMHC.IGF-1Ea at selected time points after MI. $^{*}P < 0.05$, $^{**}P < 0.005$.

the αMHC.IGF-1Ea hearts is associated with improved heart function. Studies abrogating monocyte recruitment using a selective CCR2 inhibitor have resulted in reduced IL-1β, IL-6, MCP-1, and TNFα levels [37]. IGF-1Ea influences macrophage polarisation in skeletal muscle [38] and we similarly found reduced Ly6C^{high} monocyte and Ly6C+ macrophage normalised cell numbers while CD206+ macrophage numbers were increased by day 7, suggesting that IGF-1Ea promoted a quick progression to the reparative phase of repair by modulating macrophage phenotype. In

the αMHC.IGF-1Ea hearts we observed a decrease in MCP-1, which is the chemokine for CCR2, recently shown to distinguish infiltrating monocytes from the resident macrophage population which has a different embryological origin and expresses CD206 [39–41]. It is therefore possible that, in our αMHC.IGF-1Ea transgenic mouse model, IGF-1Ea reduces the infiltration of inflammatory Ly6C^{high} monocytes by preventing upregulation of MCP-1 while still allowing for expansion of the resident macrophage population.

In summary, we show that the αMHC.IGF-1Ea mouse model can modulate several associated aspects of the cellular repair process after MI, including immune cell recruitment, cytokine expression, and matrix turnover. All of these changes occur within the first 7 days after infarct, at which time a functional improvement can already be measured in αMHC.IGF-1Ea hearts compared to WT controls. These data provide new insights into the mechanism of IGF-1Ea and suggest that early immunomodulation is key to successful cardiac repair after injury.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Robert D. Sampson and Susanne Sattler contributed equally to this work.

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