Immune response to systemic inflammation in the intestinal microcirculation

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1. ABSTRACT

Systemic inflammation is characterized by acute or chronic dysregulation of the host immune response. The intestine plays an important role in systemic inflammation. Disturbances in the intestinal microcirculation due to infiltration of immune cells during systemic inflammation can increase bacterial translocation from the gut to the circulation and aggravate the pathological condition. Therefore, the intestinal microcirculation is relevant with respect to two aspects – as pathophysiological trigger and therapeutic target in systemic inflammation. Experimental intravital microscopy represents a unique method to study the immune response in organs and tissues *in vivo*. Novel non-invasive imaging technologies facilitate the examination of the human microcirculation. Future developments are needed to miniaturize the imaging technologies and automate the time-consuming analyses of the *in vivo* data in order to make the intestinal microcirculation accessible for routine diagnostics and therapeutic monitoring.

2. INTRODUCTION

Systemic inflammation is characterized by acute or chronic activation and dysregulation of the immune response not restricted to a local inflammatory process such as in sepsis or autoimmune diseases (1). Sepsis is the dysregulated immune response to an acute infection (2). Infiltration of immune cells and unbalanced release of inflammatory mediators in primarily unaffected areas ("horror autotoxicus") lead to multiple organ dysfunction and often result in the death of patients with sepsis (3).

Impairment of the microcirculation represents the key event in the pathophysiology of sepsis (4). Effects on all components of microcirculation, including smooth muscle cells, endothelial cells, platelets, leukocytes and red blood cells have been identified (5). The intestinal microcirculation (IMC) is involved early in the disease (Figure 1A). Intestinal mucosal hypoperfusion can cause damage to the epithelial barrier (Figure 1B) thus releasing bacteria and their toxins into the systemic circulation (bacterial translocation) and creating a "gut-derived" septic state (6). Particularly in the later phases of sepsis with predominant immunosuppression, this infectious challenge can be extremely detrimental. Therefore, the IMC has been suggested to act as the "motor" of multiple organ failure and death in sepsis (7). Consequently, studies in this area are relevant with respect to two different aspects – the IMC as pathophysiological origin and as therapeutic target in severe systemic inflammation, such as sepsis.

3. INTESTINAL IMMUNE RESPONSE

Unlike most the organs, the gastrointestinal (GI) tract is constantly being exposed to a plethora of environmental stimuli that includes dietary products, commensal microbiota, and potentially dangerous pathogens. The GI tract is equipped with an abundance of highly specialized innate and adaptive immune cells that reside closely with over 100 trillion commensal microorganisms (8). Intestinal epithelial cells form a single layer of cells joined together by tight junctions, creating a selectively permeable barrier. Located directly below this layer is the lamina propria, where
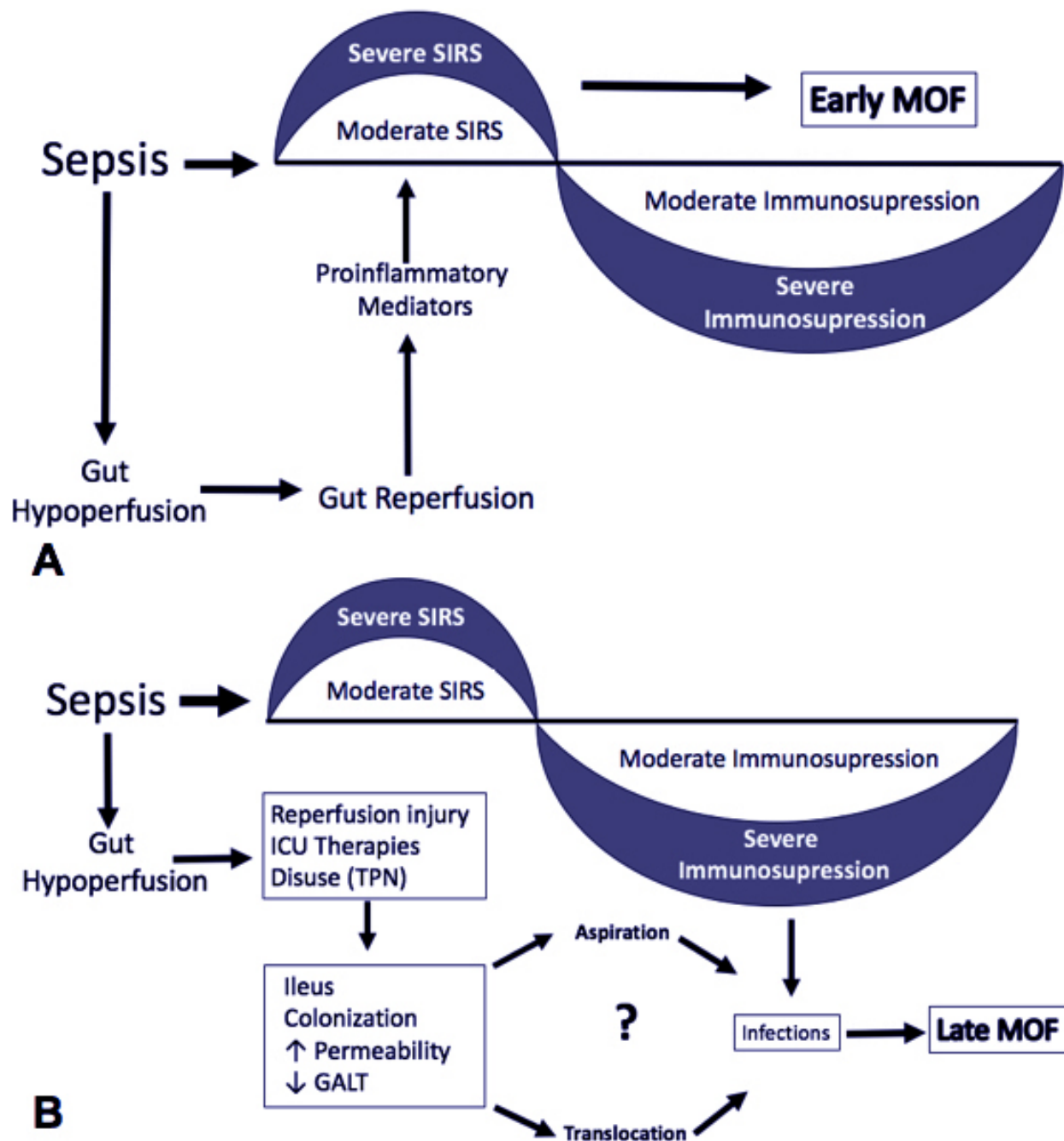
Figure 1. Gut = motor of multiple organ failure (MOF) in inflammation, sepsis and trauma. A – early phase. B – late phase. Following initial systemic inflammatory response syndrome (SIRS), subjects with immuno-suppression are threatened by translocated gut bacteria (65). TPN: Total parenteral nutrition; GALT: Gut-associated lymphoid tissue.

populations of intestinal immune cells reside and is responsible for regulating host-microbial interactions and maintaining tissue homeostasis (9). The effector functions of B and T lymphocytes are not relied upon as much in the intestinal immune system because rapid effector functions provided by the innate immune system are more essential in quickly responding to encountered antigens and maintaining intestinal homeostasis (10).

Of all the cells involved in the innate immune response of the intestine, polymorphonuclear neutrophils (PMNs) are one of the most important, representing an essential component of the first line of defense against invading microorganisms. Inactive PMNs are primed by bacterial derived mediators and pro-inflammatory cytokines/chemokines through pattern recognition receptors, such as Toll-like receptors (TLRs), NOD-like receptors (NLRs), and

C-type lectin receptors (CLRs) (11). These stimuli include but are not limited to: tumour necrosis factor-α (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-8 and interferon (IFN)-γ (12,13). Observations indicate that PMNs are the predominant infiltrating cells during the initial responses to inflammation, providing an important source of pro-inflammatory mediators to regulate the communication, migration and activation of other immune cells of both the innate and adaptive immune responses. PMN mediated killing of invading microbes present in mucosal tissues is accomplished by rapid phagocytosis and degranulation. Pathogens are destroyed through nicotinamide adenine dinucleotide phosphate (NADPH) oxygenase dependent mechanisms (e.g. reactive oxygen species) and the release of antibacterial proteins such as cathepsins, defensins, lactoferrin, and lysozyme (13). Continuous migration of PMNs across mucosal epithelial and the release of oxidizing radicals, inflammatory mediators, and other cytotoxic compounds can cause damage to adjacent epithelial cells and disruption of the epithelial barrier, exacerbating mucosal inflammation, representing a tradeoff of efficient microbial killing. The formation of neutrophil extracellular traps (NETs) represents another important antimicrobial defense mechanism (14). In response to activation by the stimuli mentioned previously, neutrophils generate extracellular fibers composed of DNA, histones and granules such as neutrophil elastase, and cathepsin G (14,15). These fibrous structures provide a high local concentration of antimicrobial components to kill microbes extracellularly, and in addition, these structures may serve as a physical barrier to prevent the spread of pathogens (14,16). Although NETs contribute to the antimicrobial response, the uncontrolled or excessive release of NETs can damage host cells and contribute to disease pathophysiology. In a study by Gao *et al.*, it was demonstrated that the release of NETs contributed to the intestinal damage in LPS-induced experimental sepsis (17). In this study, injury to endothelial cells by NET formation increased microvascular permeability, tissue edema and oxygen supply impairment (17,18). Tanaka *et al.* observed that NETs are associated with the formation of platelet and leukocyte-platelet aggregates in the microcirculation of septic mice, contributing to microcirculatory disturbances (19).

The migration of leukocytes from the blood to the inflamed tissue requires a series of adhesive and signaling events (Figure 2). The initial contact of leukocytes with the endothelium is initiated by leukocyte margination, which draws leukocytes close to the endothelial surface rather than in the central blood stream (20). This step is enhanced in the small postcapillary venules, which serves as the main location of leukocyte recruitment. Leukocyte rolling is mediated by selectins and represents the initial process of active leukocyte recruitment. The initial tethering and rolling of leukocytes is predominately mediated by the glycoprotein P-selectin Glycoprotein Ligand- (PSGL-1) binding to P- and E-selectins expressed on the surface of endothelial cells (21,22). The next step in leukocyte migration is the firm adherence of leukocytes to the endothelium. This first requires integrins to undergo a conformational change to gain full adhesive functions (21). Chemokines induced by inflammatory stimuli can transcytose from the inflamed environment to associate with G-protein coupled receptors on rolling leukocytes and through an inside-out signaling cascade, activate leukocyte integrins for tighter adhesion to the endothelium (23–25). In neutrophils, the binding of PSGL-1 to selectins can activate β2-integrins macrophage-1 antigen (Mac-1) and Lymphocyte function-associated antigen 1 (LFA-1) for subsequent firm adhesion by binding with the endothelial counter receptors of the intercellular adhesion molecule (ICAM) family such as ICAM-1 and ICAM-2 (26,27). In contrast, lymphocytes and monocytes express the β1-integrin Very Late Antigen-4 (VLA-4), which is bound by endothelial vascular cell adhesion molecule 1 (VCAM-1) (28). Firm adhesion of the leukocyte to the surface of the endothelium is key for the final step of transmigration (Figure 3). The mechanism by which leukocytes transmigrate is incompletely understood (13). Migration can occur either through a paracellular or transcellular manner, although it is well accepted that leukocytes cross the endothelium between cell borders (paracellular) (29). Leukocytes are able to extend protrusions through junctions between adjacent endothelial cells and pass the endothelial layer. The passage of leukocytes through endothelial junctions may be facilitated by vascular endothelial cadherin gap formation, which is stimulated by ICAM-1 mediated adhesion to the endothelium (21). After leukocytes have transmigrated, they move into the interstitium along a chemoattractant gradient toward the inflamed tissue (27).

Dendritic cells (DCs) are likely among the first cells to detect invading microbes since they reside in high numbers in the lamina propria. Their role is to activate and mobilize innate immune defenses. This is accomplished by rapid secretion of pro-inflammatory cytokines, which stimulates and coordinates antimicrobial activity of tissue resident cells, as well as induces DCs to migrate to mesenteric lymph nodes to initiate adaptive responses (30). Monocytes represent only a small fraction of the cells residing in the lamina propria, however during inflammation, large numbers of monocytes are recruited from the blood into the intestinal mucosa. Innate lymphoid cells (ILCs) are a mucosal tissue resident cell, located in the lamina propria. ILCs are constitutively present in tissue and are poised for rapid secretion of cytokines for host defense and tissue repair (10). Group 1 ILCs (ILC1s) and group 3 ILCs (ILC3s) are important in promoting innate immune defenses against viruses, bacteria,
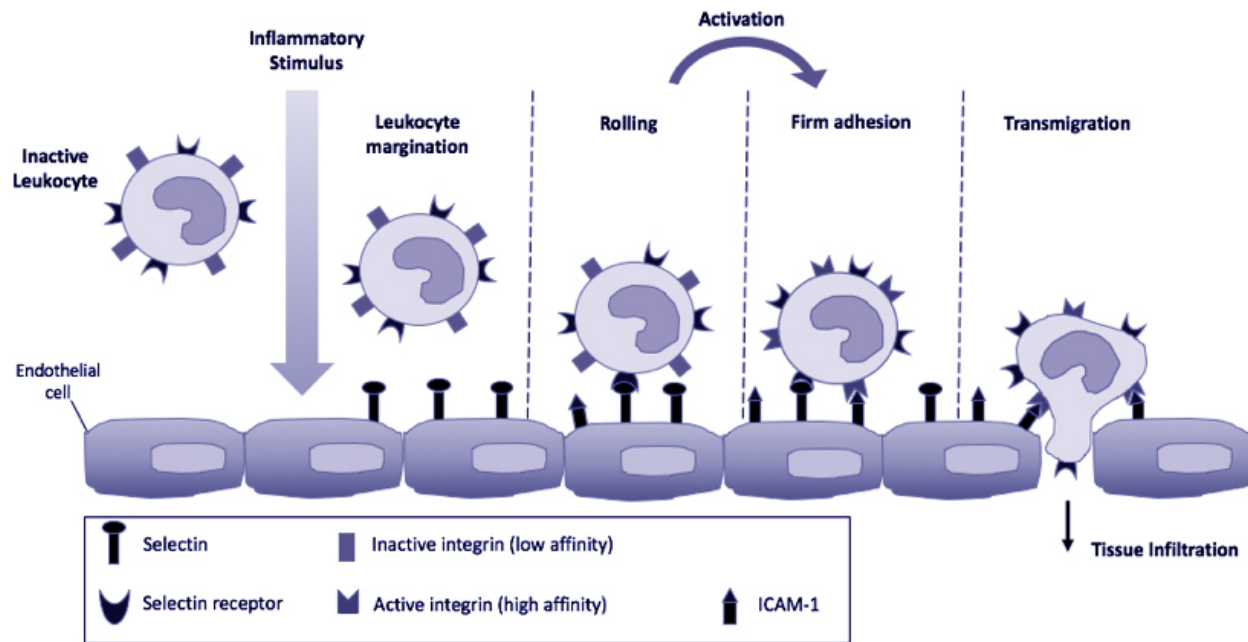
Figure 2. Leukocyte endothelial interactions - Cellular mechanisms that enable leukocyte migration into the inflamed tissue is a multistep process. The process starts with an inflammatory stimulus, which brings the inactive leukocyte closer to the endothelium surface through the process of margination. Selectins on the endothelium surface interact with receptors on the leukocyte to tether the leukocyte and begin the rolling process. The integrins then become activated on the leukocytes surface, firmly adhering the cell to the endothelium and allowing for transmigration to the inflamed tissue.

parasites and fungi (31). ILC1s produce interferon-γ (IFN-γ) to provide immunity to viruses, intracellular bacteria and parasites, while ILC3s produce IL-17A, IL-22, lymphotoxin, and tumour necrosis factor (TNF) to help control against extracellular bacteria (32–34).

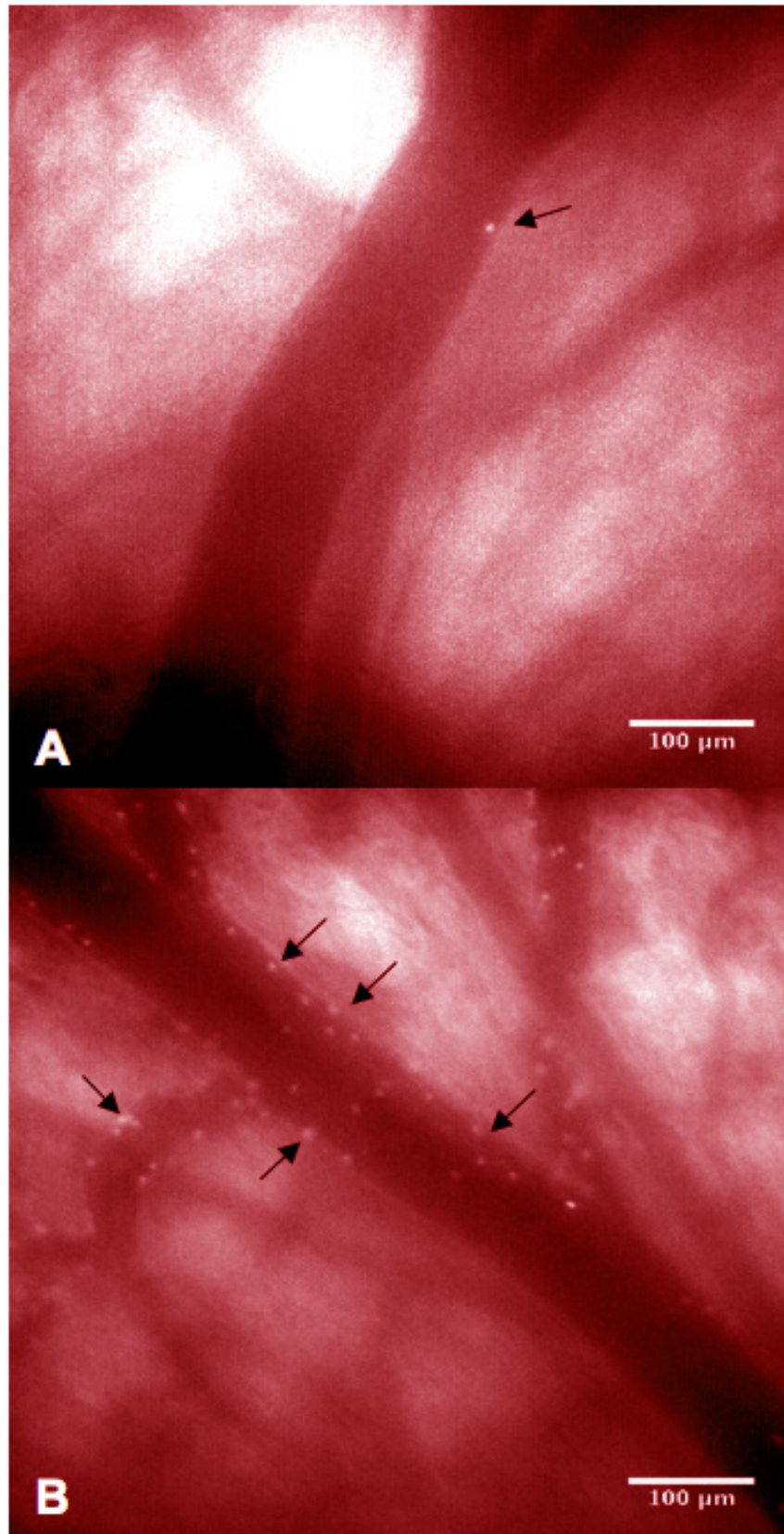## 4. INTESTINAL MICROVASCULAR ANATOMY

The intestine is divided into two major layers, the muscular and the mucosal layer, each containing their own microvasculatures which are connected at the submucosa level (35). The microcirculation consists of arterioles, venules and capillaries (Figure 4), which form a branching network to serve important roles in the absorptive, secretory and protective functions of the intestine. The vessels are organized in a manner that facilitates oxygen and nutrient delivery to the tissue, as well as aids the proper functioning of the immune system. The intestine is supplied with blood through the inferior and superior mesenteric artery (IMA, SMA) and branches of the celiac trunk, which enter the serosal surface of the gut at the mesenteric border. Seventy percent of the blood flow entering the gut is distributed to the mucosa and submucosa while the remainder is supplied to the muscular and serosal layers. The smaller branches of the SMA pierce the muscle layer through to the submucosa where they give rise to 1st order arterioles (1A). Second order arterioles (2A) derive from 1A, ultimately giving rise to 3rd order arterioles (3A), which proceed into the mucosa to the tip of the villi where they form a

mesh-like pattern of capillaries. These capillaries drain into the postcapillary venules (3V), which pass straight down to enter the submucosa where they drain into submucosal collecting venules (1V). This nutrient rich blood drains into the superior mesenteric vein (SMV), which connects with the hepatic portal vein.

Using intravital microscopy (IVM) real time videos can be collected of the microcirculation to study leukocyte endothelial interactions (LEI) in 1V and 3V venules. By addition of fluorescent markers and staining of individual cells, the multistep process of LEI can be visualized under physiological conditions and quantified to assess degree of immune stimulation by measurements of rolling, adhesion and transmigration. IVM is unique because it allows for *in vivo* studying of these dynamic processes. IVM can be useful to study LEI in many areas of the body including the intestine, eye, brain, heart, bladder, muscle, etc (36–39).

5. INTESTINAL BLOOD FLOW

The dysregulated immune response in systemic inflammation has significant impact on the microvascular blood flow in the intestine and other organs (40–42). This is directly related to the interaction of immune cells with the endothelium but also indirect factors, such as platelet activation leading to intravascular coagulation or changes in protein composition of plasma, contributing to the disturbances within the microcirculation in sepsis

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Figure 3. Leukocyte adhesion: Intravital microscopy of intestinal submucosal collecting venules (1V) in a mouse. (A) control animal, (B) Endotoxin-induced leukocyte endothelial interactions (see arrows).
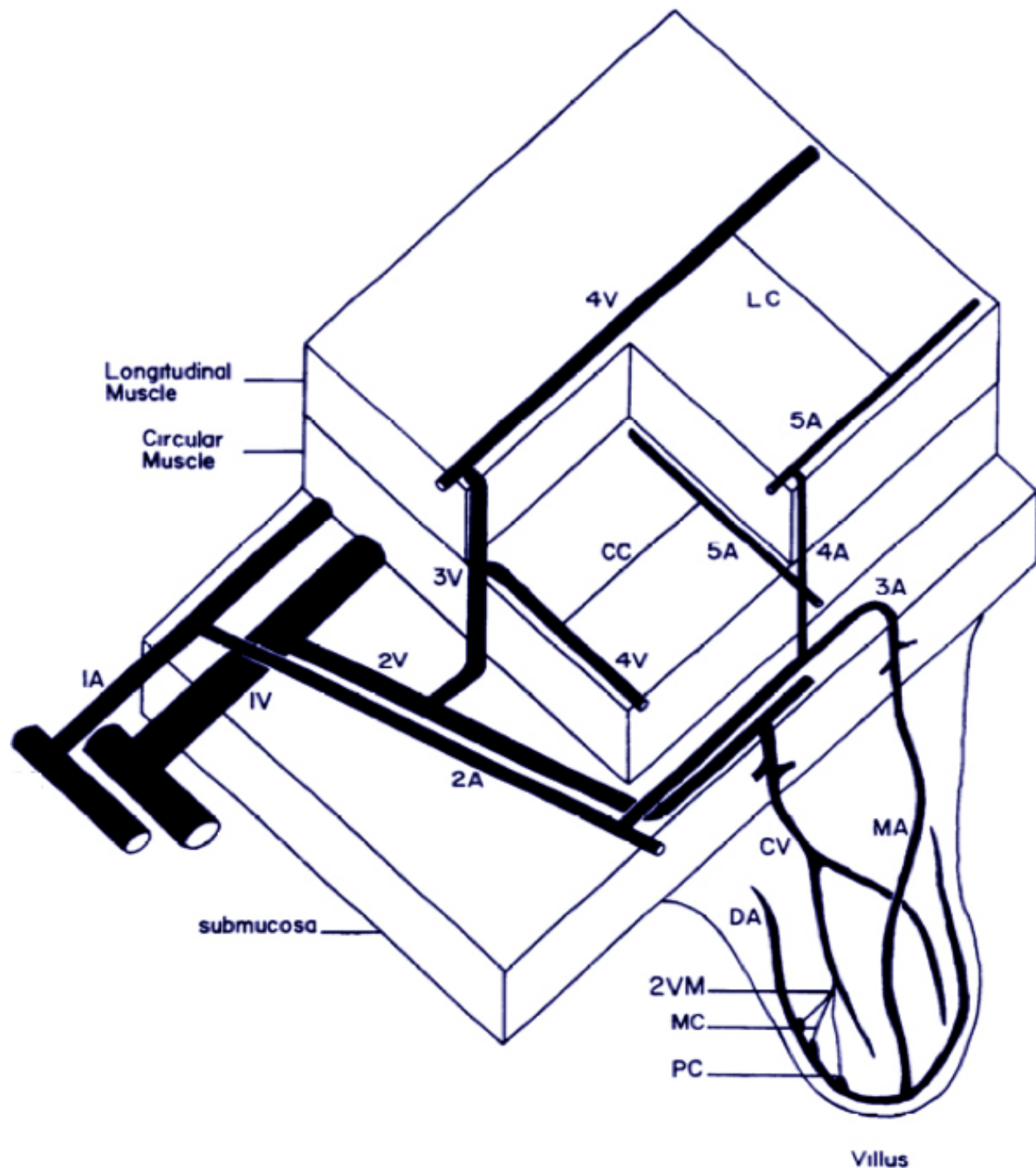
Figure 4. Intestinal microanatomy - Diagram of a single microvasculature unit in a rat intestine showing microcirculatory connections in the muscle layer, submucosa, and mucosal villi. The microvessels are classified numerically according to their degree of branching. 1A through 5A represents the 5 major arterial branches. 4V through 1V represent the major venular branches. Circular muscle capillaries (CC) and longitudinal muscle capillaries (LC) run parallel to muscle fibers. The mucosal villus is supplied blood from the base of the villus to the tip by the main arteriole (MA), which then branches into distributing arterioles (DA). Blood from DA pass through precapillary sphincters (PC) before entering the mucosal capillaries (MC) in that villus. These capillaries drain into second-order mucosal venules (2VM), which then empty into collecting venules (CV). For a more detailed description see (35).

(Figure 5). Persistence of severe alterations within the microvasculature have been associated with both the development of multiple organ failure and mortality (43). De Backer *et al.* observed that both vascular density and proportion of perfused small vessels were significantly reduced in patients with severe sepsis. More severe microcirculatory alterations were seen in non-survivors and patients with worse outcome (44).
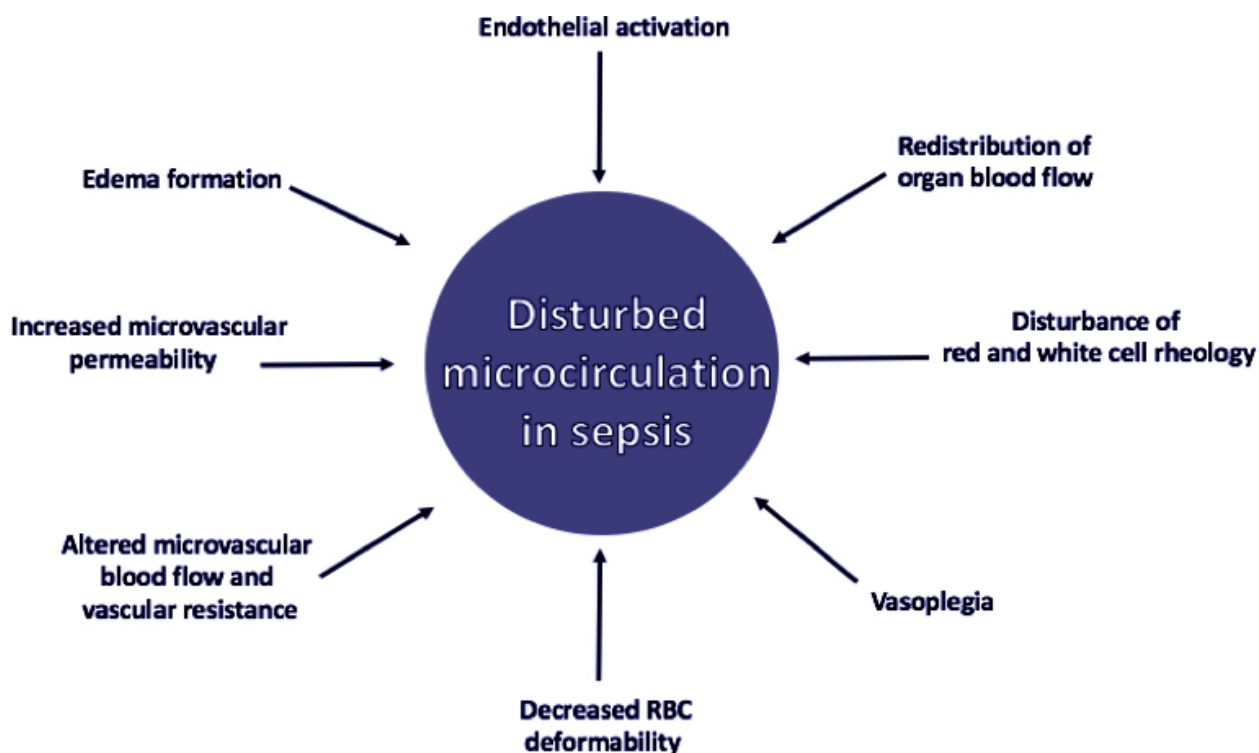
Figure 5. Factors contributing to disturbances of the microcirculation in sepsis; modified from: (66).

Additionally, Sakr *et al.* observed that microcirculatory alterations improve rapidly in septic shock survivors but not in patients dying from multiple organ failure. Also, microcirculatory alterations and not global hemodynamics and oxygenation were related to the occurance and the severity of multiple organ failure (43). This makes it critical to include the assessment of the microvascular blood flow as part of patient management since it facilitates the identification of patients with higher risk of detrimental outcome.

In addition to outcome prediction, IVM parameters can also be used for monitoring of specific treatment approaches. For example, since leukocyte recruitment in the microcirculation is one of the main mechanisms of innate immune response, its consequences on microvascular blood flow can be used to monitor patients treated with immune-modulatory therapies. In this context IVM can also address some of the limitations to current biomarkers of inflammation. E.g., measuring plasma levels of c-reactive protein (CRP), a non-specific biochemical marker of inflammation, is a common practice to determine the inflammatory status. Due to CRP's long plasma half-life, CRP is not accurate for monitoring of treatment efficacy as its levels remain high initially (45). Since IVM is an *in vivo* method, its parameters respond in real time and are more useful for therapeutic monitoring.

It is important to distinguish between different vessel subpopulations when assessing tissue blood flow (see Figure 4). Although assessment of larger vessels is important as well, tissue perfusion mainly depends on capillary blood flow. The capillaries are the vessels that contribute predominantly to oxygen and nutrients supply to the tissues. Commonly used imaging techniques in clinical settings for visualization of microcirculation in various tissues at bed-side are contrast-enhanced ultrasound (CEUS; (46)) and side stream dark field imaging (SDF; (47)). CEUS uses specific microbubbles that are administered intravenously. Although it is a non-invasive method of imaging, the microbubbles' short prescence in circulation and their instability at different ultrasound frequencies are some of its disadvantages (48). SDF technology works by visualizing red blood cells (RBC). Green polarized light is used for SDF and the scattered light is absorbed by the hemoglobin of RBCs of superficial vessels making them appear black (40). Although the blood vessel walls are not visualized, as RBCs flow next to each other most of the time, it becomes possible to visualize the vessel walls. Plasma gaps, which appear white, can be used to identify and quantify the microvascular blood flow. A downside to SDF imaging is that it only allows microcirculatory analysis of superficial mucosal surfaces (e.g., rectum, tongue). SDF penetration depth is up to 500μm (49).

General variables to consider when characterizing microvascular perfusion are total vessel density (TVD), proportion of perfused vessels (PPV), microvascular flow index (MFI), and perfused vessel density (PVD). TVD can be calculated by drawing three equidistant horizontal and three vertical lines on the image field and dividing the number of all vessels crossing the lines by the total length of the lines. To characterize the perfusion observed in the vessels, perfusion can be classified as present (continuous flow for at least 20 s), absent (no flow for at least 20 s), or intermittent (at least 50% of the time with no flow). The PPV can then be calculated by subtracting the number of vessels with intermittent/no flow from total number of vessels, dividing it by total number of vessels and converting it into a percentage (multiplying by 100; (40). PVD can be calculated by multiplying TVD with PPV. For the calculation of the MFI the image field is divided into four quadrants and each quadrant is given a value depending on the flow characteristics: absent (0), intermittent (1), sluggish (2), or normal (3). The final score is then based on the average of the four values measured.

In patients with ileostomy or colostomy, mucosal blood flow in intestinal villi can be assessed by SDF imaging. MFI measurement can be simplified by estimation of blood flow in single villi and by using a modified scale: 3 - continuous flow; 2 - hypoperfused (i.e., sluggish or intermittent flow); or 1 -no flow. The sum of all single villi MFIs is divided by the number of the villi (i.e., averaged MFI; (41). We can also describe the perfused vessel density of intestinal villi by dividing the number of villi with normal flow by the total number of villi in the visual field.

When analyzing intestinal microcirculation in experimental sepsis, capillary perfusion can be analyzed separately in the villi and the muscular layers by IVM. Using image analysis software, the length of functional, dysfunctional and non-functional capillaries per area is measured and presented as functional capillary density (FCD), dysfunctional capillary density (dFCD) and nonfunctional capillary density (NCD) (50). FCD measures capillaries with continuous flow regardless of the speed of the flowing cells (Figure 6) whereas dFCD, as the name implies, includes only capillaries with intermittent/interrupted flow. Presenting FCD an dFCD in one graph (Figure 7) can be used as a novel approach to describe changes of intestinal microvascular blood flow (51). A right shift of the FCD/dFCD ratio is the result of capillary recruitment which increases the total number of capillaries. This increase in FCD and or DCD is most likely due to the opening of arterio-venous shunts, which are closed under physiological conditions, and has been observed in different experimental models of sepsis (51,52).

In addition to IVM imaging, other methods exists to quantify microvascular blood flow in the IMC such as laser Doppler flowmetry, laser speckle contrast imaging and thermography (53). However those methods do not have the ability to visualize single microvessels, are diagnostically less conclusive and should be replaced with IVM when feasible.

Several factors contribute to the disturbance of intestinal microvascular blood flow in systemic inflammation, such as sepsis (Figure 5). Red blood cells, the most abundant cell type found in blood, play critical role in microcirculation as they are directly involved in oxygen delivery to tissues and organs. They are involved in autoregulation of microvascular blood flow as they detect changes in local oxygen levels and have the ability to coordinate a response to it (54,55) During sepsis, RBCs can lose their deformability which leads to an aggregation within the microvasculature (56). Endothelial cells are another cell type that play critical roles in regulating microcirculation. They perform a diverse set of functions including regulating capillary permeability and recruitment, regulating leukocyte adhesion and migration, and setting the vasomotor tone of the blood vessels. Under normal conditions, endothelial cells produce nitric oxide (NO) which stimulates the relaxation of arteriolar smooth muscle cells causing vasodilation of blood vessels and an increase in blood flow (57). During sepsis, inducible NO synthase (iNOS), which has an immune modulatory role, is heterogeneously expressed in different vasculature beds leading to the pathological shunting of microvasculature blood flow. Additionally, due to the differential expression of iNOS, some areas become vasodilated and become hyper-perfused (58,59). The smooth muscles cells also are affected in sepsis since they lose their tonus and adrenergic sensitivity possibly due to the excess NO production by iNOS further contributing to the perfusion dysregulation (55,60). Upon receiving strong pro-inflammatory signals, activated leukocytes leave the microvasculature, damaging the endothelium and tissues, and further adding to plasma extravasation damage. This fluid leakage into tissues impairs oxygen delivery even more, adding to the severity of hypoxia (61). Glycocalyx shedding in septic shock is associated with a pro-coagulant state as the induce the expression of adhesion molecules on leukocytes and higher mortality rate (60,62)

6. OUTLOOK

Studies of the intestinal microcirculation are a suitable tool to elucidate the immune response in systemic inflammation because of the experimental accessibility and clinical relevance of the GI tract. Therapeutic protection of the intestinal microcirculation represents a promising approach to improve the outcome of patients with
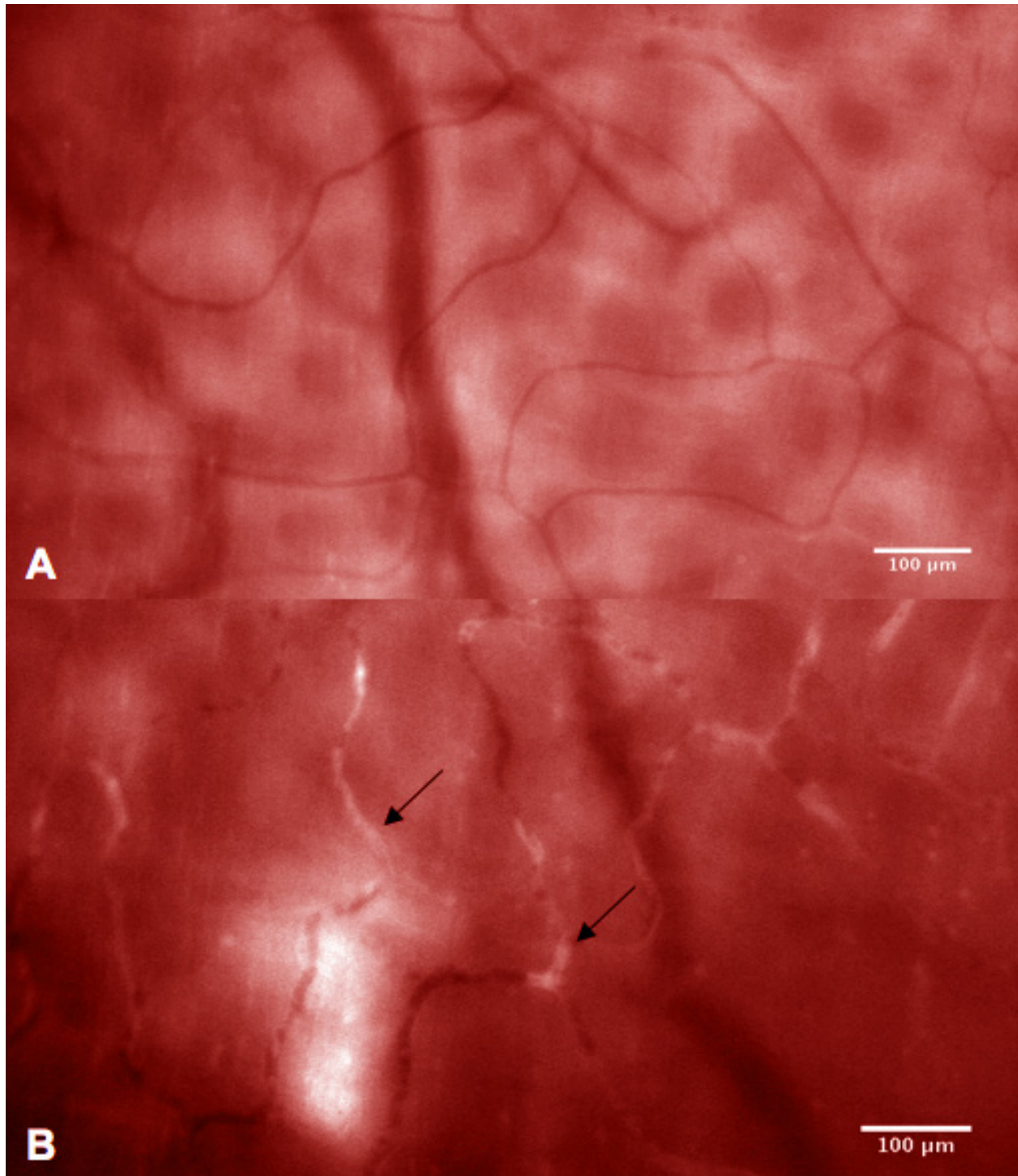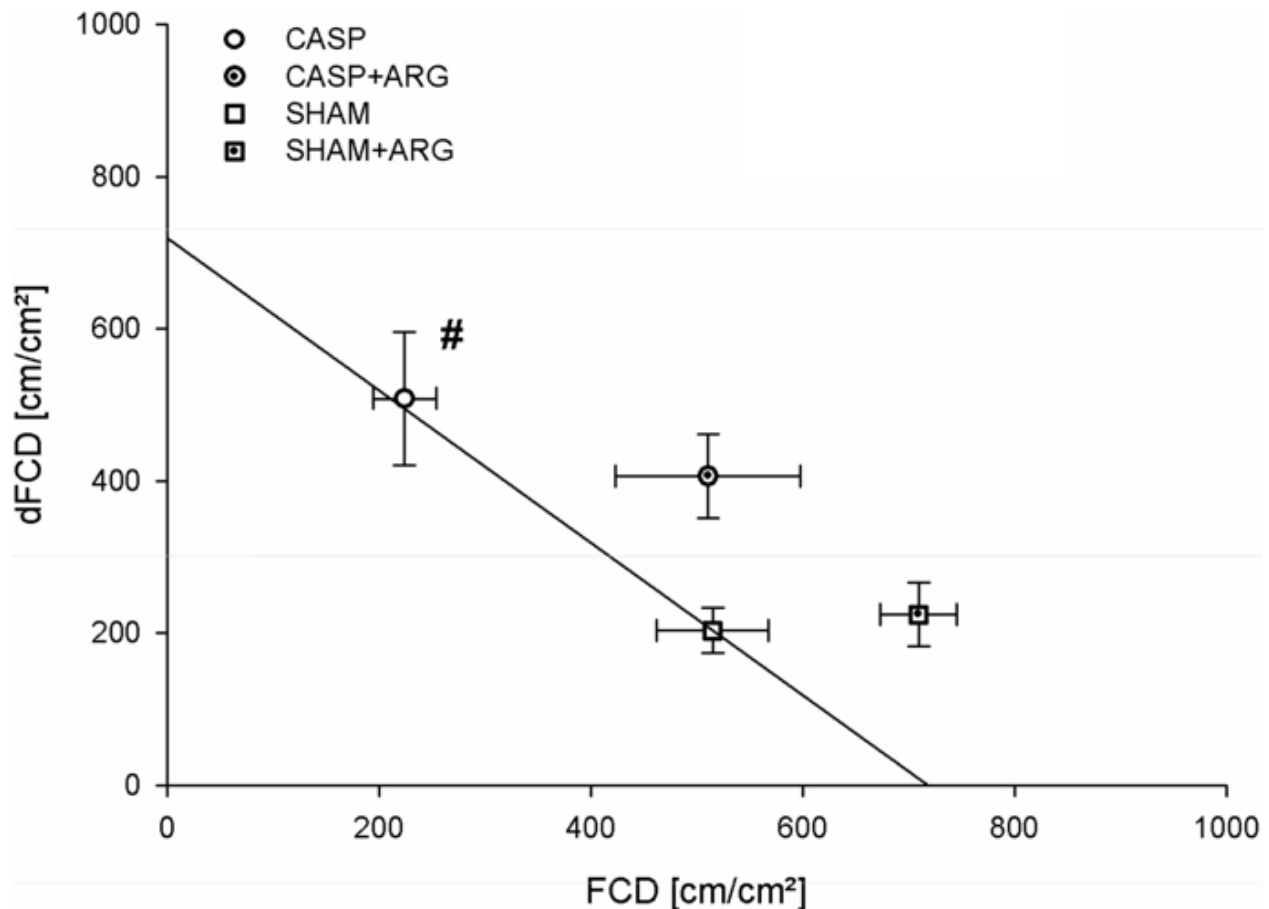
Figure 6. Functional capillary density in the muscular layer of the terminal ileum in a mouse. (A) Control animal, perfused vessels with uninterrupted flow are visualized as black (B) Endotoxin-induced sepsis, plasma gaps (seen in white and indicated by arrows) and intermittent/absent flow indicate microvascular disruptions.

systemic inflammation, such as sepsis. To make the intestinal microcirculation clinically accessible for routine diagnostics and therapeutic monitoring miniaturization of the imaging technologies and

automation of the time-consuming analyses of the *in vivo* data is necessary. In the last decade, significant advances were achieved in this regard (63,64). However, validation of the newly developed

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Figure 7. Functional (FCD) vs dysfunctional capillary density (dFCD) in the mucosa of the terminal ileum in colon ascendens stent peritonitis-induced (CASP) murine sepsis. Administration of argatroban (ARG) in both sham surgery (SHAM) and CASP shifted the data to the right of the line (representing constant total number of capillaries) based on control animals. $p < .05$, mean ± SE; with permission from: (51).

methods and devices is now needed before they can be utilized to translate the experimental findings into clinical practice. Experimentally, there is still a significant gap in knowledge regarding fundamental mechanisms of systemic inflammation. Without detailed knowledge of the molecular pathways involved in systemic inflammation, it will be difficult to improve the therapeutic options. The biggest hurdle is the exact diagnostic of the dysregulated immune response (activation vs. suppression).

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