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# On leukocyte recruitment in colonic ischemia-reperfusion

Stefan Santén



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Som med vederbörligt tillstånd av Medicinska Fakulteten vid Lunds Universitet för avläggande av doktorexamen i medicinsk vetenskap kommer att offentlig försvaras i aulan, CRC, ingång 72, Universitetssjukhuset i Malmö, onsdagen den 4 juni 2008, kl.09.15.

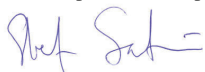
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Abstract <p>Leukocyte recruitment is a rate-limiting step in inflammatory disease. Tissue accumulation of leukocytes is a multi-step process comprising leukocyte rolling, adhesion and transmigration across the vascular endothelium. The aim of this thesis was to investigate the mechanisms underlying ischemia-reperfusion (I/R)-induced leukocyte-endothelial cell interactions in the colon. For this purpose, intravital microscopy of the colonic microcirculation was adopted. It was found that CXC chemokine and I/R-induced leukocyte recruitment in postcapillary venules is primarily mediated by PSGL-1 and P- but not E-selectin. Moreover, it was found that mast cells exert a dual function in CXC chemokine and I/R-provoked colonic leukocyte recruitment. On one hand, upstream chemokines trigger leukocyte recruitment via local mast cells and on the other hand, mast cells regulate downstream formation of chemokines, which in turn provoke leukocyte recruitment. SB 239063, a selective p38 MAPK inhibitor, has been implicated to exert a protective effect in I/R-induced tissue injury. Herein, it was found that SB 239063 protected against I/R-induced leukocyte recruitment and mast cell-generated TNF-<math>\alpha</math>-provoked P-selectin-mediated leukocyte adhesion. Interference with Rho-kinase signalling in I/R-induced tissue injury has indicated beneficial effects in decreasing leukocyte recruitment and pro-inflammatory mediators. Fasudil and Y27632, selective Rho-kinase inhibitors, were found to decrease colonic leukocyte recruitment, CXC chemokine and TNF-<math>\alpha</math> formation and the production of oxidative stress metabolites. This thesis identifies fundamental mechanisms regulating colonic I/R-generated leukocyte recruitment, which may help to understand the pathophysiology of inflammatory disease and be of assistance in developing more specific therapies in the future.</p>		
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## **1. LIST OF ORIGINAL PAPERS**

This thesis is based upon the following papers and manuscripts, which are referred in the text to by their Roman numerals:

- I. **Santen S**, Schramm. R, Menger M.D, Wang Y, Jeppsson B, and Thorlacius H. P-selectin glycoprotein ligand-1 regulates chemokine-dependent leukocyte recruitment in colonic ischemia-reperfusion. *Inflammation Research* 2007;56(11):452-8
  
- II. **Santen S**, Wang Y, Menger M.D, Jeppsson B, and Thorlacius H. Mast-cell-dependent secretion of CXC chemokines regulates ischemia-reperfusion-induced leukocyte recruitment in the colon. *International Journal of Colorectal Disease* 2008;23(5):527-34
  
- III. **Santen S**, Mihaescu A, Menger M.D, Wang Y, Jeppsson B, and Thorlacius H. Inhibition of p38 MAPK ameliorates ischemia-reperfusion-induced leukocyte recruitment in the colon. Submitted to *Surgery*
  
- IV. **Santen S**, Wang Y, Menger M.D, Jeppsson B, and Thorlacius H. Protective effect of Rho-kinase inhibition in colonic ischemia-reperfusion. Manuscript



## **2. ABBREVIATIONS**

ADP	adenosine diphosphate
ATF	activating transcription factor
AT-II	angiotensin-II
ATP	adenosine triphosphate
DNP	dinitrophenyl group
5-HT	5-hydroxytryptamin (serotonin)
ICAM	intercellular adhesion molecule
IL	interleukin
IFN- $\gamma$	interferon- $\gamma$
L-NAME	N <sup>G</sup> -nitro-L-arginine methyl ester
LPS	lipopolysaccharide
MCP-1	monocyte chemoattractant protein-1
PAF	platelet activating factor
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
VCAM-1	vascular cell adhesion molecule-1

### **3. INTRODUCTION**

Inflammation is a local and sometimes systemic response to an invading pathogen. It promotes the body discriminate self from non-self whereby the invading factor is effectively eliminated. Mechanical trauma, toxins, neoplasia and ischemia-reperfusion (I/R) are other triggers of inflammatory reactions and reflect the response from the vascular system to noxious stimuli in order to circumscribe the pathological process. The local inflammatory symptoms (swelling, redness, heat and pain) are initiated by host defence mechanisms rather than by the invading pathogen or the trauma itself. The inflammatory reaction is a complex series of events involving many of the body's defence systems. This sometimes develops into the systemic inflammatory response syndrome (SIRS) characterized by changes in peripheral leukocyte count, body temperature, heart rate and respiratory function. Patients suffering from this are at greater risk of developing acute lung injury (ARDS) and multiple organ dysfunction syndrome (MODS), which is the leading cause of death in intensive care units in the western world [1].

Leukocyte recruitment plays a major role in the inflammatory response and is regarded as a crucial and rate limiting step in this process. There are a number of leukocyte subclasses each with its specific role in the pathophysiology of inflammatory disease but polymorphonuclear leukocytes (PMNL) are

considered to be especially important in the reaction against invading pathogens. However, it has become evident that PMNL are important not only in this process but also in other inflammatory reactions such as I/R, inflammatory bowel disease and in the pathophysiology of organ rejection after transplantation [2], [3], [4]. Of bone marrow origin, it takes about 5 days for the PMNL to differentiate into fully mature, functioning cells. Upon intravascular release, the PMNL circulate for about 10 hours before the cells reside in an organ where it survives for another 5 days. After appropriate stimulation, PMNL are then able to extravasate across the vascular barrier and migrate into the tissue and thereby escalate the inflammatory response by releasing a number of cytotoxic substances.

The colon is an important organ regulating much of the intestinal water and ion absorption and also acts as a reservoir for a huge amount of enteric bacteria allowing a controlled elimination of the feces. In humans, the colon is about 1.5 meters long and it's ascending, transverse, descending and sigmoid portions connect the small intestine with the rectum. The large intestinal wall contains five distinct layers: the mucosa, submucosa, inner and outer muscular layers and serosa. In comparison with the small intestine, the colon is a functionally and morphologically distinct entity. The blood supply

to the colon is regulated by the superior mesenteric artery (SMA), which mainly supply the ascending and first part of the transverse colon, and the inferior mesenteric artery that basically supports the distal part of the transverse and the descending colon. Importantly, in humans there is an existing collateral blood supply between the two main arterial branches. In rodents however, the superior mesenteric artery is of unequalled importance for the colonic blood supply [5]. The colon is considered less susceptible to ischemic injury compared to the small intestine and the metabolic turnover rate is also less in

the large bowel [6]. Colonic ischemia-reperfusion (I/R) is a common clinical problem in trauma, bowel strangulation and during abdominal aortic aneurysm repair. It enables disruption of the physical protective barrier allowing bacterial translocation across the colonic wall. This in turn could severely aggravate the clinical condition and acts as a super secondary inflammatory hit after the initial reperfusion-induced inflammatory response. The goal of this thesis is to highlight and clarify some of the mechanisms regulating I/R-provoked leukocyte recruitment in the colon.

## 4. BACKGROUND

### 4.1.1 Shock

Shock is the condition in which the transport of oxygenated blood to the end organ is insufficient to meet the metabolic demand [7]. The symptoms and clinical findings in this life-threatening condition are low blood pressure, weak and rapid pulse, cold skin, lethargy and anxiety. It can be triggered by different aetiological entities like, for instance, sepsis, major burns, blunt trauma, dehydration and bleeding and is classified into four categories according to the cause of initiation: hypovolemic (loss of intravascular volume), cardiogenic (impaired cardiac pump function), obstructive (mechanical impairment of the heart and large vessels) and distributive (venous dilation with normal circulating volume) [8]. The inflammatory response during shock is an adaptive set of events where a mild to moderate state of systemic hyperinflammation (SIRS) is initiated in order to maximize the healing potential of the organism [9]. The intensity of the SIRS development depends upon the amount of tissue involved, the severity of shock and a variety of host factors whereby the initiation of severe SIRS can expand into an early MODS [10], [11]. During shock, a series of compensatory mechanisms evolves like, for example, securing the blood flow to the heart and brain. As a consequence, pooling of the circulating blood volume occurs resulting in splanchnic hypoperfusion. Much at-

tention has been focused on the mechanisms that regulate prolonged gut hypoperfusion, subsequent bacterial translocation and the development of MODS as a consequence of shock [11]. Interestingly, leukocyte inflammatory recruitment after hypovolemic shock (i.e. exsanguination followed by resuscitation) is mediated through endothelial and leukocyte cell adhesion molecules [12], [13], which also supports leukocyte recruitment in other experimental models such as peritonitis and colitis [14], [15]. Moreover, it has been shown that 45 min of SMA occlusion followed by 2 hours of reperfusion renders a rise in the levels of pro-inflammatory mediators and PMNL extravasation in the lung, which in turn induces an acute lung injury mimicking ARDS [16]. However, this seems to be independent of gram negative bacterial-derived LPS since LPS also was detected in sham operated healthy animals. Furthermore, culture of lymph nodes adjacent to the SMA did not show bacterial growth. Importantly, this experimental study shows that signs of inflammatory-induced remote lung injury are not only dependent on bacterial aetiology but might evolve out of other pathophysiological mechanisms.

#### 4.1.2 Ischemia-reperfusion and tissue injury

Although the consequences of depriving a tissue of its blood supply have been recognized for many years, the diseases that are initiated by tissue ischemia remain the main cause of death in the Western world. I/R-induced tissue injury is encountered within many fields of medicine e.g. orthopaedics, vascular surgery, neurology and also in general surgery and there are many different ways in which I/R-provoked injuries present themselves in the clinical situation. In general, the ischemic injury develops in two temporal phases: acute as in arterial embolus formation and sub-acute as in the development of the abdominal compartment syndrome. Although the hypoxic tolerance amongst cell types differs depending on the metabolic rate and adaptive mechanisms, cell death by necrosis or apoptosis inevitably follows after longer periods of ischemia [17]. During the ischemic phase oxygen depletion causes multiple cellular metabolic and ultra structural changes, such as acidosis, altered membrane potential, altered ion distribution, ATP-depletion, cellular swelling and ultimately irreversible cell damage and cellular death occurs. Furthermore, during ischemic periods there is an intracellular accumulation of degraded ATP that forms hypoxanthine, which is subsequently converted into highly reactive oxygen species (ROS) during reperfusion [18].

It is widely held that endothelial cells, especially those lining the venu-

lar microvascular bed, are vulnerable to I/R and within the endothelium itself, ischemia promotes the expression of pro-inflammatory mediators, such as leukocyte adhesion molecules, cytokines and leukotrienes while the production of protective gene products such as NO-synthase and thrombomodulin are repressed [19]. The ultimate goal in the treatment of ischemia is to restore blood flow to the affected organ. However, paradoxically, post ischemic reperfusion of the affected tissue is sometimes associated with greater damage to the organism than the actual ischemic phase itself [20]. Reperfusion derived inflammatory mediators and activated leukocytes, released from the reoxygenated organ, sometimes create injury, not only locally, but also in remote organs, such as in the heart, liver, lungs and kidneys, which in turn could lead to organ dysfunction and ultimately MODS [21], [22].

Inflammatory leukocyte recruitment is initiated and regulated by the cross-talk between the microvascular endothelium, leukocytes and adjacent tissue resident cells like macrophages and mast cells that are lining the microvascular bed [23], [24], [25]. In the gastrointestinal tract, under normal physiological conditions, the intestinal barrier protects the host from the potentially harmful content of the bowel lumen. However, during splanchnic I/R barrier disruption might result in bacterial translocation into the abdominal cavity and circulation. Moreover,

tissue reperfusion is also associated with further aggravation of the endothelial cell injury initiated during the ischemic phase. Reintroduction of oxygen into the tissue leads to conversion of hypoxanthine into ROS such as hydrogen peroxide ( $H_2O_2$ ), superoxide ( $O_2^-$ ), hydroxyl radicals ( $OH^-$ ) and hypochlorous acid (HOCl). These highly cytotoxic and cytoreactive substances, some of which ( $H_2O_2$  and  $O_2^-$ ) normally act as messengers in intracellular signalling have the ability to interact with intracellular components such as cellular membranes, proteins, transcription factors such as NF- $\kappa$ B and DNA whereby the ultra-structure and possibly function of these components are altered [18].

After the reintroduction of oxygenated blood into a previous ischemic organ it has been observed that the number of leukocytes traversing the vascular barrier, primarily in post capillary venules, increase drastically [26]. The mechanisms behind inflammatory leukocyte recruitment is a fine tuned interplay between cells of the interstitial compartment, like mast cells and macrophages, leukocytes and the endothelium in the affected organ [27], [28]. Upon activation, during the reperfusion phase, mast cells and macrophages release a number of substances that activate leukocytes and the products released also act as a chemical gradient, over the vascular barrier, for leukocytes to detect and migrate towards [29]. Moreover, activation of the endothelium by locally produced cytokines,

leukotrienes, PAF, and ROS makes the endothelial cells increase the expression of adhesion molecules like selectins, which takes place within minutes after initiation of reperfusion [19]. The expression of adhesion molecules on both endothelial cells and leukocytes are necessary to fulfil the first step in the capturing of free flowing leukocytes from the blood stream by the endothelium. I/R also triggers the endothelial cells to release chemokines which in turn acts as leukocyte activators and allows the activated leukocytes to come into close proximity with the adhesion-molecule-expressing endothelium enabling the white blood cells to start rolling along the vascular endothelial lining. This initial contact between leukocytes and the vascular endothelial cells is the first action in a series of well defined steps characterized by leukocyte rolling on the endothelium, firm adherence and finally leukocyte transmigration across the vascular barrier [30]. The activated, migrated leukocytes are then able to release a number of toxic substances such as proteases, elastases and ROS as a line of defence in the inflammatory process, which in turn results in increased microvascular permeability, oedema and thrombosis that could further compromise restoration of oxygen and nutrient supply to the I/R-injured tissue [31], [32].

It has been observed that blood flow to an ischemic organ is often not immediately fully restored during the reperfusion phase. This is manifested as a reduced number of perfused ves-

sels and consequently, tissue hypoxia develops during the initial reperfusion phase [19]. The possible mechanism behind this phenomenon includes plugging of capillaries by stiffer activated leukocytes, increased platelet-leukocyte aggregation, leukocyte-endothelial cell adhesion and partially detached endothelial cells [33], [34]. Together with the leukocyte dependent increase in plasma leakage and oedema formation these mechanisms lead to mechanical compression of the microvascular vessels which in turn act as a steric hindrance for the blood to freely flow in the microvasculature whereby the resulting relative ischemia (hypoxia) prevents immediate full restoration of the affected organ [35].

#### 4.1.3 Mediators in ischemia-reperfusion

##### **Tumor necrosis factor-alpha**

Tumor necrosis factor-alpha (TNF- $\alpha$ ) is a potent regulator and initiator of many inflammatory conditions. This 26 kD protein is produced and released by most cells of the inflammatory system, including macrophages, mast cells, monocytes and T-cells in response to stimuli, such as bacteria and bacterial toxins, activated complement factors and cytokines [36]. Initially recognized as an inducer of necrosis in certain tumor cell lines, this protein plays an important role in many clinical conditions, such as rheumatoid arthritis, Crohn's disease and psoriatic arthritis [37], [38]. TNF- $\alpha$  is expressed

as a transmembrane precursor (26 kD), which undergoes proteolytic cleavage and processing forming a soluble trimer (monomeric form 17 kD) where both the membrane bound and the soluble form is bioactive [36]. Upon stimulation of Toll-like receptors by a diversity of pathogen-associated molecules or the activation of various cytokine receptors on cell membranes, this starts a sequential induction of intracellular signalling pathways whereby the down stream activation of the nuclear factor kappa B (NF- $\kappa$ B) is initiated. This increases the cellular transcription factors for and the release of TNF- $\alpha$  [39].

Two high affinity cell-surface receptors for TNF- $\alpha$  have been identified: tumor necrosis factor receptor 1 (TNFR1) and TNFR 2, which are principally expressed on all cells except erythrocytes [40]. The primary role of TNF- $\alpha$  is in the regulation of immune cells and the immune response. However, once bound to its receptor, TNF- $\alpha$  also has multiple other effects in target cells like stimulation of apoptosis, regulation of cellular proliferation and differentiation, cytoskeletal rearrangement and viral replication [40], [41]. For example, TNFR-1 contains a death domain motif that is primarily involved in signalling for apoptosis. Although much less studied, TNFR-2 is believed to be activated by the membrane-bound, 26 kD form of TNF- $\alpha$  and is only indirectly involved in the cellular apoptotic process by secondary activation of TNFR-1 [41].

Upon stimulation with TNF- $\alpha$  and other pro-inflammatory cytokines, the vascular endothelium responds by increased production of adhesion molecules like selectins (P- and E-selectin), ICAMs and VCAM-1, which in turn are able to interact with their respective leukocyte counter-receptor promoting leukocyte-endothelium interactions [42], [43]. During experimentally induced I/R in the liver, heart and intestine it has been observed that the serum levels of TNF- $\alpha$  are increased [44], [45], [46]. It has also been shown that TNF- $\alpha$  produced in the liver and by the small intestine during I/R, induces leukocyte sequestration and enhanced plasma leakage in the lung leading to lung injury similar to that observed in ARDS [47], [48]. Pre-treatment with anti-TNF- $\alpha$  antibody [49] and TNF- $\alpha$  anti-serum [48] protects against not only local I/R-induced tissue injury but also against the distant TNF- $\alpha$ -generated neutrophil alveolar infiltration and lung damage [49]. Clinical observations in vascular surgery and in trauma patients show a similar pattern of initial systemic rise in pro-inflammatory parameters including TNF- $\alpha$ , IL-1 and IL-6 [50], [51]. Anti-TNF- $\alpha$  treatment is regularly used in patients with Crohn's disease, rheumatoid arthritis and psoriasis. In patients with sepsis, limited data from clinical trials has shown a non-significant increase in survival in patients undergoing anti-TNF- $\alpha$  therapy [52]. The clinical use of TNF- $\alpha$  immunoblockade in I/R has not been evaluated.

## Complement

The complement system can be activated by one of three pathways: the antibody-dependent classical pathway, the alternative pathway and the mannose-binding lectin/mannose-binding lectin-associated serine protease (MBL/MASP) pathway [53]. The primary functions of the complement systems are; inducing cell lysis in invading pathogens by polymerization of factor C5b-9 whereby the integrity of the invading organisms cell membrane is disturbed, activation of the inflammatory cascade in response to anaphylatoxins (C3a, C4a and C5a) and promoting humoral immune responses by aiding in antigen presentation to lymphocytes. Factor C3a and C5a mediates mast cell, eosinophil and neutrophil migration and are also amplifiers of inflammatory reactions by inducing the release of various mediators from mast cells and leukocytes [54], [55]. Complement factor C5a and C3a also promotes cellular adhesiveness, PMNL lysozyme release and also increase the production of endothelial derived ROS [56], [57].

Activation of the complement cascade and its impact on inflammatory changes is well characterized in myocardial I/R-generated tissue injury and was proposed already some 30 years ago [58]. The pathophysiological mechanisms of complement activation in intestinal I/R are somewhat unclear. One possible triggering mechanism is ischemic damage to endothelium with antigen exposure on cellular sur-



faces, which in turn induces binding of circulating IgM natural antibody. This immune complex then causes C1 binding and initiation of complement activation. However, the activation of complement during bowel I/R may be initiated through both the classical and alternative pathways as indicated in two recent studies [59, 60]. Using receptor blockade, monoclonal antibodies against factor C5a and factor C5 knock-out mice, indicated that complement factor C5a had the ability to activate leukocytes and upregulate the endothelial cell expression of adhesion molecules like P-selectin and  $\beta_2$ -integrins [61], [62], [63].

Clinical use of complement inhibitors showed promising results initially [64], [65] where C1 esterase inhibitor were used in patients undergoing coronary bypass operation. However, in randomized controlled trials (RCT) there was no demonstrable benefit on survival after anti-C1-treatment [66], [67]. Interesting initial results on the reduction of myocardial infarct size and mortality was observed in patients going through coronary bypass surgery by using recombinant antibody against factor C5 [65]. However, a RCT containing more than 900 patients was not able to confirm these results [68].

### **Chemokines**

Chemokines or *chemo*attractant cytokines are a large family of relatively small (7-15 kD) proteins that are divided into four different subfamilies

(CXC, CC, C or CX<sub>3</sub>C) depending on their arrangement of their N-terminal cysteine residues [69]. Structurally, CXC chemokines have a single amino acid separating the first two cysteins whereas CC chemokines have their N-terminal cystein amino acids adjacent to each other [70]. Unlike classical chemoattractants such as complement factors, PAF and bacterial derived fMLP, chemokines are quite diverse in their selectivity and specificity of target cells. For instance, CXC chemokines have the potential to activate and attract PMNLs and T-lymphocytes whereas CC chemokines have the ability to be active on multiple leukocyte subclasses like, monocytes, eosinophils, dendritic cells, natural killer (NK)-cells and PMNLs. The C chemokines exert their action on T-lymphocytes and NK-cells whereas CX<sub>3</sub>C chemokines stimulate NK-cells, T-lymphocytes, monocytes and PMNLs. Currently there are over 40 human and 30 murine chemokines identified, acting through seven transmembrane G-protein-coupled receptors on target cells. Their action is primarily to recruit leukocytes into inflammatory sites but emerging evidence indicates an important role in angiogenesis and immune functions as well [71]. Expressed and released by a wide variety of immune-regulating cells, (mast cells, leukocytes, macrophages) and non-immune cells, (endothelial and epithelial cells) chemokines are formed and produced by the stimulation of inflammatory cytokines like PAF, TNF- $\alpha$  and IL-1

but also by complement factor C5a and bacterial toxins like LPS [70], [71].

Members of the CXC chemokine family, like IL-8 (murine homologues macrophage inflammatory protein-2 (MIP-2) and cytokine-induced neutrophil chemoattractant (KC)), have been implicated as important mediators in the pathogenesis of several clinical conditions, such as glomerulonephritis and bacterial meningitis and have also been demonstrated to be potent chemoattractants for PMNLs [72], [73]. Most chemokine receptors interact with multiple chemokine ligands and most chemokines interact with more than one receptor. However, it is important to notice that CXC chemokines interact exclusively with CXC chemokine receptors and the CXC chemokine IL-8, one of the best characterized chemokines, acts through its corresponding high affinity receptors CXCR1 and CXCR2 in humans. In mice, the function of the CXC chemokines, such as MIP-2 and KC, are mediated through the CXCR2 receptor whereas CXCR1, or its homologue, has not yet been identified [74]. Emerging evidence suggests an important pathophysiological role for the CXC chemokines MIP-2 and KC in I/R-provoked leukocyte recruitment in the heart, liver and striated muscle [75], [76] [77] and a role for the CXC chemokines in I/R-induced leukocyte recruitment in the colon have recently been forwarded [78].

## Leukotrienes

Leukotrienes (LTs) are a novel group of biologically active metabolites of arachidonic acid origin that are produced through 5-lipoxygenase enzymatic activity. LTs are thought to play a role in inflammatory leukocyte recruitment and are divided into two separate classes on structural basis [79]. Depending on whether they have cysteine amino acid residues or hydroxyl groups they are divided into cysteinyl-LT (LTC<sub>4</sub>, LTD<sub>4</sub>, and LTE<sub>4</sub>) and LTB<sub>4</sub>, respectively. Initial observations suggested that LTB<sub>4</sub> were mainly involved in leukocyte chemotaxis whereas cysteinyl-LTs primarily were associated with bronchoconstriction and asthma. However it has become increasingly evident that LTs also induce profound effects within the vascular wall enabling activation of smooth muscle and endothelial cells [80], [81].

Early observations of distal ileum isolates from dogs revealed that production of LTB<sub>4</sub>, LTC<sub>4</sub>, LTD<sub>4</sub> and LTE<sub>4</sub> were unaffected by ischemia alone but were significantly increased after subsequent reperfusion [82]. Further investigations by Zimmerman *et al* and Karasawa *et al* suggested a protective role of selective LTB<sub>4</sub> receptor blockade in intestinal I/R-induced leukocyte recruitment [83], [84]. However, the origin of the intestinal reperfusion-derived LTs are not clear, although some evidence indicate the mucosa [85]. Recently, studies in 5-lipoxygenase knock-out mice has shown reduced local and distant

organ damage after experimentally induced bowel I/R underlining the importance of 5-lipoxygenase derived metabolites in the generation of I/R-provoked tissue injury [86].

### **Nitric oxide**

Nitric oxide (NO) has been proposed as a mediator in a vast number of physiological processes. It is widely accepted that NO has a regulatory role in vascular tone, platelet aggregation, neuronal transmission and as a protective cytotoxic mediator in microbial invasion [87]. NO is produced by the enzyme NO synthase (NOS) using L-arginine and molecular oxygen as substrates and there are three isoforms identified. The two constitutively expressed calcium dependent isoforms are neuronal NOS (nNOS), primarily located in neurons, and constitutive NOS (eNOS), predominantly expressed in endothelial cells. The third NOS isoform, with a demonstrable activity in endothelial cells, vascular smooth muscle cells, skeletal muscle cells, cardiac myocytes, intestinal epithelial cells and PMNLs, is the inducible NOS (iNOS) that is upregulated during inflammatory processes [87]. The role of NO in leukocyte-endothelial interactions in the microvasculature was initially observed by Kubes et al. [88]. By using L-NAME, an eNOS inhibitor, it was concluded that small quantities of eNOS-derived NO has a protective effect against leukocyte rolling in the cat mesentery. It has also been postulated that constitutively pro-

duced NO decreases leukocyte recruitment during LPS and H<sub>2</sub>O<sub>2</sub> exposure, indicating that endogenous production of NO acts as a regulatory effector during certain types of inflammatory responses [89], [90]. Different mechanisms have been proposed regarding the ability of continuously produced NO to decrease leukocyte-endothelium interactions during certain types of inflammatory reactions. One possible mechanism suggested by Miles et al. [91] is inhibition of ROS production. This observation was further supported by the fact that eNOS inhibition induces oxidative stress both *in vivo* and *in vitro* [92], [93]. There have also been observations of NO inhibiting NF- $\kappa$ B, which in turn decrease the production of pro-inflammatory cytokines and endothelial adhesion molecules [94].

In non-inflamed tissue iNOS is normally not expressed but upon stimulation by inflammatory cytokines like TNF- $\alpha$ , interferon- $\gamma$  and IL-1, as well as microbes and microbial derivatives like LPS, iNOS becomes activated producing large quantities of NO. Using pharmacological inhibition of iNOS, it has been argued whether iNOS-derived NO is able to decrease the inflammatory response or not [95], [96]. The disparate findings in these studies are probably related to the different inflammatory mechanisms involved in each model but, importantly, it demonstrates that iNOS derived NO does not inhibit leukocyte recruitment in every experimental model. Using iNOS (iNOS<sup>-/-</sup>)-deficient

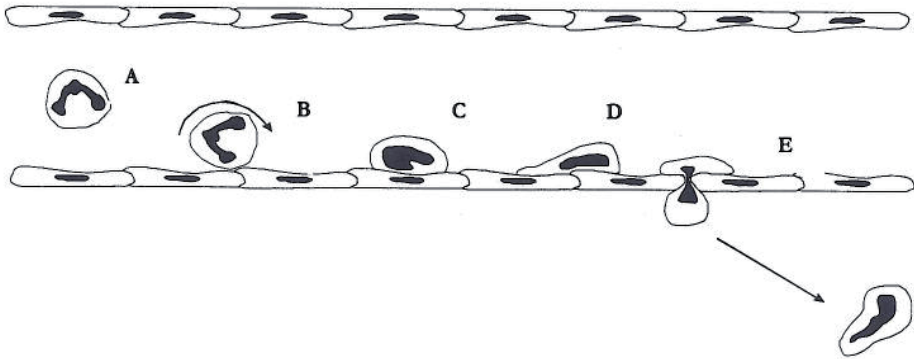
mice to further clarify the molecular basis of NO in inflammatory leukocyte recruitment, McCafferty et al. [97], [98] observed that iNOS<sup>-/-</sup> deficiency indeed increased the levels of PMNL infiltration in a model of acute colitis. In iNOS<sup>-/-</sup> deficient mice subjected to i.p. challenge with LPS it was observed that leukocyte rolling and adhesion increased compared to wild type animals. However, this observation has not been confirmed by others [99], [100].

In the intestine, it has been observed that both eNOS and in particular iNOS, enhance its activity after initiation of I/R, increasing the production of NO [101]. It has also been shown that clamping of the SMA enhance leukocyte-endothelial cell interactions in the liver and small intestine [102]. The pathophysiological mechanism behind this observation is probably due to increased expression of adhesion molecules on endothelium [102]. Interestingly, pretreatment with the NO donor diethylenetriamine/NO (DETA-NO), a NO-donor with long half-life, reversed the observed increase in leukocyte-endothelium interaction and adhesion molecule expression. The observation that administration of DETA-NO is an effective strategy in preventing I/R-provoked tissue injury has later been confirmed by others [103].

## 4.2 Inflammatory leukocyte recruitment

### 4.2.1 Multistep paradigm of leukocyte recruitment

Leukocyte extravascular recruitment into sites of inflammatory changes is a hallmark and a rate-limiting process in inflammation. Governed by specific interactions between leukocytes and endothelial cells, the emigration process, which mainly takes place in small post-capillary venules, involves a series of tightly regulated interdependent interactions between adhesion molecules expressed on the cell surfaces of leukocytes and endothelium [30]. There are three subsets of adhesion molecules that are primarily involved in the leukocyte recruitment process; the selectins, the integrins and the immunoglobulin supergene family resulting in leukocyte rolling, firm adhesion and endothelial transmigration (Fig. 1) [26]. In this process the shift from rolling to firm leukocyte adhesion is considered a committed step and the transition is regulated by the presence of appropriate activation signals mediated by chemoattractants such as chemokines and PAF [69].



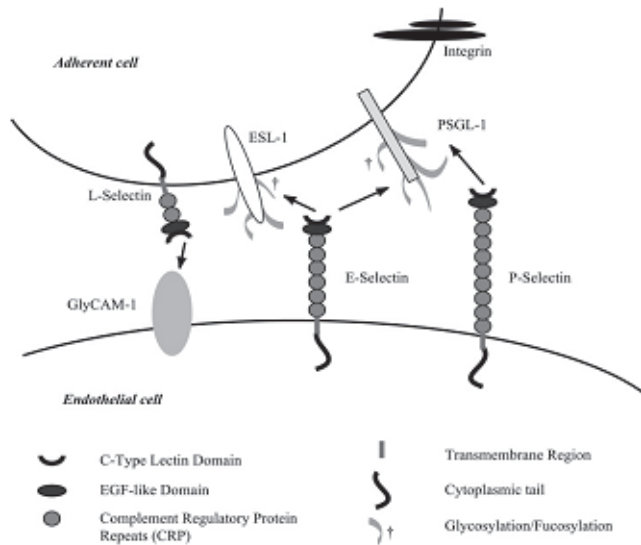
**Figure 1.** Leukocyte recruitment process with **A)** free-flowing leukocytes that are captured by endothelial cell adhesion molecules, resulting in **B)** leukocyte rolling, activation **C)**, subsequent firm leukocyte adhesion **D)** and trans- or para-cellular endothelial diapedesis **E)** into the tissue. Modified from Klintman D, *On leukocyte recruitment in endotoxemic liver injury*, PhD thesis, Lund University, 2002

#### 4.2.2 Leukocyte rolling

Margination of leukocytes is the mechanism by which leukocytes tend to move into a position close to the endothelial surface due to hemodynamic factors in the microcirculation, rather than in the central blood stream [104]. This enables the leukocytes to come into close proximity of the endothelium allowing leukocyte rolling, which is primarily supported by the selectins. The selectin family of adhesion molecules comprise three members i.e. L-selectin, E-selectin and P-selectin and represents a class of calcium-dependent glycoproteins that are composed of an NH<sub>2</sub>-terminal lectin domain that binds to carbohydrate ligands, a single epidermal growth factor-type repeat and variable number of consensus repeats. The selectins also have a single transmembrane region and a short cytoplasmic tail (Fig 2) [105].

#### L-selectin

L-selectin (CD62L, LAM-1) was first suggested as a unique lymphocyte homing receptor, identified by the use of a monoclonal antibody which blocked the binding of lymphocytes to high endothelial venules (HEV) in lymph node tissue [106]. Constitutively expressed by all classes of leukocytes, this 75-110 kD molecule was later on also shown to promote leukocyte rolling in other *in vivo* models [107], [108]. This observation has later on been questioned since further investigations also found that leukocyte recruitment in these models was a P-selectin mediated event [109], [110]. L-selectin has also been suggested to be a critical molecule for initial capture of leukocytes by endothelial cells thus enabling subsequent P-selectin mediated leukocyte rolling [109]. Activation of neutrophils with chemokines or by exposure to the activated endothelium induces shedding



**Figure 2.** Leukocyte and endothelial cell adhesion molecules supporting inflammatory leukocyte recruitment. From Laferriere et al, *Ann N Y Acad Sci*, 2002 (973): 562-72.

of L-selectin into the plasma [111]. This is an endoproteolytic enzymatic activity at a membrane proximal site whereby the extracellular domains are released and detected as changed plasma levels during different inflammatory conditions [112], [113].

### E-selectin

E-selectin (CD62E, ELAM-1) was originally identified as an IL-1 inducible adhesion molecule in human umbilical vein endothelial cells (HUVEC) [114] but TNF- $\alpha$ , TNF- $\beta$  and LPS were soon also found to induce transcription and expression of E-selectin [115]. A 64 kD core protein with glycosylation sites was later identified [116] and a peak with maximal levels of cell surface expression arises 4-6 hours after stimula-

tion and rapidly declines to basal levels after another 12-16 hours. Both in vitro assays and in vivo trials have shown that E-selectin functions as a rolling receptor for PMNLs [117], [118] as well as other cells of the immune system such as T-lymphocytes [119]. By the use of monoclonal antibodies against E-selectin the importance of E-selectin in leukocyte recruitment has been suggested in multiple animal experimental models such as in inflammatory bowel disease and peritonitis but also in experimentally induced I/R [120] [121], [122]. The relative importance of initial E-selectin mediated leukocyte-endothelial interactions in leukocyte inflammatory recruitment has later been suggested to be of minor significance since studies showed that E-selectin may be of more

importance in downstream events in the extravasation process [123].

E-selectin deficient mice have been observed to have no major abnormalities in their inflammatory response and are not more susceptible to bacterial infection than their wild type littermates [124]. A possible explanation for this observation could be that E-selectin is not a critical molecule in all models of inflammatory leukocyte recruitment, alternatively compensatory mechanisms like over-expression of P-selectin could compensate for E-selectin deficiency [125]. However, E- and P-selectin double mutant mice show deficiency of leukocyte rolling and are prone to develop severe infections [126].

### **P-selectin**

P-selectin (CD62P, PADGEM) was initially described in  $\alpha$ -granules of platelets and in Weibel-Palade bodies of endothelial cells, hence designated as platelet-dependent granule-external membrane protein [127], [128]. Fusion of the Weibel-Palade granules with the plasma membrane is induced by pro-inflammatory mediators such as histamine, thrombin and ROS [129], [130] and takes place within minutes after stimulus initiation. After initial exposure, it is down regulated by internalization but can recycle into granules [131], [132]. P-selectin exposure on cell membranes has been observed to be bimodal where cytokines like TNF- $\alpha$  and IL-4 may induce *de novo* P-selectin synthesis promoting leukocyte-endothelium

interactions up to 24 hours after initial stimulation [13], [133].

Initial *in vitro* data showed that leukocytes are able to roll along endothelial cells expressing P-selectin. The importance of P-selectin mediated leukocyte recruitment was later confirmed in *in vivo* observations [134], [27], [109]. Functional blocking of P-selectin with monoclonal antibodies reduced leukocyte rolling in a cremaster model, DSS-colitis and LPS-induced liver damage, indicating that P-selectin-dependent rolling is an important pathophysiological mechanism supporting inflammatory leukocyte recruitment in various models [133], [15], [135]. In I/R-provoked leukocyte recruitment, P-selectin mediated rolling has been found to be of major importance in the lung, cremaster and small intestine [136], [137]. Moreover, a recent study also implicates the importance of P-selectin in I/R-provoked colonic leukocyte recruitment [138]. Interestingly, P-selectin has also been observed to mediate experimentally induced venular leukocyte rolling in hemorrhagic shock, where leukocyte-endothelial interactions were blocked using i.v. monoclonal antibody treatment directed against P-selectin and by the use of P-selectin interference the number of surviving animals were improved [12], [139].

### **Selectin ligands**

Unlike most other cell adhesion molecules that interact with their ligands on the basis of protein-protein interac-

tions, the selectins are dependent on protein-glucosylation on their ligands to be able to exert their action [140]. In addition, selectins bind to glycolipids and proteoglycans but leukocyte recruitment due to these ligands has not been shown [141], [142]. The importance of proper selectin ligand glucosylation is exemplified by the leukocyte adhesion deficiency syndrome-2 (LAD-2) where patients get severe recurrent infections since PMNLs are unable to interact with vascular endothelial selectins and hence extravascular leukocyte recruitment fails [143], [144].

Four sulphated, sialylated and fucosylated L-selectin glycoprotein ligands have been identified so far: Glycam-1, CD34, MadCAM-1 and sulphated glycoprotein 200 (sgp 200) which are expressed in lymph node HEV [140]. L-selectin also interact with other ligands, such as P-selectin glycoprotein ligand-1 (PSGL-1), albeit with a low affinity, hereby supporting leukocyte-leukocyte interactions [145], [146]. The implications of this finding are however unclear.

E-selectin ligand-1 (ESL-1), a 150 kD glycoprotein, is a high affinity ligand for E-selectin [147], [148]. The protein is expressed in many different cell types including fibroblasts, epithelial cells and endothelial cells but ESL-1, in the E-selectin binding form, is only expressed on cells of myeloid origin. E-selectin also has the ability to interact with PSGL-1 and L-selectin but the pathophysiological role of these observations is

uncertain [140].

PSGL-1 is the best-characterized selectin ligand and is considered a major ligand to P-selectin on neutrophils and stimulated T-cells [149], [150]. The functional importance of P-selectin-PSGL-1-mediated leukocyte rolling and subsequent leukocyte recruitment has been demonstrated *in vivo* and *in vitro* by using monoclonal antibodies directed against PSGL-1 [149], [151]. It has later been confirmed to support the majority of biologically important P-selectin interactions in peritonitis, traumatic shock and colitis [152], [153] [154]. The role of PSGL-1 in I/R-induced leukocyte rolling and recruitment are poorly understood and available data are limited. However, a protective effect of soluble PSGL-1 was observed in I/R of the heart and liver [155], [156].

Heat-stable antigen (HSA/CD24), a small heavily glucosylated polypeptide of approximately 70 kD has also been reported to bind to P-selectin albeit with a much lower affinity than PSGL-1 [140]. The role of HSA in the pathophysiology of leukocyte inflammatory recruitment has not been established but notably, HSA-P-selectin interaction has been shown to mediate rolling in carcinoma cells [157].

#### 4.2.3 Leukocyte firm adhesion

Integrins are type 1 transmembrane glycoproteins comprised of associated alpha and beta subunits. Leukocytes are able to express about half of the 24 known heterodimers where the most



relevant integrins for leukocyte migration are the  $\beta_2$ -integrin subfamily (CD18) including CD11a (CD11a/CD18 or LFA1), CD11b (CD11b/CD18 or MAC1), CD11c (CD11c/CD18 or P150,95) and CD11d (CD11d/CD18 or  $\alpha_4\beta_2$ ). PMNLs express primarily  $\beta_2$ -integrins but also low levels of  $\alpha_4\beta_1$ , a member of the  $\beta_1$ -integrin subfamily [158], [159]. By antibody blocking and the use of integrin-deficient mice, LFA-1 and MAC-1 are suggested to be the primary integrins that mediate firm leukocyte adhesion in the inflammatory leukocyte recruitment processes [160], [138], [24]. The expression and binding activity of  $\beta_2$ -integrin on PMNLs increase as the cells marginate from the blood stream and begin to roll and then firmly adhere at sites of inflammation. During inflammation, endothelial cells are activated by pro-inflammatory cytokines to synthesize chemokines and lipid chemoattractants, such as leukotrienes, that are presented on their luminal surface. Activated endothelial cells also transport chemokines, like IL-8, from their abluminal side to the luminal where they are presented to the leukocytes on the endothelial cell surfaces [161]. When leukocytes roll on endothelial cells expressing high levels of chemokines, leukocytes are further activated and up-regulate or increase the avidity of integrins and a transition from a low-affinity to a high-affinity state takes place within minutes [162], [163]. Specificity in leukocyte arrest is thus ascribed to the differential expres-

sion of integrins and their ligands and of chemokines and their receptors.

$\beta_2$ -integrins interact with endothelial cell surface ligands (intercellular adhesion molecule-1-3, ICAM-1-3) that belong to the immunoglobulin gene superfamily. The ICAMs are also constitutively expressed on other cell types like, fibroblasts, dendritic cells, lymphocytes and platelets [164]. ICAM-1 and -2 are markedly upregulated by a variety of pro-inflammatory mediators [165], [166].

Leukocyte adhesion deficiency syndrome 1 (LAD-1) is a rare autosomal recessive trait characterized by chronic or recurrent bacterial and some times fungal infections without pus formation despite granulocytosis [167]. These patients have a mutation in the  $\beta_2$ -integrin gene whereby a defect or absent production of CD18 occurs. PMNLs from LAD-1 patients show initial rolling along endothelium but are unable to switch to a high-avidity state for further ligand binding which makes leukocyte arrest and subsequent transmigration impossible.

#### 4.2.4 Leukocyte transendothelial migration

Leukocyte transmigration into inflamed tissue occurs through the venular walls with minimal disruption of the structures. Utilizing pseudopodia, the leukocytes crawl along the endothelium towards sites of transmigration identifying junctional clefts between endothelial cells [168], [169]. This paracellular,

transmigratory route is considered the most frequently used in inflammatory leukocyte recruitment although a trans-cellular route has also been proposed [170]. Platelet endothelial cell adhesion molecule-1 (PECAM-1, CD 31) is a member of the immunoglobulin supergene family and is constitutively expressed on endothelium, neutrophils and platelets [171]. Various inflammatory models have indicated that PECAM-1 supports leukocyte transendothelial migration and other experimental evidence also suggest a role for VCAM-1 and ICAM-1 [172], [173], [174].

### 4.3 Intracellular signalling in inflammation

#### 4.3.1 p38 MAPK

The mitogen-activated protein kinase (MAPK)-signalling pathway is one of the major systems used by eukaryotic cells to transduce extracellular stimuli into intracellular and intranuclear responses [175]. The MAPK superfamily is comprised of three family members; extracellular signal-regulated kinase (ERK), c-JUN NH<sub>2</sub>-terminal kinase (JNK) and p38 that all exert their action, sometimes in parallel, in response to a wide variety of stimuli including osmotic shock, ionizing radiation, microbial infection and ischemic injury [175]. Through sequential phosphorylation of threonine and tyrosine residues MAPKs are activated whereby coordination of profound cell activities including gene

transcription, cell differentiation and protein synthesis are performed [176], [177].

p38 MAPK was originally described as a 38 kD polypeptide (p38 $\alpha$ , SAPK2a) that underwent tyrosine phosphorylation in response to endotoxin treatment [178]. Later, three additional MAPK isoforms were identified; p38 $\beta$  (SAPK2b), p38 $\gamma$  (SAPK3) and p38 $\delta$  (SAPK4) that constitute splice variants of the p38 gene transcript [179], [180], [181], [182]. In endothelial cells, p38 $\alpha$  and p38 $\beta$  are ubiquitously expressed whereas the p38 $\delta$  isoform expression is inducible and stimuli dependent. Moreover, p38 activation in endothelial cells has been described after TNF- $\alpha$  and IL-1 stimulation [183], [184].

The role of p38 MAPK activity in inflammation has been extensively characterized by the use of pyridinyl-imidazole anti-inflammatory drugs such as SB203580 and SB239063 [185, 186], which are selective inhibitors of p38 $\alpha$  and p38 $\beta$  [187]. Hence, an important regulatory role for p38 MAPK in the production of pro-inflammatory mediators such as IL-1, TNF- $\alpha$  and IL-6 has been suggested [188], [189] and a decrease in TNF- $\alpha$  and CXC chemokine production in endotoxemic liver injury has been observed after p38 MAPK inhibition [190]. Interestingly, p38 signalling also regulates the expression of vascular adhesion molecules, NO production acting as a regulator of iNOS activity and p38 MAPK has also been described to play a role in TNF-medi-

ated apoptosis in endothelial cells [191], [192], [193], [194].

p38 MAPK regulates phosphorylation, i.e. activation, of protein kinases and has a wide range of down-stream substrates including transcription factors like ATF-1, -2 and c-myc. It also activates AP-1, which is important in the subsequent activation of stress responsive gene-products including IL-1, IL-2 and TNF- $\alpha$  [175]. Saccani et al. has also reported a p38 $\alpha$ -dependent phosphorylation of histone H3 promoters implicating a role for p38 MAPK in NF- $\kappa$ B recruitment [195].

Studies of p38 MAPK activity in I/R-provoked tissue injury have mainly been restricted to the heart but limited data are also available for liver, kidney, cremaster and for the intestine. In general, the induction of I/R activates p38 MAPK within minutes of the reperfusion onset [196], [197]. Attenuation of I/R-induced adhesion molecule expression (P-selectin and ICAM-1) together with reduced PMNL-infiltration, infarction size and leukocyte infiltration was observed after pre-treatment with SB239063 following coronary obstruction in rats [196]. In mice, reduced coronary infarction size after prophylactic SB239063 treatment was observed by Kaiser et.al, but interestingly, the same group were unable to repeat their findings in a porcine model [198]. Zheng et.al found a protective role for SB203580 in reperfusion-induced small bowel apoptosis and a decreased lactate production after 45 min

of superior mesenteric artery occlusion [199]. Johns et.al were not able to detect any reduction in I/R-generated leukocyte-endothelium interaction in the cremaster [200]. These studies are not coherent and in somewhat contradictory. A possible explanation for the discrepancies is that activation of the p38 MAPK signaling pathway is species as well as organ specific. Thus, a plausible pathophysiological mechanism could be that specific p38 isoforms dominates the p38 activity in various organs and I/R-models and that p38 MAPK interference by SB239063 and SB203580 selectively only inhibits the p38 $\alpha$  and p38 $\beta$  isoforms.

#### 4.3.2 Rho-kinase

Small GTPases of the Rho family are essential regulators of several central aspects of cell functions, such as cell motility, proliferation and apoptosis. Rho-kinases (ROCKS) were the first effectors of Rho to be studied and characterized for their roles in mediating phosphorylation of myosin light chain, thereby regulating actomyosin contractility in non-striated cells [201], [202]. Two ROCK isoforms have been identified: ROCK I (Rho-kinase  $\beta^3$ /ROK $\beta^4$ ) and ROCK II (Rho-kinase  $\alpha^3$ /ROK $\alpha^4$ ), which are universally expressed in tissue although ROCK II are most abundant in the cytoplasm of striated muscle cells [203], [201], [204]. With a molecular mass of 160 kD, ROCKS consist of 3 domains including a catalytic N-terminal domain, a coiled-coil and C-

terminal domain, the latter also acting as a Rho binding site whereupon activation occurs [203].

Activation of the Rho/Rho-kinase-mediated pathway plays an important role in signal transduction and is initiated by many inflammatory agonists, such as AT-II, IL-1, 5-HT, PDGF and ATP/ADP whereby the expression of ROCK is regulated through the PKC/NF- $\kappa$ B pathway [205]. ROCK downstream activity includes upregulation of pro-inflammatory mediators including IL-6, MCP-1, IFN- $\gamma$  and PAF and two recent studies also suggests a regulatory role of ROCK in TNF- $\alpha$ , MIP-2 and KC expression [205], [206]. In contrast, ROCK mediates hypoxia-induced down-regulation of endothelial nitric oxide synthase (eNOS) [207]. Interestingly, observations of a regulatory role of ROCK-activity in LPS-provoked leukocyte adhesion in the liver and large vessels have been reported indicating an important role for ROCK in LFA-1 mediated leukocyte-endothelial cell interactions [206], [208].

Hypoxia and ROS are well-documented potent inducers of ROCK activation [209], [210]. Studies with fasudil and the structurally unrelated inhibitor Y27632 have indicated beneficial effects of selective ROCK inhibition in experimentally induced I/R of the heart. For example, Bao et al. reported that Y27632 given to mice prior to coronary occlusion resulted in a dose-dependent reduction of infarction size, decreased numbers of accumulated PMNLs and

attenuated levels of circulating IL-6 and KC [211]. A cardio-protective effect of fasudil in I/R has also been reported by Wolfrum et al. and recently published data from Versteilen et al. indicate that *in vivo* inhibition of ROCK in renal I/R preserves renal blood flow by improving eNOS function [212], [213]. A beneficial effect on ROS production, inflammatory cytokine production and survival in rats pre-treated with fasudil before subjected to liver transplantation has also been observed [214]. Interestingly, initial clinical trials treating patients with either fasudil or Y27632 have shown promising results in patients with pulmonary hypertension, coronary artery events and cerebro-vascular injury [215], [216], [217].

#### 4.4 Mast cells

Mast cells are potent effector cells playing a key role in IgE-mediated hypersensitivity, allergic disorders, immune responses and in different inflammatory conditions. Mast cells originates from hematopoietic pluripotent CD 34<sup>+</sup> progenitor cells, which is also the origin of PMNLs, macrophages, erythrocytes and platelets [218]. Based on different metachromatic staining properties and variable tissue distribution, mast cells are further subdivided into connective tissue and mucosal mast cells where the latter are abundant in the gastrointestinal tract. Mast cell development *in vivo* occurs mainly in non-hematopoietic environment and is dependent on the synergistic promotion of various stimuli

of which some, such as stem cell factor (SCF) and IL-3, are crucial, whereas others like IL-4, IL-9 and IL-10 acts in synergy with SCF and IL-3 [219]. Thus, development of functioning mast cells is critically dependent on the expression of SCF and its receptor (kit/CD117) [220]. WBB6F1 mice, which were used in the present study, lack mast cells due to a mutation in the kit oncogene for the SCF receptor.

Mast cells are found closely apposed to the vasculature in essentially all tissue and are sensitive to subtle changes in the surrounding environment. Once activated through various kinds of stimuli like proteins, complement factors, neuropeptides, ROS and bacterial toxins (Table 1), mast cells are capable of

releasing a large variety of pro-inflammatory mediators like cytokines, histamine, PAF, leukotrienes which in turn has the capacity to induce endothelial and leukocyte activation and adhesion molecule expression (Table 2).

Mast cell activity has primarily been associated with IgE-mediated reactions but as stated above, emerging data implicates mast cells as central regulators of various other pathophysiological processes. However, their localization in high concentrations adjacent to microvascular vessels, at sites where the internal and external environment meet (gut, skin and respiratory tract) probably reflects their main function *i.e.* to regulate vascular function, initiate inflammatory response and to ac-

**Table 1.** *Mast cell activators*

Proteins	IgE via FcεR1
Complement factors	C3a, C5a
Neuropeptides	Substance P, VIP, Somatostatin
ROS	O <sub>2</sub> -
Lipids	PAF, LTB <sub>4</sub> , LTC <sub>4</sub>
Others	LPS, Compound 48/80, histamine, SCF

**Table 2.** *Mast cell products*

Vasoactive	Adenosine, NO, prostaglandins
Amines	Histamine, 5-HT
Cyto/chemokines	IL-1, IL-6, TNF-α
Lipids	Leukotrienes, PAF
Others	ROS

tivate other cells. Using activators, such as mast cell degranulating agent compound 48/80, emerging data implicates that mast cells are able to mediate leukocyte rolling, adhesion and extravascular recruitment [221], [222] [223].

There is a growing body of evidence suggesting mast cells involvement in I/R-generated leukocyte recruitment. Existing data shows that mast cells are activated *i.e.* degranulate, during I/R. For example, Boros et al. have reported increased ROS-generated histamine release from post-ischemic tissue and histamine may participate in the leukocyte recruitment by generating endothelial P-selectin expression [224], [28]. Although histamine could be produced by numerous other cells, convincing

data of mast cell degranulation during I/R has later been presented by Kanwar et al. who also showed that pre-treatment with superoxide dismutase, a well known antioxidant, reduced the I/R-generated histamine-mediated leukocyte rolling and adhesion in the small bowel [225]. Activation of complement factors C3a and C5a has also been shown to play an important role after histamine release from cardiac mast cells [226] and these complement factors in turn have also been shown to contribute to I/R-induced tissue injury of the heart [227]. Additionally, mast cells are able to store synthesized TNF- $\alpha$ , which in turn has been shown to support leukocyte recruitment in the cremaster, a P-selectin mediated event [25], [228].

## **5. AIMS OF THE THESIS**

- I. To define the molecular basis of CXC chemokine-induced leukocyte recruitment in the colon.
  
- II. To investigate the role of mast cell in chemokine- and ischemia-reperfusion-provoked colonic leukocyte recruitment.
  
- III. To analyze the molecular mechanisms of p38 MAPK-regulated leukocyte recruitment in colonic ischemia-reperfusion.
  
- IV. To study the effect and mechanisms of action behind Rho-kinase-supported ischemia-reperfusion-induced leukocyte recruitment in the large intestine.
  
- V. To analyze the effects of NO-supplementation in colonic I/R-generated leukocyte recruitment.

## 6. MATERIALS AND METHODS

### 6.1 Animals

This chapter briefly outlines the animals, methods and analyses used in this study. More detailed information and protocols are found in the papers.

Adult male Balb/c (I & II), mast cell-deficient (WBB6F1, Jackson Laboratory, Bar Harbor, Maine, USA) (II) and C57/Bl6 (III, IV & Appendix) mice weighing between 22 and 28 grams were used. Animals were kept on a 12-12 hour light-dark cycle with free access to pellet food and tap water. After anaesthesia and analgesia, the right jugular vein was cannulated with a polyethylene catheter for intravenous administration of test substances, fluorescent dyes and additional anaesthesia. The ethical committee at Lund University approved all experiments.

### 6.2 Cells

The following cell-lines and protocols were used in the *in vitro* studies (III).

#### 6.2.1 Bone marrow mast cells

Mice were sacrificed and the bone shafts of tibias and femurs were flushed with sterile medium repeatedly and the bone marrow suspension were passed through a sterile filter to remove debris. The cells were centrifuged at 800 r.p.m. for 20 minutes at 4°C and then cultured at a concentration of  $0.5-1.0 \times 10^6$  cells/ml in RPMI 1640 with 10% fetal

calf serum (FCS), 50  $\mu$ M 2-mercaptoethanol and 20 ng/ml of IL-3. After 3 weeks, 40 ng/ml of SCF was added to the medium. The bone marrow mast cells (BMMC) medium was replaced 3 times a week and control of BMMC purity was performed after 4 weeks by 1% Toluidine blue staining of fixed cytocentrifuge preparations. Once the BMMC reached a purity of > 99% (after 5-10 weeks) the cells were used in subsequent experiments.

#### 6.2.2 Neutrophil isolation

Neutrophils were freshly extracted from C57/Bl6 mice by aseptically flushing the bone marrow of femurs and humeri with ice-cold PBS and then subsequently isolated by using Ficoll-Paque™ Research Grade (Amersham Pharmacia Biotech, Uppsala, Sweden). The purity of the isolated neutrophils was higher than 70% as assessed in a haematocytometer. The neutrophils were resuspended in culture medium until used in the adhesion assay.

#### 6.2.3 Endothelial cells

The polyoma transformed murine endothelial cell line eEnd.2 was cultured in DMEM with 10% FCS, L-glutamine, penicillin and streptomycin at 37°C and 5% CO<sub>2</sub>. The cells were subcultured twice weekly as previously described [229].



### **6.3 Surgical procedure**

In anaesthetized animals, a midline laparotomy was performed and colonic ischemia was induced by placement of a non-traumatic clamp over the superior mesenteric artery (SMA) at its aortic origin. Following 30 minutes of ischemia and 120 minutes of reperfusion the colon was gently exteriorized and analysis of colonic microcirculation by inverted intravital fluorescence microscopy was performed. An equilibration period of 5 minutes was allowed before initiation of microscopical observations.

After intravital observation, blood was drawn from the tail vein for analysis of systemic leukocyte counts, including polymorphonuclear leukocytes and monomorphonuclear leukocytes. After excision of a 5-6 cm long segment of the colon, the bowel was cut open along the antimesenteric border, gently rinsed and further analysed.

### **6.4 Intravital microscopy**

Observations of the colonic microcirculation were made using an inverted Olympus microscope (IX70, Olympus Optical Co. GmbH, Hamburg Germany) equipped with different lenses ( $\times 10/\text{NA } 0.25$  and  $\times 40/\text{NA } 0.60$ ). The microscopic images were televised using a charge coupled device video camera (FK6990 Cohu, Pieper GmbH, Schwerte, Germany) and recorded on videotape (Sony SVT-S3000PS-VHS recorder) for subsequent off-line analysis. Analysis of leukocyte-endothelium interactions (rolling and adhesion)

was made in venules (inner diameter 15-35  $\mu\text{m}$ ) in the submucosa with stable resting blood flow. Blood perfusion within individual micro-vessels was studied after contrast enhancement by *i.v.* administration of fluorescein isothiocyanate-labelled dextran 150000 (0.05 ml, 5 mg/ml, Sigma Chemical Co.). *In vivo* labelling of leukocytes with rhodamine 6-G (0.1 ml, 0.5 mg/ml, Sigma Chemical Co) enabled quantitative analysis of leukocyte flow behaviour in the colonic microcirculation. Quantification of microcirculatory parameters was performed off-line by frame-to-frame analysis of the videotaped images. Leukocyte rolling was determined by counting the number of leukocytes passing a reference point in the venule per 30 s and expressed as cells per min. Firm adhesion was measured by counting the number of cells adhering to the venular endothelium (200-300  $\mu\text{m}$  long segments) and remained stationary for 30 s and is given as cells per millimetre venule length. Blood flow velocities were measured off-line by use of CapImage<sup>®</sup> software (Zeintl, Heidelberg, Germany). The velocity was calculated as a mean value from five to eight measurements per venule and is expressed as mm per s. Venular wall shear rate was determined based on the Newtonian definition: wall shear rate =  $8 \cdot [\text{red blood cell velocity}/1.6]/\text{venular diameter}$  as described previously [230].

## 6.5 ELISA

For enzyme-linked immunoabsorbent assay (ELISA), a 3 cm long segment of colonic tissue was incubated in culture medium for 24 hours at 37°C. The medium was then stored in -20°C until ELISA analysis was performed. The levels of immunoreactive protein in the supernatant of the CXC chemokines MIP-2 and KC as well as TNF- $\alpha$  was determined by means of double-antibody specific Quantikine ELISA kits using recombinant MIP-2, KC (I – IV, Appendix) and TNF- $\alpha$  (IV) as standards (R&D Systems Europe, Abingdon, Oxon, U.K). Additionally, *in vitro* production of mast cell-derived TNF- $\alpha$  was quantified (R&D Systems Europe, U.K) after IgE and DNP challenge (III).

## 6.6 Reverse transcription polymerase chain reaction

Using Rneasy<sup>®</sup> Minikit (Quiagen GmbH, Hilden, Germany) total RNA was extracted from a segment of colonic tissue, treated with DNase (Amersham Pharmacia Biotech, Sollentuna, Sweden) and amplified with SuperScript One-Step RT-PCR system (Gibco BRL Life Technologies, Grand Island, NY) using the following primers:  $\beta$ -actin (f) 5'-ATG TTT GAG ACC TTC AAC ACC-3',  $\beta$ -actin (r) 5'-TCT CCA GGG AGG AAG AGG AT-3'; P-selectin (f) 5'-ACG AGC TGG ACG GAC CCG-3'; P-selectin (r) 5'-GGC TGG CAC TCA AAT TTA CAG-3'; E-selectin (f) 5'-GGT AGT TGC ACT TTC TGC GG-3'; E-selectin

(r) -CCT TCT GTG GCA GCA TGT TC-3'. Amplicons were subsequently separated on agarose gel (I & II).

## 6.7 Quantitative polymerase chain reaction

Colon samples were harvested, rinsed and kept in RNA isolation reagent (RNAlater) at -20°C. Total RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Next, samples were treated with RNase-free DNase (DNase I; Amersham pharmacy Biotech, Sollentuna, Sweden) to remove potential genomic DNA contaminants. RNA concentrations were determined by measuring the absorbance at 260 nm spectrophotometrically. Each cDNA was synthesized by reverse transcription from 5  $\mu$ g of total RNA using Stratascript<sup>®</sup> First-Strand Synthesis System (Stratagene, AH diagnostics, Stockholm, Sweden) and random hexamer primers. Quantitative PCR analysis was performed in a total volume of 25  $\mu$ l where each sample contained 125 ng cDNA using Brilliant SYBRgreen QPCR master mix and MX 3000 P QPCR detection system (Stratagene). The thermal cycling conditions entailed 40 cycles: denaturing at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min. All samples were tested in duplicate and normalized on the basis of their  $\beta$ -actin content. The specific primers were as follows:  $\beta$ -actin (f) 5'-ATG TTT GAG ACC TTC

AAC ACC-3',  $\beta$ -actin (r) 5'-TCT CCA GGG AGG AAG AGG AT-3'; P-selectin (f) 5'-ACG AGC TGG ACG GAC CCG-3'; P-selectin (r) 5'-GGC TGG CAC TCA AAT TTA CAG-3'; E-selectin (f) 5'-TAC TGT CAG CGG GC TAC AC-3'; E-selectin (r) 5'-GGC ACT TGC AGG TGT AAC TAT TG-3'; ICAM-1 (f) 5'-GTG ATG CTC AGG TAT CCA TCC A-3'; (r) 5'-CAC AGT TCT CAA AGC ACA GCG-3' (IV).

### **6.8 Western blot**

ATF-2 is a downstream intracellular substrate of activated *i.e.* phosphorylated p38 MAPK (pp38 MAPK). The activity of pp38 MAPK in endothelial cells challenged with TNF- $\alpha$  for 4 hours was assayed by incubating ATF-2 and immunoprecipitated pp38 MAPK with SB239063 or not (III).

### **6.9 MPO analysis**

A segment of colonic tissue was rinsed, homogenized and stored in  $-80^{\circ}\text{C}$ . Upon analysis, the supernatant of the freeze-thawed samples was assessed by spectrophotometry for MPO-activity by determination of the MPO-catalyzed change in absorbance of  $\text{H}_2\text{O}_2$  at 460 nm and  $25^{\circ}\text{C}$  (IV).

### **6.10 MDA analysis**

Lipid peroxidation is well known mechanism of reactive oxygen species-induced cellular injury and is used as and indicator of oxidative stress. Polyunsaturated fatty acid peroxides generate

malondialdehyde (MDA) upon decomposition. LPO-586<sup>TM</sup> is a colorimetric assay designed to quantify MDA. Colonic tissue were collected, rinsed and homogenized and prepared according to the manufacturers recommendation. Next, the absorbance of the supernatant was measured by spectrophotometry at 586 nm (IV).

### **6.11 Cell adhesion assay**

Subcultured endothelial cells (eEND. 2-origin) were plated at a density of  $2 \times 10^4$  cells per well in 96-well plates. When confluent, the cells were pre-treated with SB 239063 in different doses (1 - 100  $\mu\text{M}$ ) for 30 minutes prior to TNF- $\alpha$  challenge. The endothelial cells were stimulated with negative control medium or recombinant TNF- $\alpha$  (100 ng/ml, R&D Systems Europe) for 3.5 hours before exposure to neutrophils. Next,  $2 \times 10^4$  neutrophils were coincubated for 20 minutes and allowed to interact with the confluent endothelial cells. Subsequently, the wells were gently washed with PBS three times in order to remove non-adherent cells. The MPO activity was determined spectrophotometrically as the MPO-catalyzed change in absorbance occurring in the redox reaction of  $\text{H}_2\text{O}_2$ -tetramethylbenzidine (460 nm,  $25^{\circ}\text{C}$ ) (III).

### **6.12 Flow cytometry**

Subcultured endothelial cells (eEnd. 2-origin) were pre-treated with different doses (1 - 100  $\mu\text{M}$ ) of SB 239063 for 30

min and subsequently challenged with TNF- $\alpha$  at a final concentration of 100 ng/ml for 4 hours. The endothelial cells were then isolated by trypsination and isolated cells were centrifuged, washed with PBS and mixed with fluorescein isothiocyanate-labelled antibody directed against P-selectin (RB40.34, rat IgG, Pharmingen) or a fluorescein isothiocyanate-labelled control antibody (R3-34, rat IgG, Pharmingen) for 20 min in the dark at 4°C. Washed and resuspended cells were kept on ice in the dark until analysis in a flow cytometer (Coulter Epics XL-MCL Beckman Coulter) was performed (III).

### 6.13 Drugs, antibodies and reagents

To induce anaesthesia and analgesia animals received an i.p. injection of 7.5 mg ketamine hydrochloride and 2.5 mg xylazine per 100 g body weight (I – IV, Appendix). CXC chemokine and TNF- $\alpha$ -induced leukocyte rolling and adhesion were analyzed by injecting 3 – 300 ng of MIP-2, KC (I – III) and TNF- $\alpha$  (II) (in 0.2 ml PBS) (R&D Systems Europe) *i.p.* 3 hours prior to intravital microscopy. Immunoneutralization of selectins was achieved by injecting 20 – 100  $\mu$ g of a monoclonal antibody directed against murine P- (rat IgG RB40.34, R&D Systems Europe), E-selectin (rat IgG 10E9.6, R&D Systems Europe), PSGL-1 (rat IgG 2PH1, R&D Systems Europe) and an isotype-matched control antibody (rat IgG R3-34,

R&D Systems Europe) *i.v.* 5 minutes prior to *i.p.* chemokine, TNF- $\alpha$  challenge or clamping of the SMA.

To characterize p38 MAPK, Rho-kinase activity and NO-supplementation in I/R-induced leukocyte recruitment, *i.v.* and/or *i.p.* and *s.c.* pre-treatment with 0.01 – 1.0 mg/kg of SB239063 (*trans*-1-(4-Hydroxycyclohexyl)-4-(4-fluorophenyl)-5-(2-methoxypyridimidin-4-yl)imidazole), (Sigma Chemical Co, St Louis, USA), 4 – 40 mg/kg of fasudil (HA-1077 dihydrochloride, 1-(5-Isoquinolinylulfonyl)homopiperazine dihydrochloride) (Sigma Chemical Co) and 1 – 10 mg/kg of Y27632 (Y27632 dihydrochloride monohydrat, (*R*)-(+)-*trans*-4-(1-Aminoethyl)-N-(4-Pyridyl) cyclohexanecarboxamide dihydrochloride monohydrat, (Sigma Chemical Co), 0.1 – 10 mg/kg of DETA-NO (NOC 18, 3,3-Bis (aminoethyl)-1-hydroxy-2-oxo-1-triazene), (Sigma Chemical Co) was performed (III, IV, Appendix).

### 6.14 Statistical analyses

Data are given as mean values  $\pm$  SEM and *n* represents the number of animals per experimental group. A computer package program (SigmaStat 3.5, Jandel Scientific, USA) was used to assess statistically significant differences between groups. Statistical evaluations were performed using Kruskal-Wallis one-way ANOVA on ranks for unpaired samples (Dunn's post hoc test). Differences between groups were considered statistically significant when  $p < 0.05$ .

## **7. RESULTS AND DISCUSSION**

### **7.1 PSGL-1-mediated leukocyte recruitment in the colon (I)**

Convincing evidence has demonstrated that leukocyte recruitment is a rate-limiting step in inflammatory intestinal disease [120], [15]. Moreover, it has been shown that infiltration of leukocytes comprise a multistep process, where an initial rolling is a prerequisite to leukocyte firm adhesion and subsequent transmigration [30]. Leukocyte rolling along the vascular endothelium is critically dependent on endothelial cell-expression of P-selectin although other candidates have been suggested [123], [133]. The selectin ligands, including P-selectin glycoprotein ligand-1 (PSGL-1), are in general poorly investigated with regards to their selectin-specificity but PSGL-1 has been proposed as a high-affinity receptor for P-selectin although data implicating E-selectin affinity also exist [231], [231]. It has recently been shown that I/R-generated leukocyte rolling in colonic microvasculature is supported by the selectin family of adhesion molecules, especially P-selectin [138]. Additionally, emerging evidence suggests that chemokines are fundamental mediators in orchestrating tissue specific localization of leukocytes [70]. However, the role of selectins and their ligands in mediating leukocyte recruitment in the colonic microcirculation is elusive and partly contradictory.

The objective of the first study of this thesis was to clarify the molecular mechanisms behind CXC and I/R-induced leukocyte rolling and adhesion in the colon.

By using intravital microscopy, analysis of leukocyte recruitment in the colonic microvascular bed challenged with the CXC chemokine MIP-2 showed a dose-dependent increase in leukocyte rolling and adhesion. Inhibition of P-selectin-mediated rolling by the use of a monoclonal antibody decreased MIP-2-provoked leukocyte rolling and adhesion by 96% and 81%, respectively. Administration of an anti-E-selectin antibody had no effect on leukocyte recruitment after MIP-2 challenge. These observations indicate that MIP-2-induced leukocyte rolling is a P-selectin mediated event and that MIP-2 also regulates leukocyte adhesion, which is considered as a down-stream event of leukocyte rolling [43]. Knowing that CXC chemokines plays a key role in P-selectin supported leukocyte rolling and adhesion in colonic I/R [78], it was also of interest to examine the function of PSGL-1 in MIP-2 and I/R-induced leukocyte recruitment. Hence, subjecting MIP-2 and I/R-challenged mice to pretreatment with a monoclonal antibody towards PSGL-1 decreased leukocyte rolling and adhesion by more than 85% and 93%, respectively. This conclusion expands on previous studies showing

a dominant role of PSGL-1 in mediating leukocyte rolling in venules of the heart and liver [155], [156]. However, it should also be noted that P-selectin and PSGL-1 did not completely abolish CXC- and I/R-induced leukocyte rolling in the colon indicating that other adhesion molecules to mediate the residual P-selectin-PSGL-1-independent leukocyte-endothelial cell interactions.

In summary, study I suggests an important function for PSGL-1 and P-selectin in CXC chemokine and I/R-induced leukocyte-endothelial cell interaction in colonic microcirculation. These findings indicate that PSGL-1/P-selectin not only mediate leukocyte rolling in colonic inflammation, but might also constitute a potential target in the development of new therapies directed against pathological inflammation in the colon.

## **7.2 Mast cell-regulated CXC chemokine and I/R-induced leukocyte recruitment (II)**

Mast cells have the capacity to release chemokines and other pro-inflammatory mediators as a response to certain stimuli like ROS, bacterial toxins and complement factors and may play a role in inflammatory leukocyte recruitment, which has been suggested previously [222], [25]. The generation of oxygen free radicals is a key component in the pathogenesis of I/R-induced tissue damage in the gastrointestinal tract [233], [234]. Recently published data indicate

that ROS produced during I/R promote CXC chemokine-induced leukocyte rolling and adhesion in the colon [78]. The aim of the second study was to determine the regulatory role of mast cells in CXC- and I/R-provoked leukocyte recruitment.

Intraperitoneal (*i.p.*) challenge with 300 ng MIP-2, KC and TNF- $\alpha$ , in wild type mice, induced a significant increase in the number of rolling and adhering leukocytes in the colonic microvascular bed, as compared to PBS treated animals. Moreover, TNF- $\alpha$  induced a clear-cut increase in the number of adhering leukocytes in mast cell-deficient mice. On the other hand, neither MIP-2 nor KC was able to increase leukocyte rolling and adhesion. In fact, leukocyte rolling and adhesion decreased by more than 90% in mast cell-deficient animals. These results indicate that CXC chemokine-provoked leukocyte rolling and adhesion in the colonic microvasculature is indeed regulated by mast cells.

To further investigate the molecular mechanisms behind I/R-generated leukocyte recruitment, wild type and mast cell-deficient mice were subjected to colonic I/R. Accordingly, 30/120 minutes of I/R rendered a significant increase in the number of rolling and adhering leukocytes in wild type animals, as compared to sham operated animals. Interestingly, leukocyte-endothelial cell interactions in mast cell-deficient animals subjected to I/R were decreased by more than 57%. Additio-

nally, I/R increased the colonic formation of MIP-2 and KC in wild type but not in mast cell-deficient mice. These results indicate that up-regulation of CXC chemokines in colonic I/R may stimulate the expression of adhesion molecules such as P-selectin, which in turn promote leukocyte-endothelium interactions. However, genetic modified mice lacking mast cells are unable to initiate leukocyte recruitment as a response to CXC chemokine- and I/R-treatment. Knowing that endothelial cells lack the capacity to express the receptor (CXCR2) for MIP-2 and KC, direct activation of endothelium by CXC chemokines are not possible [235], [228]. On the other hand, TNF- $\alpha$  treatment in mast cell-deficient mice increased firm leukocyte adhesion, which shows that endothelial cells in mast cell-deficient animals indeed are able to support leukocyte-endothelial cell interactions. Mast cells, on the other hand, do express CXCR2 and activated mast cells are able to release a number of pro-inflammatory substances, like histamine, TNF- $\alpha$ , PAF and chemokines, which in turn are able to not only activate endothelial cells but also act as chemoattractants for activated leukocytes [223].

In summary, study II suggests that mast cells are important regulators of CXC chemokine and I/R-provoked leukocyte rolling and adhesion in the colon. Thus, inhibiting mast cell activation could be an effective therapy to reduce tissue injury in CXC and I/R-generated inflammation in the colon.

### **7.3 p38 MAPK activity in colonic ischemia-reperfusion (III)**

p38 MAPK is one of three major mitogen-activated protein kinase pathways transmitting extracellular stimuli, such as microbial infections, cytokines and I/R-derived pro-inflammatory mediators into intracellular responses within the cytoplasm and nucleus [236]. The detailed mechanisms behind p38 MAPK activity are not known. It has been suggested that p38 MAPK is an important regulator in the generation of pro-inflammatory mediators such as chemokines and TNF- $\alpha$  even though the detailed molecular mechanisms behind these observations are not known [237], [238]. Interference with p38 MAPK has been proposed to have a protective role in various inflammatory disease models of the heart, intestine and liver [196], [199], [239]. However, the role of p38 MAPK activity in I/R-induced colonic leukocyte recruitment is elusive. The objective of the third study was to define the role of p38 MAPK activity in I/R- and CXC chemokine-induced leukocyte rolling and adhesion in the colon as well as in TNF- $\alpha$ -induced neutrophil adhesion and P-selectin expression on endothelial cells *in vitro*.

In accordance with previous studies, induction of I/R by clamping of the SMA induced a significant increase in the number of rolling and adhering leukocytes as compared to sham-operated animals. Intravenous pre-treatment with the selective p38 MAPK inhibi-

tor SB239063 2 hours before I/R, decreased I/R-provoked leukocyte rolling and adhesion by 91% and 98%, respectively. This was also observed in animals challenged with *i.p.* pre-treatment of SB239063 for 30 and 240 minutes before induction of I/R. Interestingly, administration of SB239063 had no effect on I/R-generated MIP-2 and KC in the colon. Knowing that MIP-2 and KC are potent inducers of leukocyte recruitment, i.e. rolling and adhesion, in the colon, it was also of great interest to evaluate the role of p38 MAPK in chemokine-generated leukocyte recruitment in the colon. Challenge with 300 ng MIP-2 and KC for 3 hours induced leukocyte rolling and adhesion in the colonic microvasculature as previously observed [240]. However, *i.v.* pre-treatment with SB239063 30 minutes before induction of I/R decreased CXC-induced leukocyte rolling and adhesion significantly, as compared to PBS treated animals.

To further elucidate the molecular mechanisms behind the regulatory role of p38 MAPK in leukocyte recruitment, a series of *in vitro* assays were performed. Since I/R-induced leukocyte recruitment is dependent on mast cells and the fact that mast cells are potent producers of TNF- $\alpha$ , which in turn has the capacity to activate endothelial cells, lead us to question whether p38 MAPK may regulate TNF- $\alpha$  generation from activated mast cells. Hence, IgE/DNP stimulation of mast cells induced a significant increase in TNF- $\alpha$  production,

which was effectively inhibited by p38 MAPK interference. Next, the capacity of endothelial cells to increase their P-selectin expression after TNF- $\alpha$  stimulation was investigated. It was found that the P-selectin expression increased from 27% in PBS treated to 73% in TNF- $\alpha$  treated endothelial cells but pre-treatment with SB239063 effectively decreased the TNF- $\alpha$ -generated increase in P-selectin expression. Moreover, we examined the effect of p38 MAPK inhibition in TNF- $\alpha$ -generated neutrophil-endothelial interactions, in which MPO was used as a marker for neutrophil adhesion. p38 MAPK inhibition did indeed reduce TNF- $\alpha$ -provoked neutrophil-endothelial cell adhesion, as detected by decreased MPO levels after SB239063 pre-treatment, significantly.

In summary, study III suggests an important regulatory role of p38 MAPK in I/R- and CXC chemokine-induced leukocyte recruitment in the colon. Reduction of mast cell derived TNF- $\alpha$  as well as decreased levels of TNF- $\alpha$ -generated endothelial cell expression of P-selectin is a possible explanation behind the findings in this study. Thus, p38 MAPK inhibition could be an effective treatment to prevent leukocyte infiltration in colonic inflammatory disease.



## **7.4 Rho-kinase inhibition in ischemia-reperfusion (IV)**

In the process of transmitting an extracellular signal into an intracellular response *in vivo* and *in vitro* studies have elucidated the potential involvement of intracellular signaling pathways mediated by small GTP-binding proteins, for example the Rho-family, which in turn activate down-stream Rho-kinases [241]. Interestingly, experimental data indicate that Rho-kinase activity regulates not only vascular tone, cytoskeleton reorganization, cellular morphology, motility and adhesion, but has also been described to be involved in the pathogenesis of I/R-injury [242], [243], [244], [245]. Selective Rho-kinase inhibition with the structurally different substances fasudil and Y27632 have indicated beneficial effects in limiting the I/R-induced tissue injury in the heart, brain and kidney [245], [246], [213]. However, the regulatory role of Rho-kinase activity in the reperfused colon has not been examined. Based on these considerations and by using the selective Rho-kinase inhibitors fasudil and Y27632, the aim of the fourth study was to investigate the molecular mechanisms behind Rho-kinase activity in colonic I/R-generated leukocyte recruitment.

Colonic leukocyte rolling and adhesion was observed to increase significantly after 30 minutes of ischemia followed by 120 minutes of reperfusion from  $3.4 \pm 1.3$  cells/min and  $2.0 \pm 0.4$  cells/mm in sham operated animals, to

$54.3 \pm 7.0$  cells/min and  $92.3 \pm 18.9$  cells/mm, after I/R. Fasudil and Y27632 *i.p.* pre-treatment decreased I/R-induced leukocyte rolling and adhesion by more than 76% and 96%, respectively. Earlier obtained data indicate that I/R-induced colonic leukocyte recruitment is mediated through the CXC chemokines MIP-2 and KC [78]. Hence, the colonic content of MIP-2 and KC were analyzed in different groups of mice subjected to sham operation, I/R and fasudil/Y27632 pre-treatment + I/R, respectively. Interestingly, *i.p.* pre-treatment with fasudil and Y27632 decreased I/R-provoked MIP-2 and KC generation in the large intestine by 78% and 68%, respectively, which also has been described by Bao et al in an acute I/R-model of the heart [211]. Moreover, it was found herein that I/R-induced colonic TNF- $\alpha$  production was decreased by 76% after fasudil and Y27632 pre-treatment, indicating that Rho-kinase inhibition not only decrease the chemotactic ability on leukocytes to extravasate by reducing the availability of the CXC chemokines MIP-2 and KC, but also interfere in the endothelial cell activation process by attenuating the levels of TNF- $\alpha$ .

Tissue MPO activity is regarded as a marker for neutrophil accumulation and has previously been observed to increase after I/R in the intestine [247]. After 30 min of ischemia followed by 120 min of reperfusion the MPO activity increased by 84%, from  $0.12 \pm 0.03$  U/g in sham operated ani-

mals to  $0.74 \pm 0.16$  U/g after I/R. Challenge with either fasudil or Y27632 for 2h decreased the I/R-provoked colonic MPO activity by 68% and 78%, respectively. Moreover, MDA-production, a marker for lipid peroxidation i.e. oxidative stress, increased by 48%, from  $5.20 \pm 0.16$  U/g to  $10.16 \pm 0.15$  U/g, in the reperfused colon. Challenge with fasudil for 2 hours decreased the I/R-generated colonic content of MDA by 42%. In an I/R model of the liver Takeda et al [248] observed not only an increased one-week survival, from 25% to 75%, along with improved histological architecture and liver function tests after Rho-kinase inhibition, but also attenuated MPO activity and MDA levels in the reperfused liver, indicating a protective role against neutrophil tissue infiltration and lipid peroxidation. Decreased levels of MPO activity, along with reduced infarction size and improved neurological function, has also been observed after fasudil challenge in I/R-induced brain injury [249]. The colonic mRNA content of P- and E-selectin and ICAM 1 was found to increase after I/R. Rho-kinase inhibition by i.p. fasudil and Y27632 pre-treatment decreased the I/R-generated mRNA production of P-, E- and ICAM-1 amplicons by more than 41%, 81% and 25%, respectively.

Taken together, study IV suggests a protective role of Rho-kinase activity in I/R-induced CXC mediated leukocyte recruitment in the colon and selective inhibition by fasudil or Y27632

could be an effective strategy in preventing I/R-generated tissue damage in the large intestine.

## **7.5 NO supplementation and colonic leukocyte recruitment (Appendix)**

I/R-provoked tissue injury is characterized by cellular oxidative stress, platelet-leukocyte aggregation, increased microvascular permeability and leukocyte recruitment into the affected tissue. During early reperfusion, profound changes in the production of nitric oxide (NO) and ROS are observed, with decreased production of the former and increased of the latter. It has been suggested by some investigators that this imbalance could be an important pathophysiological mechanism behind I/R-induced tissue damage observed in inflamed tissue [250], [251]. There are several lines of evidence implicating the bioavailability of NO as an important modulator of I/R-generated inflammatory changes in the reperfused tissue. These include; decreased activity of the  $\text{Ca}^{2+}$ -dependent NO synthase isoforms nNOS and eNOS, treatment with NO synthase inhibitors mimics the microvascular alterations seen during I/R (leukocyte recruitment, mast cell degranulation and increased vascular permeability), NO donors like diethylenetriamine/NO (DETA-NO) protects against experimentally induced I/R [101], [88], [252]. On the other hand convincing experimental evidence exist

regarding the ability of various ROS-metabolites to induce inflammatory changes in tissue during reperfusion including TNF- $\alpha$  generation, triggering of endothelial cell and leukocyte expression of adhesion molecules and increased chemokine production from endothelium and tissue resident cells like macrophages and mast cells [78], [253]. The protective role of NO supplementation during splanchnic I/R is poorly understood. Based on these considerations, the aim of this study was to analyze the effects of NO pre-treatment with DETA-NO (half-life time of  $\sim$  56 hours) on leukocyte-endothelial cell interactions in colonic I/R.

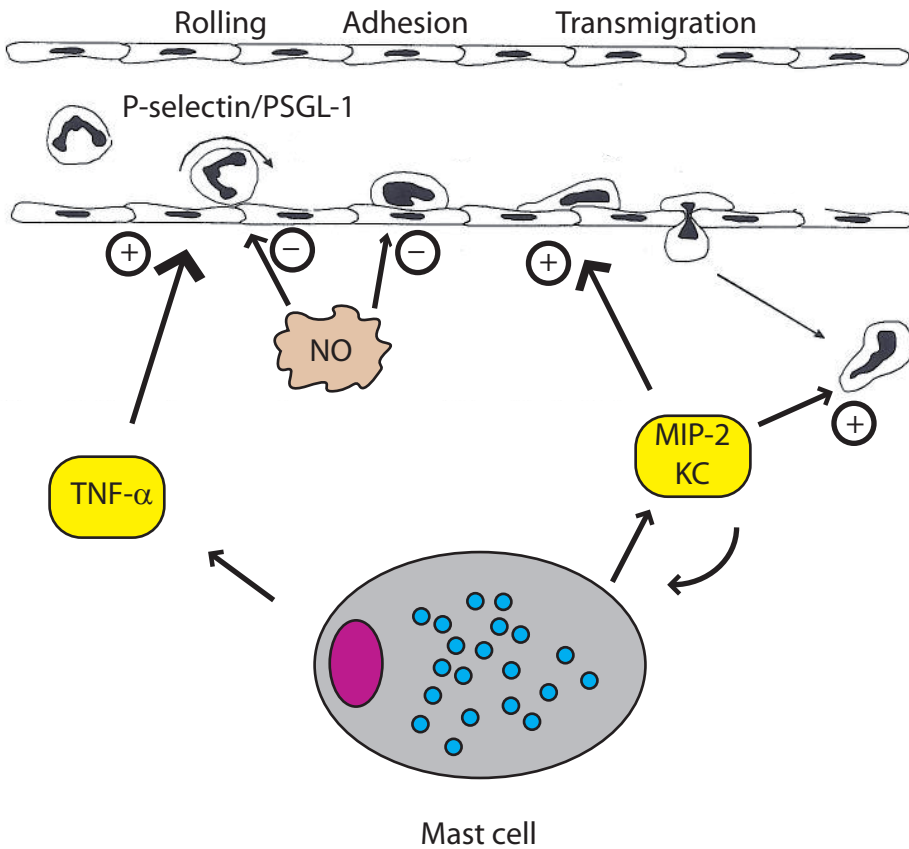
In order to induce leukocyte rolling and adhesion, 30 minutes of SMA clamping followed by 120 minutes of reperfusion was performed. The number of observed leukocytes that rolled and adhered along the microvascular endothelium increased significantly after I/R, to  $40.0 \pm 4.3$  cells/min and  $32.1 \pm 9.7$  cells/mm, respectively (Appendix, Fig 1a and b,  $P < 0.05$  vs Sham,  $n = 5-7$ ). Pre-treatment with different doses of DETA-NO (0.1 – 10 mg/kg) subcutaneously four hours before the induction of I/R reduced I/R-provoked leukocyte rolling and adhesion by 61% and 81% i.e. down to  $15.7 \pm 4.3$  cells/min (10 mg/kg) and  $6.0 \pm 1.2$  cells/mm (0.1 mg/kg), respectively (Appendix, Fig 1a and b,  $P < 0.05$  vs Vehicle,  $n = 5-7$ ). Interestingly, the colonic production of MIP-2 (Appendix, Fig 1c) and KC (Data not shown) were not reduced af-

ter DETA-NO challenge as compared to PBS (Vehicle + I/R)-treated animals, but instead showed a tendency to increase after NO pre-treatment. By using western blot analysis, phosphorylation of p38 MAPK (pp38 MAPK) i.e. p38 activation, was analyzed. Interestingly, DETA-NO pre-treatment attenuated I/R-induced phosphorylation of p38 MAPK in colonic tissue as compared to PBS treated animals (Appendix, fig 2).

In different models of I/R it has been shown that reperfusion induces production of pro-inflammatory mediators, like chemokines, leukotrienes and TNF- $\alpha$  [78], [254]. In this study a protective role of NO supplementation in colonic I/R is suggested. Hence, after pre-treatment with DETA-NO, a NO donor with enhanced half-life time, the observed numbers of rolling and adhering leukocytes were significantly decreased. The mechanisms behind this observation are unclear, but enhancing the bioavailability of NO by DETA-NO pre-treatment attenuated I/R-induced phosphorylation i.e. activation of p38 MAPK. Moreover, p38 MAPK is a well-known regulator of down-stream transcription factors (NF- $\kappa$ B) that controls the expression of pro-inflammatory cytokines, for example TNF- $\alpha$  generation [255]. In addition, it has been shown that I/R-induced leukocyte recruitment is governed by expression of the CXC chemokines MIP-2 and KC [78]. However, in this study, the detectable levels of MIP-2 and KC did not decrease in DETA-NO challenged I/R-

provoked animals but instead showed a tendency to increase.

In summary, this study shows that pre-treatment with DETA-NO decrease leukocyte rolling and adhesion in the colonic microcirculation. This observation could be the result of decreased p38 MAPK activity in the reperfused colon.



**FIGURE 3.** Suggested pathophysiological mechanisms of ischemia-reperfusion-induced leukocyte recruitment in the colon.

## **8. MAJOR CONCLUSIONS**

- I. The CXC chemokines MIP-2 and KC induce leukocyte rolling and adhesion in colonic microvasculature of mice.
- II. P-selectin glycoprotein ligand-1 and P-selectin supports ischemia-reperfusion- and CXC chemokine-induced leukocyte-endothelial interactions in the colon.
- III. Mast cells regulates CXC chemokine and ischemia-reperfusion-generated leukocyte recruitment in the large intestine.
- IV. Inhibition of p38 MAPK activity attenuates CXC- and ischemia-reperfusion-provoked leukocyte rolling and adhesion in colonic postcapillary venules.
- V. p38 MAPK regulates TNF- $\alpha$  generated P-selectin expression and neutrophil-endothelial cell adhesion *in vitro*.
- VI. Rho-kinase inhibition abrogates ischemia-reperfusion-induced leukocyte recruitment in the colon by decreasing the production of MIP-2 and KC.
- VII. NO supplementation decreases leukocyte rolling and adhesion in the reperfused colon and inhibits p38 MAPK phosphorylation.

## 9. POPULÄRVETENSKAPLIG SAMMANFATTNING

Ett effektivt immunförsvar är en förutsättning för upprätthållande av liv. Vid infektion aktiveras vårt immunförsvar vilket initierar inflammation (svullnad, rodnad, hetta och smärta) lokalt i vävnaden med uppgift att begränsa skadeeffekterna samt eliminera de främmande mikroberna. I vissa situationer kan dock inflammationen bli så uttalad att skada sker på värdens egen vävnad lokalt men även i organ som ej är direkt lokaliserade intill det primära skadeområdet. Vid sjukdomar såsom ledgångsreumatism, inflammatorisk tarmsjukdom och ms angriper immunförsvarets egna vita blodkroppar (leukocyter) kroppsegen vävnad varvid vävnadsskada kan uppstå. Ett annat sådant tillstånd är tarmischemi med efterföljande reperfusion, det vill säga avstängning och påflöde av blod till tarmen, varvid det inflammatoriska svaret kan bli så uttalat att vävnadsskada kan uppstå lokalt men även i andra organ såsom i hjärta, lever, lungor och njurar. Vid inflammatoriska processer rekryteras och aktiveras leukocyter samt celler (endotelceller) i kärlväggen av specifika signalsubstanser vilka frisätts ifrån den drabbade vävnaden. Leukocyter och endotelceller uttrycker specifika molekyler, så kallade adhesionsmolekyler, på sin yta vilket möjliggör för leukocyterna att fångas upp (rullning) och fastna (adhesion) på kärlväggen och slutligen vandra ut (migration) i vävnaden vilket

sker emellan endotelcellerna. Leukocytmigrationen utgör en begränsande faktor för inflammationens utbredning och skadeverkan i vävnaden. Målet med denna avhandling var att definiera några av mekanismerna bakom leukocytrekrytering orsakad av ischemi-reperfusion (I/R) i tjocktarmen, ett vanligt förekommande tillstånd efter trauma och kirurgiska ingrepp på kroppspulsådern.

Tidigare arbeten har indikerat att vissa specifika signalsubstanser, så kallade kemokiner, är viktiga för leukocyternas rekrytering till vävnaden. Det är dock oklart vilken betydelse dessa har för leukocytrekryteringen i tjocktarmen vid I/R. Det är ej heller klarlagt vilka adhesionsmolekyler på leukocyter och endotel som möjliggör leukocytrekryteringen men tidigare kända experimentella data indikerar att en på endotelet förekommande adhesionsmolekyl, P-selektin, kan vara av betydelse. I första och andra arbetena har således två specifika kemokiner, MIP-2 samt KC, identifierats som viktiga signalsubstanser vid leukocytrekrytering i tjocktarmen och detta möjliggörs av interaktion emellan P-selektin på endotelcellerna samt PSGL-1-molekyler på leukocyterna. I delarbete två har även mastceller, viktiga regulatoriska celler vilka ofta finns lokaliserade invid blodkärl, identifierats som möjlig ursprungscell för kemokin-medierad

leukocytrekrytering i tjocktarmen vid ischemi-reperfusionsskada.

Cellulära stimuli, såsom bakteriertoxiner, kemokiner och I/R initierar aktivering av enskilda celler genom att påverka viktiga intracellulära processer. Detta sker ofta genom så kallade signalkaskader vilka reglerar den enskilda cellens svar på yttre stimuli. I eukaryota celler finns det tre viktiga signalkaskadssystem för denna reglering och en av dessa är den så kallade mitogenaktiverade proteinkinasevägen (MAPK). I tredje arbete har således p38 MAPK, ett viktigt protein som har visat sig reglerar gentranskription, celldifferentiering och proteinsyntes, studerats vid I/R. Med hjälp av en specifik inhibitor av p38 MAPK, SB239063, kunde leukocytrullning och adhesion i vävnad vid I/R blockeras. Vidare undersökning av de molekylära mekanismerna av p38 MAPK-medierad leukocytrekrytering i endotelcells-försök visade att leukocyttadhesion regleras av TNF- $\alpha$ , producerat av mast celler, genom att denna signalsubstans ökar uttrycket av adhesionsmolekylen P-selektin på endotelcellernas yta. P-selektin uttrycket kunde förhindras med hjälp av SB239063 det vill säga p38 MAPK interferens.

Rho proteiner är en familj av små proteiner vilka initierar och aktiverar olika cellulära processer genom så kallad fosforylering, bland annat Rho-kinaser vilka i sin tur har beskrivits vara viktiga komponenter i organisation av cellskelett, -rörlighet och -adhesion. I fjärde arbetet har Rho-kinase aktive-

ring vid I/R-inducerad leukocytrekrytering undersökts. Med hjälp av två inhibitorer av Rho-kinase, fasudil och Y27632, kunde leukocytrullning och adhesion vid I/R förhindras. Efter förbehandling med fasudil eller Y27632 kunde det även konstateras att syntesen av MIP-2, KC och TNF- $\alpha$  minskades, leukocytmigrationen ut i vävnaden avtog samt att den producerade mängden fria radikaler avtog.

Kvävemonoxid (NO) är en endogent producerad gas med viktig regulatorisk funktion för bland annat kärlsammandragning och därmed blodtryck, signaltransmission i nervändar samt blodplättars förmåga att aggregera. NO produceras också vid bakteriella angrepp som försvar och har då en toxisk effekt på mikrober men även omgivande vävnad. Experimentella data finns talande för att NO har en viktig skyddande funktion vid I/R. Leukocytrekryteringen i tjocktarmen undersöktes och befanns således minska efter förbehandling med en NO-donator. Möjligen regleras detta via p38 MAPK-aktivitet.

Sammanfattningsvis har det i denna avhandling beskrivits och identifierats flera viktiga mekanismer involverade i ischemi-reperfusionens orsakad skada på tjocktarmen. P-selektin och PSGL-1 är adhesionsmolekyler vilka är essentiella vid kemokin-medierad leukocytrekryteringen i tjocktarmen. Detta arbete beskriver även mast cellers regulatoriska funktion vid I/R. Således noterades att mast celler är krusiella för initiering av

kemokin och I/R-medierad leukocytkrytering. Slutligen visade sig SB239063, fasudil samt Y27632 ha skyddande effekt emot leukocytkryteringen vid I/R i tjocktarmen. Resultaten i denna avhandling kan öka förståelsen för de patofysiologiska mekanismer vilka verkar vid ischemi-reperfusionsskada i kolon och därmed bidra till mer specifika och effektiva behandlingsstrategier vid inflammatorisk sjukdom i tjocktarmen.



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## **PAPER I**



## P-selectin glycoprotein ligand-1 regulates chemokine-dependent leukocyte recruitment in colonic ischemia-reperfusion

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**Abstract.** *Objective and design:* Leukocyte recruitment is a key feature in ischemia-reperfusion (I/R)-provoked tissue injury. This study evaluated the role of P-selectin-glycoprotein ligand-1 (PSGL-1) in CXC chemokine- and ischemia-reperfusion-induced leukocyte rolling and adhesion in the colon.

*Materials* Balb/c mice were used in an inverted intravital fluorescence microscopy study of the microvascular bed in the colon.

*Treatment:* Mice were challenged with macrophage inflammatory protein-2 (MIP-2) intraperitoneally and leukocyte-endothelium interactions were analysed 3 h later. In separate experiments, mice were exposed to I/R by clamping of the superior mesenteric artery for 30 min and leukocyte rolling and adhesion were analysed after 120 min of reperfusion.

*Results:* MIP-2 dose-dependently increased leukocyte rolling and adhesion in the colon. Pretreatment with an anti-PSGL-1 antibody reduced MIP-2-provoked leukocyte rolling and adhesion by more than 89%. I/R increased expression of MIP-2 as well as leukocyte rolling and adhesion. Immunoneutralization of PSGL-1 decreased reperfusion-induced leukocyte rolling by 85% and adhesion by 93% in colonic venules.

*Conclusions:* Our data demonstrates that PSGL-1 is a dominant adhesion molecule supporting MIP-2- and I/R-provoked leukocyte rolling. Inhibition of PSGL-1 abolished leukocyte rolling and abrogated I/R-induced leukocyte adhesion in colonic venules. These findings suggest that targeting PSGL-1 may be an effective strategy to prevent I/R-induced inflammation in the colon.

**Key words:** Adhesion – Colon – Inflammation – Ischemia – Microcirculation

### Introduction

Splanchnic ischemia is frequently encountered in cardiovascular and transplantation surgery as well as in trauma, bowel strangulation, hemorrhagic shock and in the abdominal compartment syndrome [1]. Reperfusion of the ischemic intestine is characterized by inflammatory changes, including massive recruitment of leukocytes, which in turn, cause excessive tissue damage [2]. A growing body of evidence suggests that chemokines are fundamental in orchestrating tissue specific localization of leukocytes [3]. Chemokines are divided into several different families based on structural properties. For instance, CC chemokines mainly attract monocytes and lymphocytes whereas CXC chemokines, such as macrophage inflammatory protein-2 (MIP-2) and cytokine-induced neutrophil chemoattractant (KC) stimulate migration of predominantly neutrophils [4]. Interestingly, a recent study showed that CXC-chemokines plays a key role in mediating leukocyte accumulation in ischemia/reperfusion (I/R) [5]. However, the adhesive mechanisms behind CXC chemokine-induced leukocyte recruitment in the colon remain elusive.

In general, it is widely held that leukocyte recruitment is a multistep process initiated by leukocyte rolling along activated endothelium followed by firm adhesion and transendothelial migration [6]. Several investigations have shown that leukocyte rolling is supported by the selectin family of adhesion molecules (L-, P-, and E-selectin) although their relative roles appear to be both tissue and stimulus-dependent [7–9]. Moreover, the selectin ligands are complex glycoproteins and, in terms of selectin specificity and function, are poorly understood. Nonetheless, P-selectin glycoprotein ligand-1 (PSGL-1) is a well-accepted high affinity ligand of P-selectin although PSGL-1 also binds to E-selectin [10, 11]. In addition, E-selectin ligand-1 (ESL-1) has been shown to exhibit high-affinity to E-selectin although a functional role of ESL-1 in vivo remains to be demonstrated [12]. The L-selectin ligands are mainly expressed in lymphoid organs although data support the presence of an inducible L-selectin ligand also on non-lymphoid endothelial cells [13]. Firm adhesion



and transmigration are mediated by the integrin superfamily of adhesion molecules, including  $\beta$ 1- and  $\beta$ 2-integrins [14, 15]. Notably, leukocyte rolling is a prerequisite for the subsequent firm adhesion and tissue accumulation, which makes the rolling adhesive interaction a suitable therapeutic target [16]. Importantly, the adhesive mechanisms behind leukocyte accumulation in the colon are not well-known due to a previous lack of methods to study leukocyte-endothelium interactions in the large bowel. Indeed, the fact that there are organ specific differences in the expression of adhesion molecules together with the immense colonic bacterial load underlines the importance of studying individual microvascular beds in order to clarify the specificity and characteristics of leukocyte recruitment.

Based on the above considerations, the aim of the present study was first to define the adhesive mechanisms behind MIP-2-induced leukocyte recruitment in the colonic microvasculature. Once defined, we aimed to investigate the role of such adhesive mechanisms also in I/R-induced leukocyte recruitment in the large bowel. For this purpose, we used an inverted intravital fluorescence microscopic technique, which allows detailed studies of leukocyte-endothelium interactions in the colon.

## Materials and methods

### Animals

Male Balb/c mice weighing ~23–28 g were kept under standard laboratory conditions. Animals were maintained on a 12-h light and 12-h dark cycle and were allowed free access to food and water ad libitum. All experimental procedures were approved by the Regional Ethical Committee for Animal Experimentation at Lund University.

### Anesthetic and surgical preparation

The mice were anesthetized with 7.5 mg ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 2.5 mg xylazine (Janssen Pharmaceutica, Beerse, Belgium) per 100 g body weight intraperitoneally (*i.p.*). The animals were placed in a supine position on a heating pad for maintenance of body temperature (37 °C). A polyethylene catheter (PE-10 with an internal diameter of 0.28 mm) was placed into the internal jugular vein for administration of fluorescent markers. A midline laparotomy was performed and the colon was subsequently exteriorized.

### Experimental protocol

The effects and mechanisms behind MIP-2-induced leukocyte rolling and adhesion were analyzed by injecting 3, 30 or 300 ng of MIP-2 (R&D Systems Europe, Ltd, Abingdon, Oxon, U.K) per mouse in 0.2 ml PBS *i.p.* 3 h prior to intravital microscopy. In order to delineate the role of endothelial selectins in MIP-2-induced leukocyte-endothelium interactions, an anti-P-selectin antibody (20  $\mu$ g per mouse, RB 40.34, rat IgG, R&D Systems Europe), an anti-E-selectin antibody (100  $\mu$ g per mouse, 10E9.6, rat IgG, R&D Systems Europe), an anti-PSGL-1 antibody (20 or 40  $\mu$ g per mouse, 2PH1, rat IgG, R&D Systems Europe) and an isotype-matched control antibody (40  $\mu$ g per mouse, R3-34, rat IgG, R&D Systems Europe) were given intravenously (*i.v.*) 5 min prior to *i.p.* administration of MIP-2. Sham (negative control) operated mice received only vehicle (PBS) *i.p.* and *i.v.* In separate experiments, colonic ischemia was induced by careful placement of a non-traumatic vascular clamp over the superior mesenteric artery (SMA) at the aortic origin.

Following 30 min of ischemia, the clamp was removed and reperfusion was allowed for 120 min. Notably the SMA is considered to be the most important vessel for the colonic blood supply in rodents [17]. To investigate the mechanisms behind I/R-induced leukocyte rolling and adhesion, pretreatment with the anti-P-selectin antibody (40  $\mu$ g per mouse), the anti-E-selectin antibody (40  $\mu$ g per mouse), the anti-PSGL-1 antibody (40  $\mu$ g per mouse) and the isotype matched control antibody (40  $\mu$ g per mouse) were given *i.v.* 5 min prior to SMA clamping.

### Intravital fluorescence microscopy

Observations of the colonic microcirculation were made using an inverted Olympus microscope (IX70, Olympus Optical Co.GmbH, Hamburg Germany) equipped with different lenses ( $\times$ 10/NA 0.25 and  $\times$ 40/NA0.60). The microscopic images were televised using a charge coupled device video camera (FK6990 Cohu, Pieper GmbH, Schwerte, Germany) and recorded on videotape (Sony SVT-S3000PS-VHS recorder) for subsequent off-line analysis. After positioning under the microscope, a 5-min equilibration period preceded quantitative measurements. Analysis of leukocyte-endothelium interactions (rolling and adhesion) was made in venules (inner diameter 15–35  $\mu$ m) in the submucosa with stable resting blood flow. Blood perfusion within individual microvessels was studied after contrast enhancement by *i.v.* administration of fluorescein isothiocyanate-labelled dextran 150000 (0.05 ml, 5 mg/ml, Sigma Chemical Co.). *In vivo* labelling of leukocytes with rhodamine 6-G (0.1 ml, 0.5 mg/ml, Sigma Chemical Co) enabled quantitative analysis of leukocyte flow behaviour in the colonic microcirculation. Quantification of microcirculatory parameters was performed off-line by frame-to-frame analysis of the videotaped images. Leukocyte rolling was determined by counting the number of leukocytes passing a reference point in the venule per 30 s and expressed as cells per min. Firm adhesion was measured by counting the number of cells adhering to the venular endothelium (200–300  $\mu$ m long segments) and remained stationary for 30 s and is given as cells per mm venule length. Blood flow velocities were measured off-line by use of CapImage<sup>®</sup> software (Zcintl, Heidelberg, Germany). The velocity was calculated as a mean value from five to eight measurements per venule and is expressed as mm per second. Venular wall shear rate was determined based on the Newtonian definition: wall shear rate = 8 (red blood cell velocity/1.6)/venular diameter as described previously [18].

### Systemic leukocyte counts

Twenty  $\mu$ L of blood taken from the tail vein was mixed with Turk solution (0.2 mg Gentian violet in 1 mL glacial acetic acid, 6.25% v/v) in a 1:10 dilution. Leukocytes were counted and differentiated as polymorphonuclear leukocytes (PMNLs) or mononuclear leukocytes (MNLs) in a Bürker chamber.

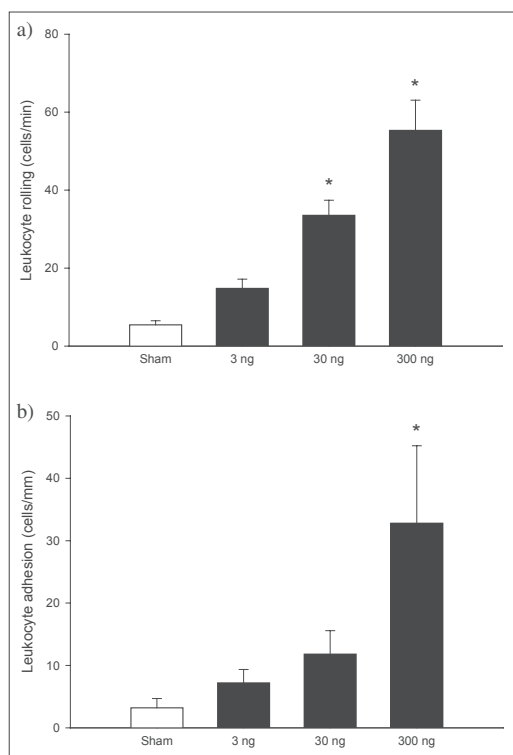
### RT-PCR

Total RNA was extracted from colonic tissue after intravital observation using Rneasy<sup>®</sup> Mini kit (Quiagen GmbH, Hilden, Germany) and treated with RNase-free DNase (Amersham Pharmacia Biotech, Sollentuna, Sweden) to remove potential genomic DNA contaminants, according to the manufacturer's handbook. RNA concentrations were determined by measuring the absorbance at 260 nm spectrophotometrically. RT-PCR was performed with the SuperScript One-Step RT-PCR system (GIBCO BRL Life Technologies, Grand Island, NY). Each reaction contained 250 ng total RNA as a template and 0.2  $\mu$ mol/L of each primer in a final volume of 50  $\mu$ L. Mouse  $\beta$ -actin served as an internal control gene. The RT-PCR profile was 1 cycle of cDNA synthesis at 50 °C for 30 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min, and 1 cycle of final extension at 72 °C for 10 min. After RT-PCR, aliquots of the RT-PCR products were separated on 2% agarose gel containing ethidium bro-

mide and photographed. The primer sequences were as follows:  $\beta$ -actin (f) 5'-ATG TTT GAG ACC TTC AAC ACC-3',  $\beta$ -actin (r) 5'-TCT CCA GGG AGG AAG AGG AT-3'; P-selectin (f) 5'-ACG AGC TGG ACG GAC CCG-3'; P-selectin (r) 5'-GGC TGG CAC TCA AAT TTA CAG-3'; E-selectin (f) 5'-GGT AGT TGC ACT TTC TGC GG-3'; E-selectin (r) 5'-CCT TCT GTG GCA TGT TC-3'.

## ELISA

At the end of the intravital observation, a 3-cm long segment of the colon was excised and cut open along the antimesenteric border. Faeces were cleared and the segment was washed in PBS containing penicillin, streptomycin and fungizon (100 U/ml) and kept cool in cold Dulbecco's Modified Eagle's Medium (DMEM). The colon was then incubated in 1 ml of DMEM solution 10% FCS and penicillin, streptomycin and fungizon for 24 h (37 °C) in a 24-well plate. The cultured medium was harvested and stored in -20 °C until analysis of MIP-2 by using double-antibody specific Quantikine ELISA kit using recombinant murine MIP-2 as standard (R&D Systems Europe).



**Fig. 1.** Venular leukocyte a) rolling and b) adhesion in the mouse colon after *i.p.* administration of indicated doses of MIP-2 (filled bars). Sham operated (open bar) animals served as negative controls. Intravital microscopy was performed 3 h after MIP-2 administration. Data are mean  $\pm$ SEM. \* $P < 0.05$  vs sham ( $n = 5$ ).

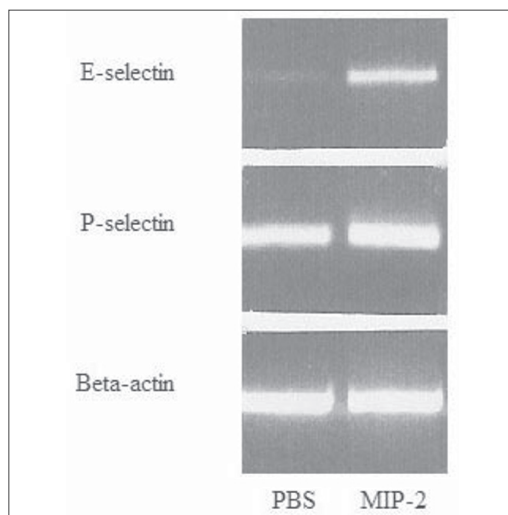
## Statistical analysis

Statistical evaluations were performed using Kruskal-Wallis one-way analysis of variance on ranks for unpaired samples (Dunn's post hoc test). The results are presented as mean  $\pm$ SEM;  $n$  represents the number of animals. Differences were considered to be significant at  $P < 0.05$ .

## Results

Leukocyte-endothelium interactions were analysed in colonic venules after an equilibration time of 5 min. It was found that the level of leukocyte rolling and adhesion in sham-operated (negative control) mice was  $5.4 \pm 1.1$  cells/min and  $3.2 \pm 1.5$  cells/mm, respectively. Challenge with MIP-2 *i.p.* significantly increased leukocyte rolling and adhesion in a dose-dependent manner and, for example, a dose of 300 ng MIP-2 resulted in  $55.3 \pm 7.7$  cells/min rolling and  $32.8 \pm 12.4$  cells/mm adherent leukocytes (Fig. 1a and b,  $P < 0.05$  vs sham). Moreover, we observed that both P- and E-selectin mRNA were expressed after MIP-2 challenge (Fig. 2).

In order to evaluate the individual role of the selectins in CXC chemokine-induced leukocyte rolling and adhesion, *i.v.* pre-treatment with monoclonal antibodies directed against P- and E-selectin were used. Injection of 20  $\mu$ g of RB40.34, an antibody against P-selectin, reduced MIP-2-provoked leukocyte rolling and adhesion by 96% and 81%, respectively (Fig. 3a and b,  $P < 0.05$  vs control ab). Interestingly, administration of 100  $\mu$ g of the anti-E-selectin (10E9.6) antibody had no effect on leukocyte rolling or adhesion in colonic venules after CXC chemokine challenge (Fig. 3a and b).

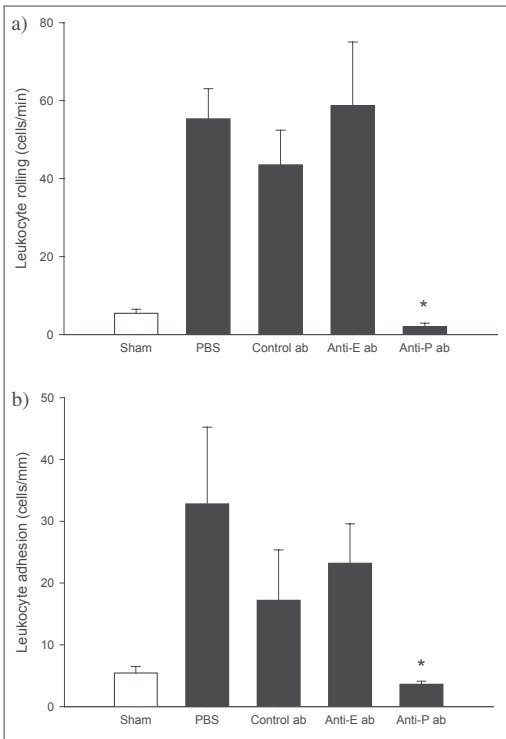


**Fig. 2.** Expression of E- and P-selectin mRNA in the colon.  $\beta$ -actin served as a housekeeping gene. RNA isolation was made 3 h after administration of 300 ng of MIP-2. The results are from one experiment, which is representative of three others performed.

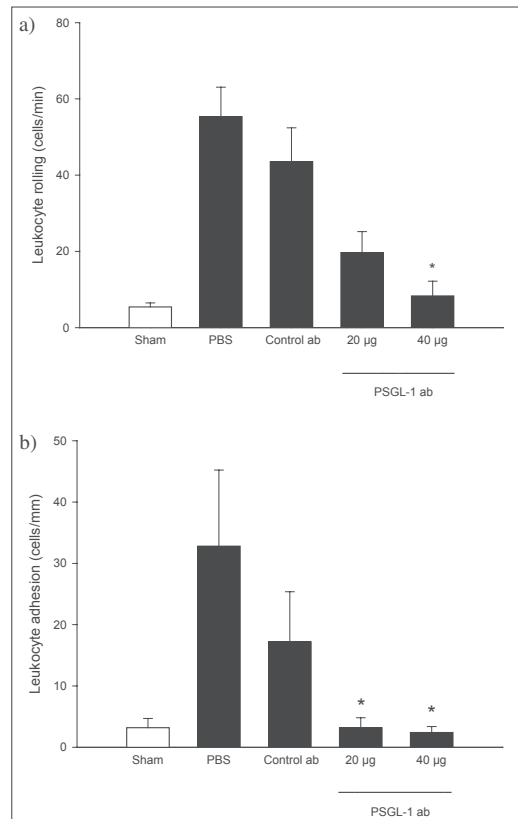
The isotyped-matched control antibody (R3-34, 40 µg) had no unspecific effect on MIP-2-induced leukocyte-endothelium interactions. To determine the role of PSGL-1 in CXC-chemokine-induced rolling and adhesion, we administered a monoclonal antibody against PSGL-1 (2PH1, 40 µg). Inhibition of PSGL-1 reduced MIP-2-induced leukocyte rolling and adhesion in the colon by 85% and 93%, respectively (Fig. 4a and b,  $P < 0.05$  vs control ab). Challenge with MIP-2 and antibodies had no significant effect on peripheral leukocyte count or hemodynamic parameters (Table 1 and 2).

Knowing that MIP-2 plays a key role in colonic I/R [19], it was of great interest to also examine the function of PSGL-1 in I/R-induced leukocyte rolling and adhesion in the colon. Indeed, the colonic content of MIP-2 increased significantly from  $4.7 \pm 0.7$  pg/mg in sham operated animals to  $65.8 \pm 7.9$  pg/mg, after I/R ( $P < 0.05$  vs sham). Induc-

tion of colonic ischemia by clamping the SMA for 30 min followed by 120 min of reperfusion evoked a clear-cut leukocyte response. Thus, I/R increased the number of rolling and adherent leukocytes to  $36.0 \pm 5.6$  cells/min and  $46.4 \pm 8.5$  cells/mm, respectively (Fig. 5a and b,  $P < 0.05$  vs sham). Immunoneutralization of PSGL-1 markedly reduced I/R-induced leukocyte rolling and adhesion by more than 89% and 96%, respectively, that is, down to  $6.3 \pm 2.3$  cells/min and  $1.2 \pm 0.5$  cells/mm (Figure 5a and b,  $P < 0.05$  vs control ab). Indeed, administration of an isotype-matched control antibody had no unspecific effect on I/R-provoked leukocyte-endothelium interactions *i.e.* leukocyte rolling was  $57.2 \pm 4.2$  cells/min and adhesion was  $30.0 \pm 8.3$  cells/mm. Administration of antibodies did not change systemic leukocyte counts or hemodynamic parameters in the colonic microcirculation (Table 1 and 2).



**Fig. 3.** Effect of antibodies directed against P-selectin (Anti-P, 20 µg *i.v.*) and E-selectin (Anti-E, 100 µg *i.v.*) and an isotype matched control antibody (Control ab, 40 µg *i.v.*) on leukocyte (a) rolling and (b) firm adhesion induced by *i.p.* challenge with MIP-2 (filled bars). The antibodies were injected 5 min prior to challenge with 300 ng of MIP-2. Intravital microscopy was performed 3 h after MIP-2 administration. Sham operated animals served as negative controls (open bar). Data are mean  $\pm$  SEM. \* $P < 0.05$  vs control antibody ( $n = 5$ ).



**Fig. 4.** Effect of an anti-PSGL-1 antibody (2PH 1, 40 µg *i.v.*) and an isotype-matched control antibody (Control ab, 40 µg *i.v.*) on leukocyte (a) rolling and (b) firm adhesion induced by challenge with 300 ng of MIP-2 (filled bars) *i.p.* Sham operated animals served as negative controls (open bar). Intravital microscopy was performed 3 h after MIP-2 administration. Data are mean  $\pm$  SEM. \* $P < 0.05$  vs control antibody ( $n = 5$ ).

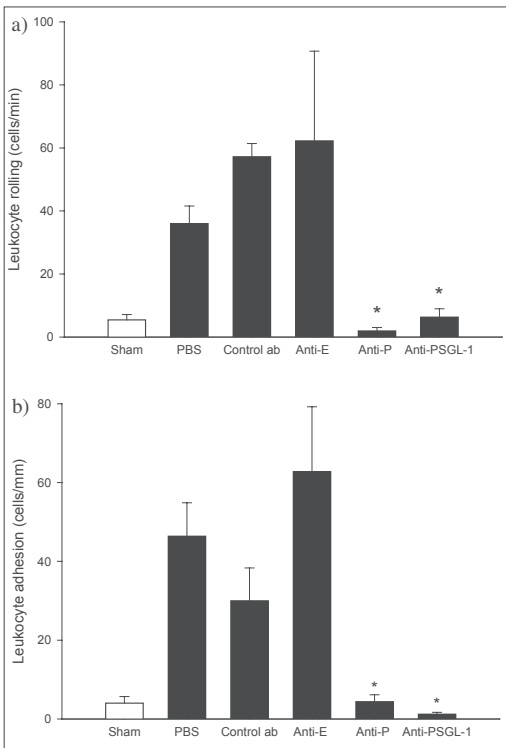
## Discussion

The present findings show that PSGL-1 is an important adhesion molecule regulating leukocyte recruitment in the colon. Indeed, our data demonstrate that PSGL-1 supports leukocyte rolling both in response to challenge with the chemokine MIP-2 and I/R in the large bowel. Inhibition of PSGL-1 blocked not only the rolling adhesive interaction but also abolished chemokine- and reperfusion-induced leukocyte adhesion in the colonic microvasculature. Taken together, our novel data suggest that PSGL-1 is a dominant adhesion molecule in supporting leukocyte recruitment and that targeting PSGL-1 may be an effective strategy in treatment of pathological inflammation in the colon.

Chemokine-dependent leukocyte recruitment is a key feature in the pathophysiology of a growing number of clinically important diseases, including glomerulonephritis [20] bacterial meningitis [21] as well as septic liver and lung injury [22, 23]. An important role of the CXC chemokines MIP-

2 and KC in colonic I/R was recently forwarded by Riaz et al. [19] although the detailed adhesive mechanisms regulating chemokine-provoked leukocyte-endothelium interactions have remained elusive. Numerous studies have shown that leukocyte recruitment is a rate-limiting step in acute inflammation [24–26]. Endothelial cell activation and surface upregulation of selectins is a precondition for extravascular accumulation of neutrophils at sites of inflammation [9]. Selectins and their ligands support early interactions between leukocytes and endothelium during inflammation [27]. We observed that challenge with MIP-2 activated endothelial cells indicated by increased E-selectin mRNA expression (P-selectin is constitutively expressed in Weibel-Palade bodies of endothelial cells). In line with this observation, administration of MIP-2 stimulated leukocyte rolling in a dose-dependent manner in the colonic microcirculation. Moreover, it was found, herein, that inhibition of P-selectin abolished leukocyte rolling induced by MIP-2 whereas immunoneutralization of E-selectin had no effect on MIP-2-induced leukocyte rolling. Indeed, inhibition of P-selectin significantly reduced MIP-2-induced leukocyte adhesion, indicating that P-selectin-dependent rolling is a precondition in chemokine-regulated accumulation of leukocytes in the large bowel. In this context, it is interesting to note that the selectin family of adhesion molecules bind to complex carbohydrates expressed on specific glycoproteins. The importance of the selectin ligands is illustrated by the leukocyte adhesion deficiency syndrome II, in which patients lack proper formation of selectin ligands and suffer from life-threatening episodes of recurrent infections [28]. Having established, herein, that P-selectin is critical in MIP-2-induced leukocyte-endothelium interactions, we wanted to study the role of PSGL-1, which is considered to be the high affinity ligand of P-selectin although PSGL-1 also binds to E-selectin albeit with much lower affinity [10, 11]. In the present study, it was found that immunoneutralization of PSGL-1 reduced MIP-2-induced rolling and adhesion by more than 85% and 96%, respectively, suggesting that PSGL-1 is a key adhesion molecule supporting chemokine-induced leukocyte-endothelium interactions in the colonic microcirculation. Considered together, these findings suggest that P-selectin and PSGL-1 are critical adhesion molecules regulating CXC chemokine-provoked leukocyte rolling and recruitment in the colon.

Knowing that splanchnic I/R upregulates expression of CXC chemokines in the colon [herein, 19], it was of great interest to evaluate the potential impact of the adhesive mechanisms behind CXC chemokine-provoked leukocyte-endothelium interactions revealed in the present study. Thus, it was found that inhibition of P-selectin, but not E-selectin reduced reperfusion-induced leukocyte rolling and adhesion in the colon, which is in agreement with previous findings [29]. Nonetheless, we observed, herein, that inhibition of PSGL-1 decreased leukocyte rolling by nearly 90% in colonic I/R. Moreover, immunoneutralization of PSGL-1 not only abolished leukocyte rolling but also decreased reperfusion-provoked leukocyte adhesion by 96% in the large bowel, suggesting that PSGL-1 is critical in mediating I/R-induced leukocyte-endothelium interaction in the colon. These findings add the colon to the list of organs, including heart [30] and liver [31] that may benefit from inhibition of PSGL-1 in conditions of low tissue perfusion and ischemia.



**Fig. 5.** Venular leukocyte (a) rolling and (b) adhesion in the mouse colon after 30 min of ischemia followed by 120 min of reperfusion (filled bars). Mice underwent *i.v.* pre-treatment with PBS, a control antibody (Control ab, 40 µg), or antibodies against E-selectin (Anti-E, 40 µg), P-selectin (Anti-P, 40 µg) and PSGL-1 (Anti-PSGL-1, 40 µg). Sham operated animals served as negative controls (open bar). Data are mean  $\pm$  SEM. \* $P < 0.05$  vs control antibody ( $n = 5$ ).

**Table 1.** Systemic leukocyte differential counts.

	PMNL ( $\times 10^6$ cells/ml)	MNL ( $\times 10^6$ cells/ml)	Total ( $\times 10^6$ cells/ml)
Sham	1.1 $\pm$ 0.5	2.1 $\pm$ 0.6	3.2 $\pm$ 1.1
3 ng MIP-2	1.8 $\pm$ 0.3	3.7 $\pm$ 0.5	5.5 $\pm$ 0.8
30 ng MIP-2	1.7 $\pm$ 0.3	3.1 $\pm$ 0.4	4.8 $\pm$ 0.7
300 ng MIP-2	2.1 $\pm$ 0.4	3.6 $\pm$ 0.5	5.7 $\pm$ 1.0
MIP-2 + Control ab	1.8 $\pm$ 0.1	1.9 $\pm$ 0.1	3.7 $\pm$ 0.2
MIP-2 + Anti-P ab	1.7 $\pm$ 0.3	3.9 $\pm$ 0.3	5.6 $\pm$ 0.6
MIP-2 + Anti-E ab	1.3 $\pm$ 0.2	3.2 $\pm$ 0.4	4.3 $\pm$ 0.6
MIP-2 + 20 $\mu$ g PSGL-1 ab	0.8 $\pm$ 0.1	1.6 $\pm$ 0.2	2.4 $\pm$ 0.3
MIP-2 + 40 $\mu$ g PSGL-1 ab	0.8 $\pm$ 0.1	1.4 $\pm$ 0.2	2.2 $\pm$ 0.3
Sham	1.4 $\pm$ 0.3	2.7 $\pm$ 0.5	4.1 $\pm$ 0.8
I/R	1.7 $\pm$ 0.3	3.0 $\pm$ 0.3	4.6 $\pm$ 0.7
I/R + Control ab	1.3 $\pm$ 0.2	2.5 $\pm$ 0.3	3.8 $\pm$ 0.5
I/R + PSGL-1 ab	1.2 $\pm$ 0.3	2.6 $\pm$ 0.3	3.9 $\pm$ 0.6

Animals were treated with PBS (Sham) and MIP-2 for 3h or exposed to 30 min of ischemia and 120 min of reperfusion. Monoclonal antibodies against P-selectin (Anti-P, 20  $\mu$ g per mouse), E-selectin (Anti-E, 100  $\mu$ g per mouse), PSGL-1 (Anti-PSGL-1, indicated doses) were administered immediately before MIP-2 challenge and initiation of ischemia. Control animals received isotype control antibodies (Control ab, 40  $\mu$ g). Blood was then collected from the tail vein and the number of PMNL and MNL were analyzed in a hemacytometer. Data are mean  $\pm$  SEM and represents  $10^6$  cells/ml ( $n = 5$ ).

**Table 2.** Hemodynamic variables.

	Diameter ( $\mu$ m)	Blood cell velocity (mm s <sup>-1</sup> )	Wall shear rate (s <sup>-1</sup> )
Sham	26.6 $\pm$ 1.9	0.8 $\pm$ 0.1	150 $\pm$ 26
3 ng MIP-2	23.9 $\pm$ 2.9	1.0 $\pm$ 0.4	209 $\pm$ 44
30 ng MIP-2	22.7 $\pm$ 1.5	1.0 $\pm$ 0.3	220 $\pm$ 33
300 ng MIP-2	24.5 $\pm$ 1.7	1.2 $\pm$ 0.2	245 $\pm$ 39
MIP-2 + Control ab	24.4 $\pm$ 2.5	0.8 $\pm$ 0.1	164 $\pm$ 28
MIP-2 + Anti-P	28.1 $\pm$ 2.2	1.2 $\pm$ 0.2	213 $\pm$ 50
MIP-2 + 40 Anti-E	29.1 $\pm$ 0.9	1.1 $\pm$ 0.3	189 $\pm$ 23
MIP-2 + 20 $\mu$ g PSGL-1 ab	28.1 $\pm$ 3.8	1.0 $\pm$ 0.1	178 $\pm$ 19
MIP-2 + 40 $\mu$ g PSGL-1 ab	25.6 $\pm$ 4.2	1.3 $\pm$ 0.2	254 $\pm$ 34
Sham	24.7 $\pm$ 1.6	1.1 $\pm$ 0.1	219 $\pm$ 21
I/R	23.6 $\pm$ 0.7	1.1 $\pm$ 0.1	233 $\pm$ 26
I/R + Control ab	26.7 $\pm$ 1.0	1.2 $\pm$ 0.3	225 $\pm$ 46
I/R + PSGL-1 ab	25.9 $\pm$ 0.8	1.0 $\pm$ 0.1	193 $\pm$ 24

Animals were treated with PBS (Sham) and MIP-2 for 3h or exposed to 30 min of ischemia and 120 min of reperfusion. Monoclonal antibodies against P-selectin (Anti-P, 40  $\mu$ g per mouse), E-selectin (Anti-E, 40  $\mu$ g per mouse), PSGL-1 (Anti-PSGL-1, indicated doses) were administered immediately before MIP-2 challenge and initiation of ischemia. Control animals received isotype control antibodies (Control ab, 40  $\mu$ g). Data are mean  $\pm$  SEM. ( $n = 5$ ).

In conclusion, this study proposes that PSGL-1 constitute a critical adhesive component in both chemokine- and I/R-induced leukocyte rolling in the colon. Indeed, inhibition of PSGL-1 also abolishes subsequent firm leukocyte adhesion provoked by MIP-2 stimulation and I/R in the large bowel. Thus, our novel findings suggest that targeting PSGL-1 may be a useful strategy to treat pathological inflammation in the colon associated with splanchnic hypoperfusion and ischemia.

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## **PAPER II**





# Mast-cell-dependent secretion of CXC chemokines regulates ischemia-reperfusion-induced leukocyte recruitment in the colon

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## Abstract

**Background and aims** Recruitment of leukocytes in the tissue microvasculature is considered to be a rate-limiting step in ischemia-reperfusion (I/R)-induced inflammation. The objective of this study was to examine the role of mast cells in CXC-chemokine- and I/R-provoked leukocyte recruitment in the colon.

**Materials and methods** Balb/c- and mast-cell-deficient mice were challenged with the CXC chemokines macrophage inflammatory protein-2 (MIP-2) and cytokine-induced neutrophil chemoattractant (KC) for 3 h. Leukocyte–endothelium interactions in the colonic microvascular bed were analyzed using an inverted intravital fluorescence microscopy technique. In separate experiments, mice were subjected to I/R by clamping of the superior mesenteric artery for 30 min followed by 120 min of reperfusion.

**Results** MIP-2 and KC induced a clear-cut increase in the number of rolling and adherent leukocytes in the colon. I/R increased the expression of MIP-2 and KC as well as leukocyte rolling and adhesion in the large bowel. Interestingly, leukocyte rolling and adhesion was reduced by more than 91% in mast-cell-deficient mice in response to CXC chemokine challenge. Moreover, I/R-induced leukocyte rolling and

adhesion was decreased by more than 57% in mast-cell-deficient animals. Administration of MIP-2 increased the colonic expression of E-selectin mRNA in wild type but not in mast-cell-deficient mice.

**Conclusion** Our data demonstrate that CXC chemokine-induced leukocyte rolling and adhesion is regulated by mast cells. Moreover, these findings also show that mast cells play a crucial role in supporting I/R-induced leukocyte rolling and adhesion in the colonic microvascular bed via secretion of CXC chemokines.

**Keywords** Adhesion · Colon · Ischemia · Mast cell · Microcirculation

## Introduction

Intestinal ischemia-reperfusion (I/R)-induced tissue injury is a common feature in aortic surgery and bowel strangulation as well as in hypovolemia and sepsis [1]. Reperfusion of the colon after a period of ischemia evokes specific changes in the microvascular bed that are similar to those observed in inflammation, such as expression of adhesion molecules on endothelial cells, leukocyte–endothelial interactions, and endothelial barrier dysfunction [2, 3]. In general, the recruitment of leukocytes to sites of inflammation is regarded as a multistep process, including leukocyte rolling, firm adhesion, and extravasation [4]. In this process, several investigations have shown that initial leukocyte rolling is mediated mainly by the selectin family of adhesion molecules (P-, E- and L-selectin) [5, 6], whereas firm adhesion is supported by  $\beta$ 1- and  $\beta$ 2-integrins [7, 8]. Numerous tissue-resident cells are needed to coordinate leukocyte recruitment, such as epithelial cells, macro-

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phages, and endothelial cells, and accumulating data has also forwarded a potential role of mast cells in regulating reperfusion-induced leukocyte recruitment in the small intestine, heart, and skeletal muscle [9–11]. In this context, it should be noted that tissue accumulation of leukocytes is both organ- and stimulus-dependent [12–14]. The colon is different from all other organs due to the enormous load of bacteria. Moreover, the colon is relatively less susceptible to ischemia when compared to, for example, the small intestine [15].

Chemokines are low-molecular-weight chemotactic cytokines that play a central role in orchestrating leukocytes to sites of tissue damage and inflammation [16]. They are divided into two major families, CXC and CC chemokines, on the basis of structural characteristics. CXC chemokines, macrophage inflammatory protein-2 (MIP-2), and cytokine-induced neutrophil chemoattractant (KC) are generally known to attract neutrophils, whereas CC chemokines exert chemotactic influence on predominately lymphocytes and monocytes [17]. Indeed, mast cells have the capacity to secrete CXC chemokines upon appropriate stimulus, although the role of mast-cell-derived CXC chemokines in I/R-induced leukocyte recruitment has not been examined previously. Interestingly, CXCR2, which is the high-affinity receptor for MIP-2 and KC [18, 19], is expressed not only on neutrophils but also on mast cells [20, 21]. Herein, we asked whether mast cells may exert a dual function in regulating recruitment of leukocytes in colonic I/R. On one hand, mast cells may be required for leukocyte recruitment induced by CXC chemokines in the colon. On the other hand, activated mast cells may release CXC chemokines, which subsequently may promote colonic recruitment of leukocytes. Thus, our hypothesis was that CXC chemokines may operate both up- and down-stream of mast cells in I/R-provoked leukocyte recruitment in the colon.

## Materials and methods

### Animals

Male Balb/c and mast-cell-deficient (WBB6F1, Jackson Laboratory, Bar Harbor, ME, USA) mice weighing ~23–28 g were kept under standard laboratory conditions. WBB6F1 mice are mast-cell deficient due to a disruption in the Kit oncogene on chromosome 5, encoding the tyrosine kinase receptor for stem cell factor, which is essential for the development of mast cells. Moreover, they exhibit impaired resistance to parasitic infection and intrinsic defects in pigment-forming cells and red blood cells. Animals were maintained on a 12-h light and 12-h dark cycle and were allowed free access to food and water ad libitum. All experimental procedures were approved by the

Regional Ethical Committee for Animal Experimentation at Lund University.

### Anesthetic and surgical preparation

Mice were anesthetized with 7.5 mg ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 2.5 mg xylazine (Janssen Pharmaceutica, Beerse, Belgium) per 100 g body weight intraperitoneally (i.p.). Animals were placed in supine position on a heating pad (37°C) for maintenance of body temperature. A polyethylene catheter (PE-10 with an internal diameter of 0.28 mm) was placed into the internal jugular vein for administration of fluorescent markers. A midline laparotomy was performed, and the colon was subsequently exteriorized.

### Experimental protocol

The effects and mechanisms behind TNF- $\alpha$ - and CXC chemokine-, i.e., MIP-2 and KC, induced leukocyte rolling and adhesion were analyzed by injecting 300 ng of TNF- $\alpha$  (R&D Systems Europe, Abingdon, Oxon, UK), MIP-2 (R&D Systems Europe) and KC (R&D Systems Europe) in 0.2 ml phosphate-buffered saline (PBS) i.p. 3 h prior to inverted intravital microscopy (IIVM). Negative controls received only PBS i.p. In separate experiments, colonic ischemia was induced by careful placement of a non-traumatic vascular clamp over the superior mesenteric artery (SMA) at the aortic origin. Following 30 min of ischemia, the clamp was removed, and reperfusion was allowed for 120 min before IIVM. Notably, the SMA is considered the most important vessel for the colonic blood supply in rodents [22]. Sham-operated animals underwent the same surgical procedure except i.p. injection of TNF- $\alpha$ , chemokines, or clamping of the SMA.

### Intravital microscopy

Observations of the colonic microcirculation were made using an inverted Olympus micro-scope (IX70, Olympus Optical GmbH, Hamburg Germany) equipped with different lenses ( $\times 10$ /NA 0.25 and  $\times 40$ /NA 0.60). The microscopic images were televised using a charge-coupled device video camera (FK6990 Cohu, Pieper GmbH, Schwerte, Germany) and recorded on videotape (Sony SVT-S3000PS-VHS recorder) for subsequent off-line analysis. After positioning under the microscope, a 5-min equilibration period preceded quantitative measurements. Analysis of leukocyte–endothelium interactions (rolling and adhesion) was made in venules (inner diameter 15–35  $\mu$ m) in the submucosa with stable resting blood flow. Blood perfusion within individual microvessels was studied after contrast enhancement by i.v. administration of fluorescein isothio-

cyanate-labeled dextran 150000 (0.05 ml, 5 mg/ml, Sigma Chemical). In vivo labeling of leukocytes with rhodamine 6-G (0.1 ml, 0.5 mg/ml, Sigma Chemical Co) enabled quantitative analysis of leukocyte-flow behavior in the colonic microcirculation. Quantification of microcirculatory parameters was performed off-line by frame-to-frame analysis of the videotaped images. Leukocyte rolling was determined by counting the number of leukocytes passing a reference point in the venule per 30 s and expressed as cells per minute. Firm adhesion was measured by counting the number of cells adhering to the venular endothelium (200–300  $\mu\text{m}$  long segments) and remained stationary for 30 s and is given as cells per millimeter venule length. Blood-flow velocities were measured off-line by use of CapImage<sup>®</sup> software (Zeintl, Heidelberg, Germany). The velocity was calculated as a mean value from five to eight measurements per venule and is expressed as millimeter per second. Venular wall shear rate was determined based on the Newtonian definition: wall shear rate =  $8[(\text{red blood cell velocity}/1.6)/\text{venular diameter}]$  as described previously [23].

#### Systemic leukocyte counts

Approximately 20  $\mu\text{l}$  of blood from the tail vein was mixed with Turks solution (0.2 mg Gentian violet in 1 ml glacial acetic acid, 6.25% v/v) in a 1:10 dilution. Leukocytes were counted and differentiated as polymorphonuclear leukocytes (PMNL) or mononuclear leukocytes (MNL) in a Bürker chamber.

#### Reverse transcription polymerase chain reaction

Total RNA was extracted from colonic tissue after intravital observation using Rneasy<sup>®</sup> Mini kit (Quiagen GmbH, Hilden, Germany) and treated with RNase-free DNase (Amersham Pharmacia Biotech, Sollentuna, Sweden) to remove potential genomic DNA contaminants, according to the manufacturer's handbook. RNA concentrations were determined by measuring the absorbance at 260 nm spectrophotometrically. Reverse transcription polymerase chain reaction (RT-PCR) was performed with the SuperScript One-Step RT-PCR system (GIBCO BRL Life Technologies, Grand Island, NY, USA). Each reaction contained 250 ng total RNA as a template and 0.2  $\mu\text{mol/l}$  of each primer in a final volume of 50  $\mu\text{l}$ . Mouse  $\beta$ -actin served as an internal control gene. The RT-PCR profile was one cycle of cDNA synthesis at 50°C for 30 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min, and one cycle of final extension at 72°C for 10 min. After RT-PCR, aliquots of the RT-PCR products were separated on 2% agarose gel containing ethidium bromide and photographed. The primer sequences were as follows:  $\beta$ -actin (f) 5'-ATG TTT GAG ACC TTC

AAC ACC-3';  $\beta$ -actin (r) 5'-TCT CCA GGG AGG AAG AGG AT-3'; P-selectin (f) 5'-ACG AGC TGG ACG GAC CCG-3'; P-selectin (r) 5'-GGC TGG CAC TCA AAT TTA CAG-3'; E-selectin (f) 5'-GGT AGT TGC ACT TTC TGC GG-3'; E-selectin (r) -CCT TCT GTG GCA GCA TGT TC-3'. Densitometry was used to analyze the intensity per square millimeter of the bands.

#### Enzyme-linked immunosorbent assay

At the end of the intravital observation, a 3-cm-long segment of the colon was excised and cut open along the antimesenteric border. Feces were cleared, and the segment was washed in PBS containing penicillin, streptomycin, and fungizon (100 U/ml) and kept cool in cold Dulbecco's Modified Eagle's Medium. The colon was then incubated in 1 ml of Dulbecco's Modified Eagle's Medium, 10% fetal calf serum and penicillin, streptomycin, and fungizon for 24 h (37°C) in a 24-well plate. The cultured medium was harvested and stored in -20°C until analysis of MIP-2 and KC by using double-antibody-specific Quantikine enzyme-linked immunosorbent assay (ELISA) kit using recombinant murine MIP-2 and KC as standards (R&D Systems Europe).

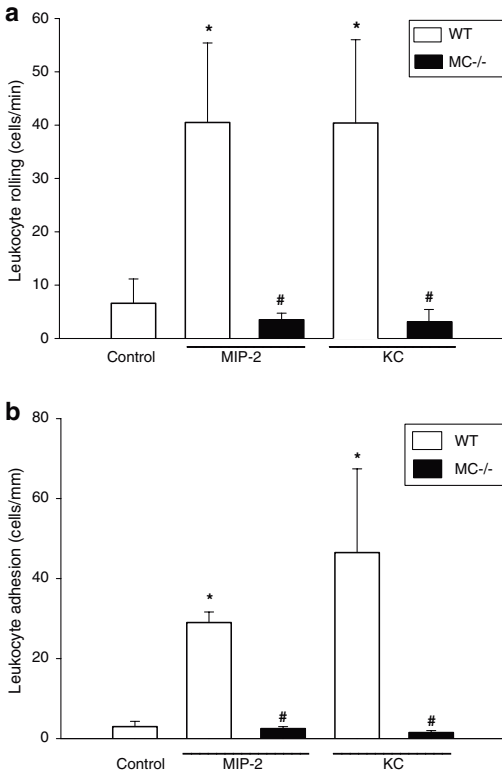
#### Statistical analysis

Statistical evaluation was performed using Kruskal–Wallis one-way analysis of variance on ranks for unpaired samples (Dunn's post hoc test was used). The results are presented as mean values  $\pm$  SEM; *n* represents the number of animals. Differences were considered to be significant at  $P < 0.05$ .

## Results

### CXC chemokine-induced leukocyte recruitment is mast-cell dependent

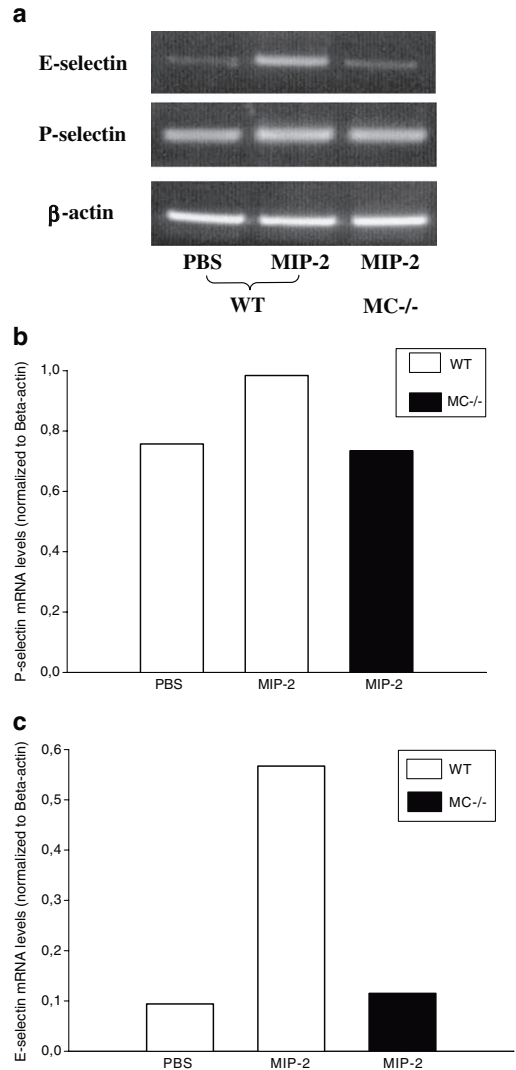
Leukocyte–endothelium interactions were analyzed in colonic venules by IIVM after an equilibration time of 5 min. It was found that the levels of leukocyte rolling and adhesion in negative control (PBS treated) mice was  $6.6 \pm 4.5$  cells/min and  $3.0 \pm 1.3$  cells/mm, respectively (Fig. 1a and b). Injection of CXC chemokines i.p. in wild-type mice significantly increased leukocyte rolling and adhesion in colonic venules (Fig. 1a and b,  $P < 0.05$  vs control). Thus, challenge with 300 ng MIP-2 resulted in  $40.5 \pm 14.9$  rolling cells/min and  $29.0 \pm 2.6$  adherent cells/mm in colonic venules (Fig. 1a and b,  $P < 0.05$  vs control) and injection of 300 ng KC increased leukocyte rolling and adhesion to  $40.4 \pm 15.6$  cells/min and  $46.5 \pm 2.1$  cells/mm, respectively (Fig. 1a and b,  $P < 0.05$  vs control). Additionally, in wild-type mice, it was observed that i.p. challenge with 300 ng



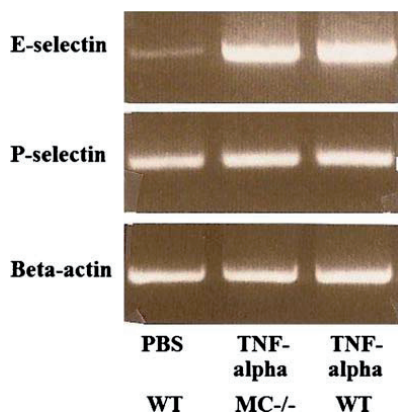
**Fig. 1** Venular leukocyte **a** rolling and **b** adhesion in the mouse colon 3 h after i.p. challenge with 300 ng MIP-2 or KC in wild-type (WT, open bars) and mast-cell-deficient mice (MC<sup>-/-</sup>, filled bars) as observed by inverted intravital microscopy. PBS-treated animals served as negative controls. Data represents mean±SEM. Asterisk  $P < 0.05$  vs control and pound sign  $P < 0.05$  vs WT + chemokine ( $n = 5$ )

TNF- $\alpha$  resulted in  $44.7 \pm 10.8$  cells/min rolling and  $81.2 \pm 8.8$  cells/mm adhering to the microvascular endothelium, which was similar in magnitude to that observed after CXC chemokine injection. Moreover, it was observed that TNF- $\alpha$  also induced a clear-cut increase in the number of adherent leukocytes, i.e.,  $83.2 \pm 12.3$  cells/mm in mast-cell-deficient mice. On the other hand, neither MIP-2 nor KC was able to increase leukocyte rolling or adhesion in mast-cell-deficient animals (Fig. 1a and b,  $P > 0.05$  vs wild type + MIP-2/KC). In fact, CXC chemokine-induced leukocyte rolling and adhesion were decreased by more than 91% in mast-cell-deficient mice (Fig. 1a and b). Administration of 300 ng MIP-2 or TNF- $\alpha$  i.p. increased the amount of colonic E-selectin mRNA as detected by RT-PCR in wild-type mice (Figs. 2a and 3). In contrast, no increase in E-selectin mRNA expression was observed after MIP-2 challenge in mast-cell-deficient mice (Fig. 2a). Notably,

administration of TNF- $\alpha$  enhanced gene expression of E-selectin in mast-cell-deficient mice (Fig. 3). No hemodynamic differences were noted between the experimental groups (Tables 1 and 2).



**Fig. 2** Expression of P- and E-selectin mRNA in mouse colon 3 h after injection of 300 ng a MIP-2 in wild-type (WT) and mast-cell-deficient (MC<sup>-/-</sup>) mice.  $\beta$ -actin served as a housekeeping gene. The results presented are from one experiment, which is representative of three others performed. RT-PCR amplicons of **b** P-selectin and **c** E-selectin were quantified in intensity unit/mm<sup>2</sup> with Quantity 1<sup>®</sup> software package from BIO-RAD and is presented as mRNA levels of P- and E-selectin normalized to  $\beta$ -actin



**Fig. 3** Expression of P- and E-selectin mRNA in mouse colon in response to i.p. administration of PBS and TNF- $\alpha$  for 3 h in wild-type (WT) and mast-cell-deficient (MC  $-/-$ ) mice.  $\beta$ -actin served as a housekeeping gene. The results presented are from one experiment, which is representative of three others performed

#### I/R-induced leukocyte recruitment is mast-cell dependent

To further elucidate the mechanisms behind I/R-provoked leukocyte recruitment in the colon, wild-type and mast-cell-deficient mice were subjected to 30 min of colonic ischemia by clamping of the SMA followed by 120 min of reperfusion. Indeed, it was observed that I/R enhanced leukocyte rolling from  $12.7 \pm 3.5$  to  $38.8 \pm 5.1$  cells/min and leukocyte adhesion from  $2.8 \pm 0.8$  to  $44.8 \pm 10.6$  cells/mm in wild-type mice (Fig. 4a and b,  $P < 0.05$  vs sham). Interestingly, we found that I/R-induced leukocyte rolling and adhesion were reduced down to  $16.8 \pm 1.4$  and  $2.0 \pm 0.8$ , respectively, in mast-cell-deficient mice (Fig. 4a and b,  $P < 0.05$  vs wild

**Table 1** Hemodynamic parameters

	Diameter ( $\mu\text{m}$ )	Blood cell velocity ( $\text{mm s}^{-1}$ )	Wall shear rate ( $\text{s}^{-1}$ )
Control	$24.5 \pm 0.7$	$1.13 \pm 0.16$	$227 \pm 26$
WT + TNF- $\alpha$	$25.0 \pm 0.8$	$1.21 \pm 0.13$	$202 \pm 20$
WT + MIP-2	$20.6 \pm 1.5$	$1.39 \pm 0.26$	$264 \pm 42$
WT + KC	$20.3 \pm 1.2$	$1.23 \pm 0.18$	$223 \pm 42$
MC $-/-$ + TNF- $\alpha$	$23.8 \pm 0.8$	$1.18 \pm 0.22$	$214 \pm 16$
MC $-/-$ + MIP-2	$21.4 \pm 0.5$	$1.31 \pm 0.24$	$276 \pm 58$
MC $-/-$ + KC	$21.9 \pm 0.8$	$1.33 \pm 0.11$	$273 \pm 32$
Sham	$22.4 \pm 0.5$	$1.04 \pm 0.06$	$217 \pm 48$
WT + I/R	$24.5 \pm 0.9$	$1.18 \pm 0.22$	$220 \pm 41$
MC $-/-$ + I/R	$24.5 \pm 1.2$	$1.06 \pm 0.31$	$220 \pm 32$

Mice were challenged i.p. with 300 ng of TNF- $\alpha$ , MIP-2, and KC or exposed to I/R (30 min of ischemia and 120 min of reperfusion) by clamping of the SMA in wild-type (WT) or mast-cell-deficient (MC  $-/-$ ) animals. Control animals received only PBS. Data are mean  $\pm$  SEM and  $n = 5$ .

**Table 2** Systemic leukocyte differential counts

	PMNL	MNL	Total
Control	$1.2 \pm 0.1$	$2.7 \pm 0.3$	$3.9 \pm 0.4$
WT + TNF- $\alpha$	$1.3 \pm 0.2$	$2.6 \pm 0.1$	$3.9 \pm 0.2$
WT + MIP-2	$1.4 \pm 0.1$	$2.4 \pm 0.2$	$3.7 \pm 0.1$
WT + KC	$1.3 \pm 0.1$	$2.4 \pm 0.1$	$3.7 \pm 0.2$
MC $-/-$ + TNF- $\alpha$	$0.9 \pm 0.1$	$2.0 \pm 0.1$	$2.9 \pm 0.2$
MC $-/-$ + MIP-2	$1.0 \pm 0.1$	$2.0 \pm 0.1$	$3.0 \pm 0.2$
MC $-/-$ + KC	$1.2 \pm 0.1$	$2.1 \pm 0.1$	$3.2 \pm 0.1$
Sham	$1.2 \pm 0.2$	$2.5 \pm 0.2$	$3.7 \pm 0.4$
WT + I/R	$1.5 \pm 0.2$	$2.2 \pm 0.5$	$3.8 \pm 0.5$
MC $-/-$ + I/R	$0.8 \pm 0.1$	$2.0 \pm 0.1$	$2.8 \pm 0.2$

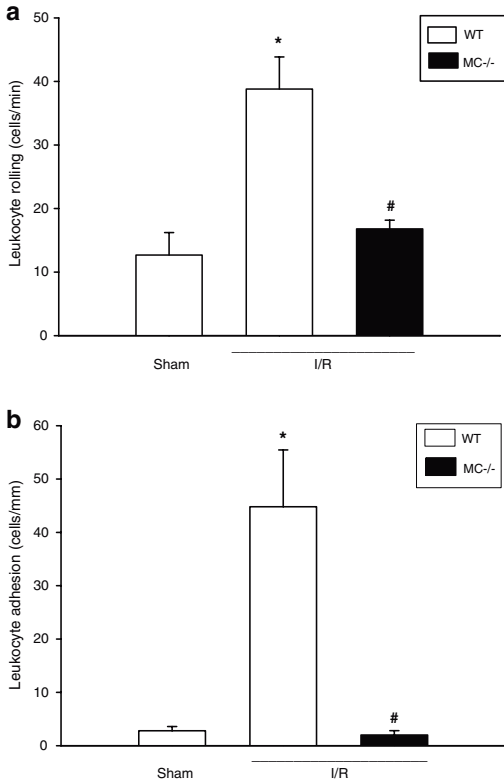
Mice were challenged i.p. with 300 ng of TNF- $\alpha$ , MIP-2, and KC or exposed to I/R (30 min of ischemia and 120 min of reperfusion) by clamping of the SMA in wild-type (WT) or mast-cell-deficient (MC  $-/-$ ) animals. Control animals received only PBS. Blood was collected from the tail vein of wild-type (WT) or mast-cell-deficient (MC  $-/-$ ) mice and analyzed in a Bürker chamber. Cells were defined as polymorphonuclear leukocytes (PMNL) or mononuclear leukocytes (MNL), and data represents  $\times 10^6$  cells per milliliter. Data are mean  $\pm$  SEM, and  $n = 5$ .

type + I/R). Thus, I/R-induced leukocyte rolling and adhesion were decreased by more than 56 and 96%, respectively, in mast-cell-deficient mice (Fig. 4a and b). Moreover, I/R significantly increased the colonic content of MIP-2 from  $4.7 \pm 0.7$  to  $36.6 \pm 7.7$  pg/mg and KC from  $15.9 \pm 1.1$  to  $71.9 \pm 12.8$  pg/mg, in wild-type animals (Fig. 5a and b,  $P < 0.05$  vs wild type + I/R). Notably, colonic CXC chemokine expression was reduced by 57% in response to I/R in mast-cell-deficient mice (Fig. 5a and b,  $P < 0.05$  vs wild type + I/R). Moreover, no significant differences in hemodynamic parameters or peripheral leukocyte counts were observed between the experimental groups (Tables 1 and 2).

#### Discussion

This study forwards a pivotal role for mast cells in I/R-induced leukocyte recruitment in the colon. Indeed, our novel data demonstrate that chemokine-induced leukocyte rolling and adhesion in the colonic microvasculature is dependent on mast cells. Moreover, I/R-provoked expression of CXC chemokines is attenuated in mast-cell-deficient animals. Thus, our data suggest that mast cells may exert a dual function in the colon; on one hand, upstream chemokines trigger leukocyte recruitment via local mast cells and on the other hand, mast cells regulate downstream formation of chemokines, which in turn provoke leukocyte recruitment. These findings help to explain the complex interplay between CXC chemokines, tissue-resident mast cells, and circulating leukocytes in the colon.

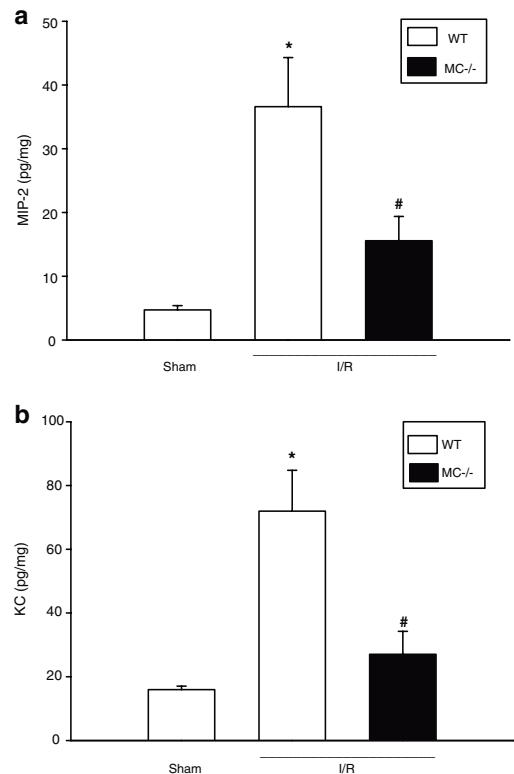
Splanchnic I/R-induced injuries are frequently encountered in cardiovascular and transplantation surgery as well



**Fig. 4** Venular leukocyte **a** rolling and **b** adhesion in wild-type (WT, open bars) and mast-cell-deficient (MC<sup>-/-</sup>, filled bar) mice after 30 min of ischemia followed by 120 min of reperfusion as detected by intravital fluorescence microscopy in the colonic microcirculation. Sham (WT + PBS)-operated animals served as negative controls. Data represents mean±SEM. Asterisk  $P < 0.05$  vs sham and pound sign  $P < 0.05$  vs WT + I/R ( $n=5$ )

as in trauma and bowel strangulation [1]. In this process, leukocyte recruitment into sites of inflammation has been implicated to be a key feature and a rate-limiting step in the pathophysiological process [24]. Recruitment of leukocytes is the result of a fine-tuned interplay between endothelial and specialized tissue-resident cells. For example, it has been shown that mast cell degranulation and the subsequent release of chemoattractant substances is one possible mechanism behind leukocyte recruitment into the extravascular space [25]. Mast cells contain a number of pro-inflammatory substances, including TNF- $\alpha$ , leukotrienes, histamine, and chemokines [26–28], but the precise role of mast cells in the crosstalk between chemokines and endothelial cells has not yet been understood. Herein, we observed that 30 min of colonic ischemia followed by 120 min of reperfusion induced a clear-cut increase in the

number of rolling and adherent leukocytes along the microvascular endothelium in the colon. Notably, these I/R-provoked leukocyte responses were abolished in mast-cell-deficient mice, suggesting a key role of mast cells in reperfusion-induced leukocyte recruitment in the colon. This observation adds the colon to the list of organs in which mast cells appear to be of importance in I/R injury, including the small intestine, the heart, and skeletal muscle [9–11]. Thus, targeting mast cell function may be a useful target in ameliorating I/R injury in the colon. In this context, it should be noted that the effect of systematic mast cell inhibition in humans is not known. Based on the phenotype of mast-cell-deficient mice, it may be concluded that mast cell depletion in humans may increase the risk of parasitic infections, but an increased risk of bacterial infections cannot be excluded.



**Fig. 5** Expression of **a** MIP-2 and **b** KC after 30 min of ischemia followed by 120 min of reperfusion in wild-type (WT, open bars) and mast-cell-deficient (MC<sup>-/-</sup>, filled bar) mice. Level of secreted MIP-2 and KC was determined by specific ELISA. Sham-operated animals served as negative controls. Data represents mean±SEM. Asterisk  $P < 0.05$  vs sham and pound sign  $P < 0.05$  vs WT + I/R ( $n=5$ )

Moreover, we found that the colonic content of the chemokines MIP-2 and KC increased significantly after I/R, which is in agreement with a previous study [29]. Indeed, several studies have shown that mast cells have the ability to secrete chemokines [28, 30, 31]. Therefore, we hypothesized that CXC chemokine formation may be dependent on tissue-resident mast cells in colonic I/R. Interestingly, it was also observed that colonic formation of CXC chemokines MIP-2 and KC, in response to I/R, was significantly reduced by 58 and 63%, respectively, in mast-cell-deficient mice, suggesting that mast cells regulate CXC chemokine secretion in I/R injury in the colon.

An accumulating body of evidence implicate that the CXC chemokines MIP-2 and KC are of importance in several clinical conditions and disease models [32, 33]. For example, a recent study demonstrated that immunoneutralization of CXC chemokines inhibits I/R-induced leukocyte rolling and adhesion in the colon [29]. However, the mechanisms behind CXC chemokine-induced leukocyte accumulation in the colon remain elusive. CXC chemokines stimulate migration of neutrophils via CXCR2, which is the high-affinity receptor for MIP-2 and KC expressed on murine neutrophils [34, 35]. In this context, it is important to note that, on one hand, numerous studies have shown that endothelial cell activation and P- and E-selectin expression are key components for effective tissue accumulation of leukocytes [36, 37] and, on the other hand, that human and murine endothelial cells do not express CXCR2 [38, 39]. Thus, knowing that mast cells express multiple chemokine receptors, including CXCR2 [40–42], we hypothesized that mast cells may constitute a critical link in CXC chemokine-induced leukocyte recruitment in the colon. Indeed, we found that both MIP-2- and KC-induced leukocyte-endothelium interactions were abolished in mast-cell-deficient mice, suggesting that CXC chemokine-induced leukocyte accumulation is dependent on tissue mast cells in the colon. Moreover, we observed that MIP-2 increased gene expression of E-selectin (a marker of endothelial cell activation) in the colon of wild-type animals, suggesting that MIP-2 has the capacity to activate colonic endothelial cells *in vivo*. Notably, this MIP-2-induced increase in E-selectin mRNA was markedly reduced in mast-cell-deficient mice, indicating that mast cells indeed constitute an important link between CXC chemokines and endothelial cell activation in the colon. An important observation was that TNF- $\alpha$  increased colonic gene expression of E-selectin in both wild-type and mast-cell-deficient animals, suggesting that the transcriptional capacity of E-selectin was intact in mast-cell-deficient animals. Combined, these data indicate that CXC chemokine-induced leukocyte rolling and adhesion in the colon is dependent on mast cells, which have the capacity, in turn, to activate endothelial cells.

In summary, our data shows that I/R-induced leukocyte rolling and adhesion in the colon is markedly reduced in mast-cell-deficient animals, suggesting that targeting mast cells may be an effective strategy against reperfusion-induced inflammation in the colon. Moreover, the present results suggest that mast cells exert a dual function in integrating the crosstalk between CXC chemokine and endothelial cells in the colon. On one hand, CXC chemokine-provoked leukocyte accumulation is dependent on mast cells for endothelial cell activation and adhesion molecule expression. On the other hand, colonic formation of CXC chemokines is dependent on mast cells. Thus, these novel findings help to explain the complex interactions mediated by chemokines in between specialized tissue-resident cells, endothelial cells, and circulating leukocytes in the colon.

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