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Adhesive mechanisms of histone-induced neutrophil-endothelium interactions in the muscle microcirculation

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SUMMARY IN GERMAN

Histone werden unter anderem durch NETosis und Zellzerstörung in den Extrazellularraum freigesetzt. Mehrfach konnte eine bakterizide Wirkung von extrazellulären Histonen nachgewiesen werden. Diese spielt eine wichtige Rolle in der primären Erregerabwehr. Es ist jedoch auch bekannt, dass extrazelluläre Histone eine nicht unbeträchtliche Zytotoxizität gegenüber körpereigenen Zellen aufzeigen. Es ergeben sich schwerwiegende Gewebeschädigungen, die wiederum mit einer erhöhten Extravasation von Leukozyten assoziiert sind und wesentlich zur Pathogenese von Krankheiten wie Sepsis, Acute Lung Injury und Glomerulonephritis beitragen können.

Das Ziel dieser Studie war es, die molekularen Grundlagen von Histon-getriggerten Wirkungen zu untersuchen. Insbesondere wurde die Rolle von spezifischen Adhäsionsmolekülen in der Histon-induzierten Extravasation der Leukozyten analysiert.

Die Studie basiert auf der Intravitalmikroskopie der Mikrozirkulation des M. cremaster der Maus. Die Intravitalmikroskopie ermöglicht eine adäquate Untersuchung der dynamischen Leukozyten-Endothel-Interaktionen am lebenden Tier.
Histone Typ 3 bzw. TNFα, welcher als positive Vergleichskontrolle genutzt wurde, oder PBS, als negative Vergleichskontrolle, wurden lokal in das Skrotum der Mäuse injiziert. Ausgewählte Adhäsionsmoleküle (P-Selektin, L-Selektin, PSGL-1, Mac-1 und LFA-1) wurden durch intravenös verabreichte, monoklonale Antikörper spezifisch geblockt. Es erfolgte eine quantitative Untersuchung des Leukozytenrollens, der Adhäsion und der Transmigration der Zellen. Weiterhin wurde mit Hilfe eines Antikörpers gegen neutrophile Granulozyten jener Zelltyp ermittelt, der primär durch die extrazellulären Histone stimuliert wird. Ergänzende Daten konnten durch hämodynamische Untersuchungen und Zählung der systemischen Leukozytenzahl gewonnen werden.

Die Injektion von extrazellulären Histonen Typ 3 führte zu einem dosisabhängigen Anstieg in der Infiltration von Leukozyten. Es konnte eine vergleichbare Wirkstärke von extrazellulären Histonen zu TNFα festgestellt werden. Da die Immunoneutralisierung von neutrophilen Granulozyten eine Aufhebung der Histon-induzierten Effekte ergab, beruht die starke proinflammatorische Wirkung vor allem auf einer Stimulation von Neutrophilen.

Die Injektion von anti-P-Selektin und anti-PSGL-1 Antikörpern reduzierte die Anzahl der rollenden, der adhärenten und der transmigrierten Leukozyten zu einem Minimum. Wurden jene Antikörper nach der Stimulation mit Histon 3 verabreicht, konnte ein Abfallen des Leukozytenrollens verzeichnet werden. Adhäsion und Transmigration der Leukozytenzahl wurden jedoch nicht beeinflusst. Es konnte geschlussfolgert werden, dass das Rollen der Neutrophilen, das durch die extrazellulären Histone provoziert wurde, vor allem durch P-Selektin/PSGL-1-Interaktionen bedingt ist. P-Selektin und PSGL-1 haben jedoch keinen direkten Einfluss auf den Histon-induzierten Vorgang der Adhärenz und Transmigration.

Die Immunoneutralisierung von LFA-1 und Mac-1 führte zu einer Aufhebung der Leukozytenadhärenz und -transmigration. Das Rollen der Leukozyten blieb durch die Antikörperbehandlung jedoch unverändert. LFA-1 und Mac-1 spielen folglich eine entscheidende Rolle in der Adhärenz von Leukozyten in Histon-getriggerten Prozessen.

Zusammenfassend konnten durch diese Studie spezifische Adhäsionsmoleküle definiert werden, welche eine entscheidende Rolle in der Signalübertragung von Histon-induzierten Wirkungen spielen. Das kann neue Ansätze in der pharmakologischen Therapie von Histon-getriggerten entzündlichen Krankheiten ermöglichen.

ABSTRACT

Extracellular histones, released during cell damage and NETosis, show a potent bactericidal effect and are known to trigger host cell cytotoxicity. Moreover, these proteins have the capacity to cause tissue injury associated with increased leukocyte accumulation.

However, molecular mechanisms regulating histone-induced leukocyte recruitment remained elusive. That is why, the objective of this study was to examine the role of adhesion molecules in histone-dependent leukocyte accumulation by use of intravital microscopy of the muscle microcirculation in mice.

Histone 3 and TNFα were administered intrascrotally and anti-P-selectin, anti-PSGL-1 and anti-L-selectin, as well as anti-Mac-1, anti-LFA-1 and neutrophil depletion antibodies were injected intravenously or intraperitoneally. Leukocyte rolling, adhesion and emigration were observed and quantified by subsequent off-line analysis. Data was completed by measurements of microvascular hemodynamic parameters and systemic leukocyte counts.

Intrascrotal injection of histone 3 increased leukocyte recruitment in a dose-dependent manner. Further, neutrophil depletion abolished histone-induced effects, suggesting that neutrophils are the dominating leukocyte subtype responding to histone stimulation. Pretreatment with anti-P-selectin or anti-PSGL-1 antibodies reduced histone-stimulated neutrophil rolling, adhesion and emigration significantly. However, when the named antibodies were administered after histone 3 stimulation, neutrophil rolling was reduced whereas the number of firmly adherent and emigrated neutrophils was unchanged. One could conclude that the inhibitory effect of P-selectin and PSGL-1 blocking on neutrophil adhesion and recruitment was due to reduction in neutrophil rolling. Moreover, pretreatment with antibodies against Mac-1 and LFA-1 had no effect on the number of rolling cells but abolished histone 3-evoked leukocyte adhesion and emigration. Importantly, antibody treatment did not influence the number of systemic leukocytes and hemodynamic parameters did not alter between the experimental groups.

Thus, the study indicates that P-selectin and PSGL-1 play an important role in histone-induced inflammatory cell recruitment by mediating neutrophil rolling which was found to be a precondition for histone-provoked firm adhesion and emigration in vivo. Moreover, it could be concluded that both Mac-1 and LFA-1 are critical in supporting

histone-induced firm adhesion of neutrophils to endothelial cells. Accordingly, these novel findings define specific selectins and integrins as potential targets for pharmacological intervention in histone-dependent inflammatory diseases.

LIST OF ABBREVIATIONS

AB	antibody
ALI	Acute Lung Injury
APC	activated protein C
BALF	bronchoalveolar lavage
CD	cluster of differentiation
cf.	confer (Latin) / compare
DAMP	damage-associated molecular pattern
DNA	deoxyribonucleic acid
e.g.	exempli gratia (Latin) / for example
ECAM	epithelial cell adhesion molecule
EGF	epidermal growth factor
ESL-1	E-selectin ligand-1
FACS	fluorescence-activated cell sorting
FITC	fluorescein isothiocyanate
H3	histone 3
H4	histone 4
HEV	high endothelial venule
i.e.	id est (Latin) / that is
ICAM	intercellular adhesion molecule
IL	interleukin
inj.	injection
IVM	intravital microscopy
JAM	junctional adhesion molecules
LFA-1	lymphocyte function antigen-1
Ly6C	lymphocyte antigen 6C
Ly6G	lymphocyte antigen 6G
M.	Musculus
Mac-1	membrane-activated complex-1
MNL	mononuclear leukocyte
n	number of animals per group
NET	neutrophil extracellular trap

NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK-cells	natural killer cells
op	operation
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
PE	phycoerythin
PECAM	platelet / endothelial cell adhesion molecule
PMNL	polymorphonuclear leukocyte
PRR	pattern recognition receptor
PSGL-1	P-selectin glycoprotein ligand-1
SEM	standard error of the mean
TLR	toll-like receptor
VCAM	vascular cell adhesion protein
VLA-4	very late antigen 4

INTRODUCTION

1.1 Inflammation

Inflammation is a stereotyped, dynamic and rather complex cellular and vascular protective tissue response occurring to eliminate the initial cause of cell injury and to initiate tissue repair. The inflammatory response can be provoked by mechanical trauma and infectious agents, amongst others. Dynamic changes of inflammatory and vasoactive mediators occur, evoking typical clinical inflammatory signs: dolor (pain), calor (heat), rubor (redness), tumor (swelling) and functio laesa (loss of function).

Sentinel cells, including macrophages, dendritic cells and vascular endothelial cells form the cellular first line response towards infection and irritation [Staros, 2005]. These cells are able to sense the initial insult by pattern recognition receptors (PRRs). Toll-like receptors (TLR) are by far the most important family of PRRs [Arancibia et al., 2007]. PRRs, presented on the surface of cells, detect exogenous pathogen-associated molecular patterns (PAMPs) and endogenous damage-associated molecular patterns (DAMPs) which leads to activation of the cells. Activated sentinel cells release proinflammatory mediators as well as vasoactive chemokines and cytokines to amplify the inflammatory response by activating the vascular and the cellular component. The vascular component of an inflammatory response is characterized by vasodilatation and extravasal movement of plasma fluid. Plasma exudation arises due to an increased vascular permeability, resulting in oedema formation [Gavins; Chatterjee, 2004]. Major cellular defense mechanisms are inter alia built by leukocytes which start to infiltrate to the site of tissue damage following cytokine attraction. Leukocyte infiltration is achieved by a multistep leukocyte recruitment cascade based on dynamic interactions of leukocytes and the adjacent vascular endothelium.

1.2 Leukocyte recruitment

1.2.1 Adhesion cascade

The leukocyte adhesion cascade, which allows leukocytes to infiltrate towards the site of inflammation, is a dynamic, multistep process. A molecular model was first published in 1991 by Drs. Von Andrian et al. [Andrian et al., 1991]. Those investigators supported a two-step cascade in which initial rolling was followed by subsequent attachment of leukocytes. Leukocyte rolling and adhesion were supposed to be separate processes. It

was claimed that the initial, reversible leukocyte rolling step is mediated by lectin adhesion molecule 1 (LECAM-1 / L-Selectin), presented on the surface of circulating, non-activated leukocytes. Leukocyte rolling was said to be a precondition for subsequent leukocyte adhesion, mediated by β_2-integrins [Andrian et al., 1991].

By the time more adhesion molecules were discovered to play an important role in leukocyte recruitment and it could be demonstrated that the vascular endothelium is an active and essential aspect in the transmigration of leukocytes, the cascade became more complex and well understood. Thereupon, the original model proposed three basic steps, including selectin-dependent rolling, chemokine-triggered activation of leukocytes and integrin-mediated stable binding to the endothelium [Butcher, 1991; Ley et al., 2007]. Nowadays, leukocyte recruitment is described as a 5-step process facilitated by specific molecules which coordinate interactions between leukocytes and endothelial cells. In short, the cascade includes: tethering and steady state rolling – slow rolling – arrest and adhesion strengthening – crawling – transmigration [McEver, 2015]. The main steps are illustrated in Figure 1 and required endothelial and neutrophilial molecules are summarized in Table 1.

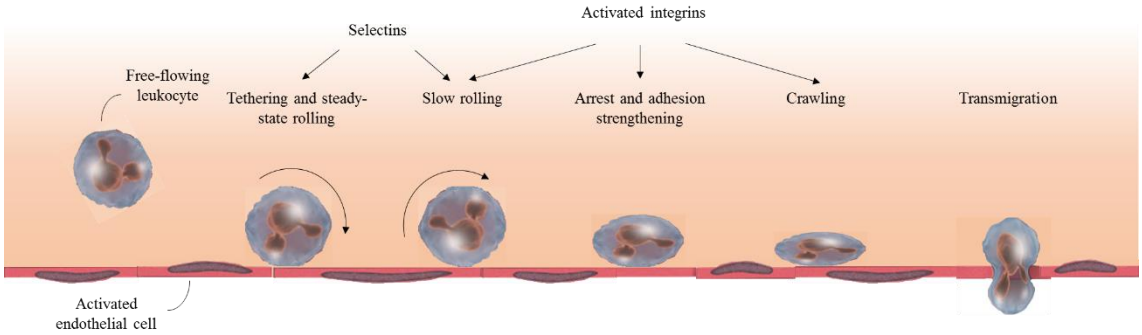

Figure 1: Leukocyte recruitment cascade. The sequential steps of leukocyte recruitment from the vascular lumen to the site of inflammation are shown. Selectin-mediated, shear sensitive leukocyte rolling in the direction of blood flow merges to shear-resistant adhesion of leukocytes, mediated by activated β_2-integrins. Subsequently, cells crawl along the endothelium in order to find their place to emigrate by passing between (paracellular) or through (transcellular) the endothelial cell layer. (Modified according to [McEver, 2015])

Leukocyte mobilization and activation of the adjacent vascular endothelium are necessary preconditions for local leukocyte transmigration towards the site of the initial insult. Activation of the vascular endothelium can be induced by many factors, including chemokines such as IL-1 and TNFα secreted by sentinel cells [Bevilacqua et al., 1985a; Bevilacqua et al. 1985b; Gamble et al., 1985] and hypoxia [Adcock et al., 1994]. Activated endothelial cells stimulate the nuclear factor kappa-light-chain-enhancer of

activated B cells (NF-κB) [Adcock et al., 1994; Adcock et al., 1995], a transcription factor found in almost all cell types. Accordingly, endothelial cells undergo profound changes in gene expression and function, resulting in an increased expression of proinflammatory cytokines, chemokines, colony stimulating factors and adhesion molecules [Krishnaswamy et al., 1999]. The activated, pro-adhesive endothelium is then able to participate in a variety of inflammatory processes and plays a key role in leukocyte recruitment.

Leukocyte mobilization from the bone marrow into the blood stream is mainly mediated by the hematopoietic cytokine granulocyte colony-stimulating factor (G-CSF) [Semerad et al., 2002]. G-CSF is secreted by activated vascular endothelial cells [Krishnaswamy et al., 1999], fibroblasts [Yang et al., 1988] and mononuclear phagocytes [Rambaldi et al., 1987] due to IL-1 and TNFα stimulated activation of the cells [Koeffler et al., 1987; Seelentag et al., 1987; Zsebo et al., 1988].

1.2.1.1 Rolling

Mobilized, circulating leukocytes are recruited to interact with the activated vascular endothelium. The initial attachment is facilitated by a rapid release of P-selectin to the surface of activated endothelial cells upon stimulation [Jung; Ley, 1997]. P-selectin interacts with P-selectin glycoprotein ligand-1 (PSGL-1), its major leukocyte counterreceptor, generating leukocyte tethering and subsequent rolling of free-flowing leukocytes [Moore et al., 1995; Norman et al., 1995; Norman et al., 2000]. It is well documented that inhibition of P-selectin and PSGL-1, respectively, effectively inhibits leukocyte recruitment in different models of inflammation, including reperfusion injury [Dold et al., 2008] and septic lung damage [Asaduzzaman et al., 2009]. The rolling process is moreover supported by E-selectin/PSGL-1 interactions [Hidalgo et al., 2007].

Leukocyte rolling, in which the cell moves along the vessel wall like a wheel, requires a rapid, balanced formation and dissociation of P-selectin/PSGL-1 bonds at the leading edge and the rear of the cell [Sundd et al., 2010]. In this process, rolling can be described as either a full (stop) or partial (go) balance of the hydrodynamic drag and adhesive forces [Hammer; Apte, 1992; Pospieszalska et al., 2009; Tözeren; Ley, 1992]. The hydrodynamic drag is caused by the blood flow causing two physical phenomena: A forward force, pushing the cell forward, and a torque, tending to rotate the cell [Hammer; Apte, 1992; Tözeren; Ley, 1992].

Summing up, tethering and subsequent steady-state rolling bring leukocytes in proximity to the endothelium, enabling them to receive signals from it. The process is predominately mediated by a fast formation and dissociation of P-selectin/PSGL-1 bindings.

Steady-state rolling and subsequent slow rolling are sequential but overlapping steps. The slow rolling process is primarily mediated by an increased expression of E-selectin following activation of endothelial cells [Jung et al., 1998; Kunkel; Ley, 1996; Ley et al., 1998]. This causes more frequent leukocyte-endothelium (E-selectin/ESL-1 and E-selectin/CD44) interactions [Hidalgo et al., 2007]. Accordingly, cells slow down; the velocity of circulating leukocytes decreases which is important to allow time for cells to detect chemotactic signals from the endothelial surface and local environment [Jung; Ley, 1999]. However, slow rolling requires not only E-selectin, but also lymphocyte function antigen-1 (LFA-1). In this regard, crosslinking of E-selectin and PSGL-1 induces activation of the β_2-integrin LFA-1 by inside-out signalling, resulting in an intermediate affinity conformational state of LFA-1 [Chesnutt et al., 2006; Kuwano et al., 2010; Salas et al., 2004]. Besides, some studies claim a L-selectin-dependent activation of LFA-1 [Sikorski et al., 1996; Stadtmann et al., 2013]. The partially activated β_2-integrin enables further leukocyte-endothelial cell interactions by binding to its counterreceptor intercellular adhesion molecule-1 (ICAM-1). Accordingly, and to sum up, slow rolling is mediated by both selectins (predominately E-selectin) and integrins (primarily LFA-1). It enhances the passage of neutrophils along the endothelium, providing more opportunities to bind to endothelial chemokines and ligands.

1.2.1.2 Adhesion

It is generally thought that initial rolling is a precondition for subsequent firm adhesion and tissue accumulation of leukocytes [Lawrence; Springer, 1991; Lindbom et al., 1992; Månsson et al., 2000]. The ability of leukocytes to arrest on endothelial cells depends on the activation status of leukocytes and thereby the conformation status of integrins, especially LFA-1. Unstimulated leukocytes show a low binding affinity, whereas high affinity conformations arise upon activation. In this regard, priming of leukocytes is described as a two-step process: initial contact with early-phase chemokines (e.g. TNFα, IL-1) or PAMPs, followed by exposure to late-phase chemoattractants (e.g. IL-8) and growth factors (e.g. GM-CSF) [Gamble et al., 1985; Klebanoff et al., 1986; McColl et al.,

1990; Sample; Czuprynski, 1991; Yuo et al., 1991]. It is the high affinity conformation of β_2-integrins which supports leukocytes to adhere to the endothelium [Constantin et al., 2000; Ding et al., 1999; Giagulli et al., 2004; Salas et al., 2002].

In order to remain in stationary contact with the endothelium, leukocytes undergo a subsequent adhesion strengthening phase mediated by outside-in signalling mechanisms. In short, binding of an endothelial cell-surface receptor to a β_2-integrin activates intracellular signalling which increases adhesion strength and induces leukocyte spreading [Totani et al., 2006; Shamri et al., 2005]. However, transmigration does not necessarily occur at the initial site of adhesion [Kolaczkowska; Kubes, 2013]. Leukocytes rather show a probing behavior (locomotion) in order to find a good place to transmigrate. That is, cells crawl actively along the vessel wall while remaining firmly attached to a single location [Ryschich et al., 2006]. The crawling process is reported frequently; the specific molecular interactions are poorly understood though. However, it is claimed that intravascular crawling is significantly dependent on Mac-1, a β_2-integrin, binding to its endothelial ligand ICAM-1 [Phillipson et al., 2006].

To sum up, leukocyte adhesion comprises leukocyte arrest and adhesion strengthening and subsequent leukocyte crawling, mediated by activated β_2-integrins. Leukocytes are in stationary, shear-resistant contact with the endothelium, a precondition for cells to transmigrate towards the site of inflammation.

1.2.1.3 Transmigration

Transmigration is the ultimate step of the leukocyte recruitment cascade towards the initial inflammatory insult. Leukocytes have to cross two layers to emigrate from the vascular lumen: the endothelial cell barrier which step takes about two to five minutes and subsequent the basement membrane which phase can take much longer (>5-15min) [Ley et al., 2007]. Leukocytes can cross the endothelium in a paracellular way at endothelial junctions (leukocytes squeeze between adjacent cells) or transcellularly (through the cells). Paracellular leukocyte emigration is revealed to be predominant [Burns et al., 1997; Phillipson et al., 2006; Shaw et al., 2001]. However, several studies prove transcellular emigration to be a sufficient way for leukocytes to pass the endothelium in vivo and in vitro [Carman; Springer, 2004; Feng et al., 1998; Millán et al., 2006; Nieminen et al., 2006; Yang et al., 2005]. Transmigration is a complex, versatile process which is not completely understood yet. However, it is known that several

adhesion molecules, including integrins and ICAM1, ICAM2, vascular cell adhesion protein 1 (VCAM1) as well as junctional proteins such as platelet/endothelial cell adhesion molecule 1 (PECAM1), junctional adhesion molecules (JAMs) and epithelial cell adhesion molecule (ECAM) are required for cells to successfully emigrate from the vascular lumen [Kolaczkowska; Kubes, 2013].

Table 1: Summary of the adhesion molecules involved in the neutrophil recruitment cascade in postcapillary venules, assigned to the main stages of the multistep cascade. (According to [Kolaczkowska; Kubes, 2013])

Stage	Endothelial molecules	Neutrophilial molecules
Tethering and Rolling	P-Selectin	PSGL-1
	E-Selectin	PSGL-1, ESL-1
	PSGL-1	L-Selectin
Slow rolling	E-Selectin	ESL-1, CD44
	ICAM-1	LFA-1
Arrest and Adhesion	ICAM-1	LFA-1
	VCAM-1	VLA-4
Crawling	ICAM-1	Mac-1
Transmigration	ICAM-1, ICAM-2	LFA-1, Mac-1
	VCAM-1	VLA-4

1.2.2 Adhesion molecules and their counter receptors

1.2.2.1 Selectins

Selectins, which are single-chain transmembrane molecules, and their ligands play a key role in leukocyte rolling. Many research groups used antibodies against the various selectins or selectin-deficient mice to study the selectin-mediated leukocyte-endothelium interactions [Kunkel et al., 1996; Kunkel; Ley, 1996; Mayadas et al., 1993; Jung; Ley, 1997].

The selectin-family comprises P-selectin, found on platelets and endothelial cells, E-selectin, expressed on the surface of the endothelium, and L-selectin, a leukocyte-specific selectin. All of which are built by a N-terminal Ca^{2+}-dependent lectin domain, an epidermal growth factor (EGF)-like domain, a series of short consensus repeats, followed by a transmembrane part and a short cytoplasmic region (Figure 2) [Bevilacqua et al., 1989; Johnston et al., 1989; Lasky et al., 1989; Siegelman et al., 1989].

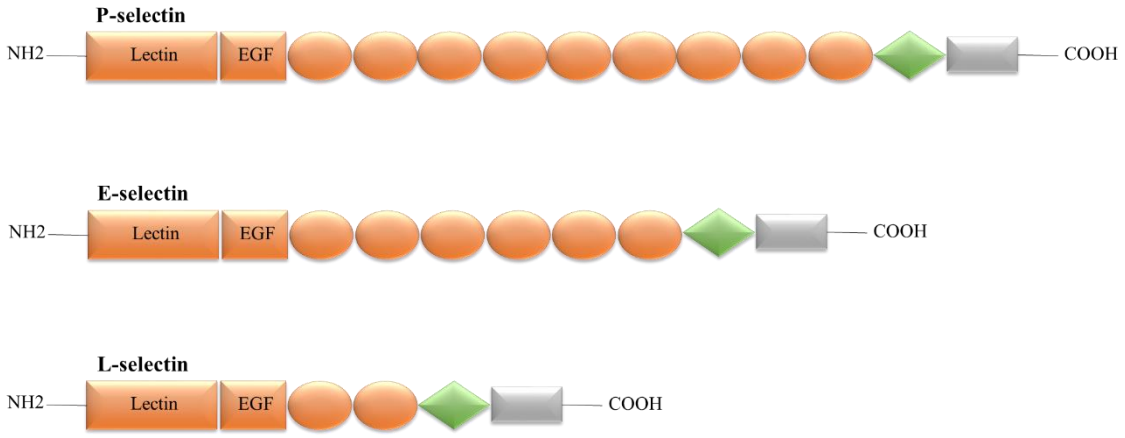

Figure 2: Molecular structure of selectins. Selectins are built by a cytoplasmic region (grey), a transmembrane part (green) and an extracellular region (orange), comprising a lectin domain, an EGF-like domain and a series of short consensus repeats. The number of consensus repeats differs between the several subtypes. (Modified according to [Bevilacqua; Nelson, 1993])

P-selectin (CD62P, PADGEM, GMP-140), first discovered in 1984 at the surface of activated platelets [Hsu-Lin et al., 1984], is expressed by vascular endothelial cells also [Geng et al., 1990; McEver et al., 1989]. The adhesion molecule, synthesized constitutively and stored in intracellular Weibel-Palade bodies [Bonfanti et al., 1989; McEver et al., 1989], is moved to the surface of endothelial cells upon stimulation by inter alia histamine, thrombin [Geng et al., 1990; Hattori et al., 1989; McEver et al., 1989] or surgical manipulation [Doré et al., 1993; Fiebig et al., 1991; Ley, 1994] within minutes [Jung; Ley, 1997]. In addition, cytokine stimulation regulates P-selectin expression at the level of transcription [Gotsch et al., 1994; Jung; Ley, 1997; Sanders et al., 1992; Weller et al., 1992], enhancing the number of available P-selectin. Due to its rapid presentation at the surface of endothelial cells, P-selectin is the dominant mediator of leukocyte rolling in the first hour of an inflammatory insult.

In contrast, E-Selectin (CD62E, ELAM-1, LECAM2) expression is regulated at the transcription level only. Following stimulation by cytokines such as IL-1 and TNFα, E-selectin is synthesized de novo and presented at the surface of endothelial cells [Bevilacqua; Gimbrone, 1987], reaching a maximum concentration after two to four hours [Bevilacqua et al., 1989]. Several studies claimed E-selectin has no or little function in rolling adhesive interactions [Frenette et al., 1996; Johnston et al., 1997; Månsson et al., 2000; Milstone et al., 1998; Norman et al., 2000; Wan et al., 2002]. In this regard, Wan et al. showed that blocking of P-selectin by antibody treatment decreases leukocyte

rolling in TNFα-treated colonic venules to a minimum, whereas inhibition of E-selectin has no effect. It was concluded that E-selectin facilitates downstream events such as activation and adhesion of leukocytes [Wan et al., 2002].

Taken together, the functions of P-selectin and E-selectin appear to be complex. Several studies revealed distinct as well as partially overlapping roles. Leukocyte recruitment was found to be inhibited in P-selectin-deficient mice at early times (initially up to four hours after inflammatory insult), whereas leukocyte rolling was normalized at later times [Bullard et al., 1996; Ley et al., 1995; Mayadas et al., 1993]. It was shown that P-selectin can initiate leukocyte rolling, while E-selectin cannot in the absence of P-selectin [Jung; Ley, 1999]. However, E-selectin can mediate slow rolling [Jung; Ley, 1999]. It becomes obvious that P-selectin is the most versatile selectin, mediating leukocyte tethering and rolling. In contrast, E-selectin plays a more important role in initiating slow rolling of leukocytes and transition to leukocyte adhesion.

L-selectin (CD62L, Leu8) is the only selectin constitutively expressed at the cell surface of most leukocytes [Kansas et al., 1985a; Kansas et al., 1985b; Lewinsohn et al., 1987]. Since its discovery as an adhesion molecule in 1983 its function in leukocyte recruitment is discussed controversially [Gallatin et al., 1983]. Some studies using L-selectin-deficient mice or functional blocking antibodies towards L-selectin reported a critical role of L-selectin in initial leukocyte rolling [Andrian et al., 1992; Arbonés et al., 1994; Jung; Ley, 1999; Ley et al., 1993; Ley et al., 1995]. However, a corresponding endothelial cell receptor could not be found. In contrast, other studies did not observe a decrease in leukocyte rolling after inhibition of L-selectin function [Jung; Ley, 1999; Weninger et al., 2000]. Moreover, recent studies claimed PSGL-1 on leukocytes to be a dominant ligand for L-selectin, facilitating secondary capture of leukocytes by L-selectin/PSGL-1-interactions [Eriksson et al., 2001; Sperandio et al., 2003; Tu et al., 1996]. Secondary capture enables the formation of leukocyte aggregates by allowing free flowing leukocytes that do not express E- or P-selectin to adhere to leukocytes on endothelial cells.

Besides, L-selectin plays a crucial role in the interaction of lymphocytes with high endothelial venules (HEVs) of lymph nodes and the spleen. By binding to glycoproteins, expressed by HEVs, L-selectin mediates lymphocyte homing to lymphatic organs [Arbonés et al., 1994; Gallatin et al., 1983].

1.2.2.2 Selectin receptors

Selectins interact with various counterreceptors, in particular PSGL-1, ESL-1 and CD44. PSGL-1 (CD162, SELPLG) is the major selectin ligand during leukocyte rolling in vivo [Hidalgo et al., 2007; Sperandio et al., 2003; Xia et al., 2002; Yang et al., 1999]. This transmembrane protein, comprising an extracellular, a transmembrane and a cytoplasmic domain, is a high affinity receptor for P-selectin, mediating leukocyte tethering and rolling [Moore et al., 1995; Norman et al., 2000; Yang et al., 1999], and a predominant ligand for L-selectin during secondary capture [Sperandio et al., 2003]. Additionally, PSGL-1 can collaborate with E-selectin (low affinity) to support leukocyte rolling [Hidalgo et al., 2007; Hirata et al., 2000; Xia et al., 2002]. PSGL-1 is constitutively expressed on the tips of microvilli of leukocytes and activated platelets [Moore et al., 1995]. Its location on the tip of microvilli increases the probability of endothelial contact.

ESL-1 (MGF-60, CFr-1) is a high-affinity, versatile ligand of E-selectin which is able to cooperate with PSGL-1 to support leukocyte rolling and with CD44 to induce slow rolling [Hidalgo et al., 2007]. It is a transmembrane glycoprotein, mainly localized in the Golgi apparatus of neutrophils [Steegmaier et al., 1997]. However, some of these molecules are expressed at the cell surface of microvilli, where ESL-1 becomes available for E-selectin [Steegmaier et al., 1997].

CD44 is a transmembrane adhesion glycoprotein, expressed by a large number of cell types. CD44 on leukocytes, a cell-specific posttranslational modified isoform of this molecule, is an E-selectin ligand, expressed at the planar cell body between microvilli of leukocytes [Andrian et al., 1995]. It plays an important role in controlling the velocity of rolling leukocytes, mediating slow rolling when microvilli are receded [Hidalgo et al., 2007; Katayama et al., 2005]. It was shown that the number of rolling leukocytes is not affected by the absence of CD44 though [Katayama et al., 2005].

To sum up, PSGL-1, ESL-1 and CD44 reveal overlapping functions, enabling leukocyte tethering, steady state rolling and slow rolling of leukocytes by interacting with P-, E- and L-Selectin (Figure 3).

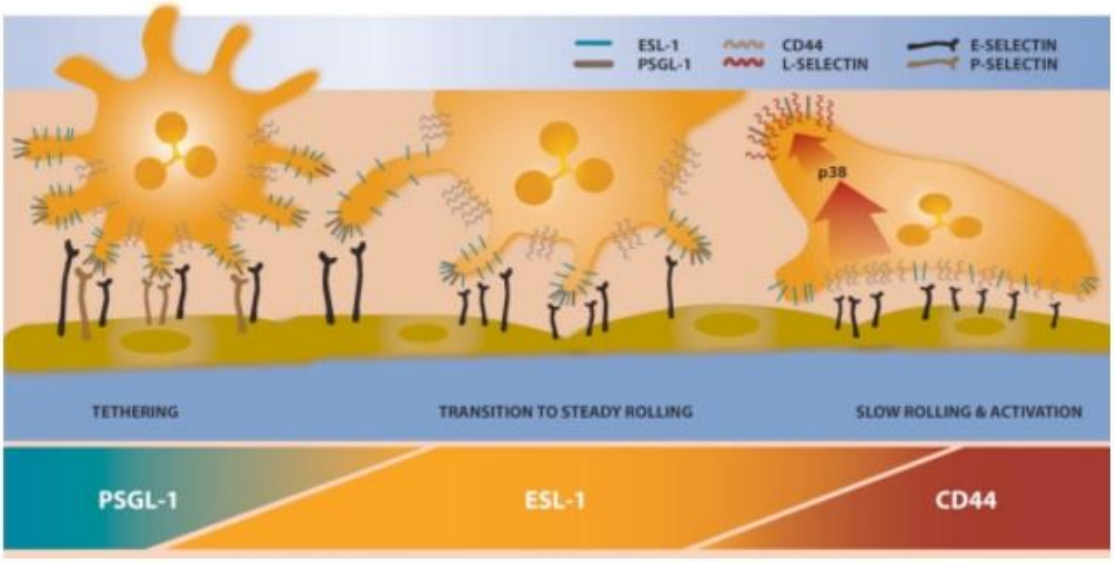

Figure 3: Selectin ligand-mediated leukocyte recruitment. PSGL-1, ESL-1 and CD44 are cooperating high-affinity receptors of selectins with overlapping functions. PSGL-1 is presented on the tips of microvilli, mediating leukocyte tethering and steady-state rolling by binding to P- and E-selectin. ESL-1, expressed at the lateral aspect of microvilli, is crucial for stabilization and rolling on E-selectin. CD44, exposed on the planar surface of leukocytes, interacts with E-selectin to reduce leukocyte rolling velocity. Leukocyte microvilli recede upon stably rolling leukocytes, exposing CD44, which initiates a p38-dependent signalling pathway, supporting secondary capture of leukocytes. (According to [Hidalgo et al., 2007])

1.2.2.3 Integrins

Integrins are a family of transmembrane cell surface receptors expressed by virtually all mammalian cells. Those integrins which are most relevant for leukocyte recruitment, namely lymphocyte function antigen-1 (LFA-1; CD11a/CD18) and membrane-activated complex-1 (Mac-1; CD11b/CD18), are exclusively expressed by leukocytes. In particular, LFA-1 is constitutively expressed on all leukocytes, whereas Mac-1 is presented at the surface of granulocytes, monocytes, macrophages and subsets of activated lymphocytes [Lefort; Ley, 2012]. Mac-1 is partially stored in intracellular granules and can be relocalized upon stimulation by chemoattractants [Jones et al., 1988]. Integrins are heterodimeric glycoproteins, formed by non-covalently paired α- and β-chains. They are divided into subfamilies according to their β-chain. Leukocyte integrins belong to the β_2-subfamily, containing a β_2 (CD18) integrin chain and a unique α-chain (CD11) [Kürzinger et al., 1982].

Leukocyte integrins are activatable receptors which can greatly increase their ligand-binding affinity via inside-out signalling pathways (outlined in Figure 4). That is, LFA-1 and Mac-1 must be activated and change their conformational state to bind to their

ligands. Inactivated LFA-1, marked by a low affinity conformation, is shaped in a bent V-structure with a closed headpiece [Larson et al., 2005; Nishida et al., 2006]. In contrast, active LFA-1 conformations show an extended extracellular domain [Nishida et al., 2006]. Extended structures differ in the conformation of the headpiece. An extended shape with a closed headpiece is often described as intermediate affinity state, whereas a high affinity conformation status is marked by an open headpiece [Luo et al., 2007]. It was shown that PSGL-1/E-selectin engagement induces a spleen tyrosine kinase (Syk)-dependent signalling pathway which results in an intermediate binding affinity [Zarbock et al., 2008]. This conformation allows leukocytes to roll slowly along the endothelium [Yago et al., 2010; Zarbock et al., 2008]. However, cells are not able to arrest on the endothelium because the ICAM-1 binding affinity remains low [Kuwano et al., 2010]. A high-affinity binding state is reached by chemokine contact. The reduced velocity of slow rolling leukocytes enables the cells to get close and hit upon chemokines and cytokines presented by endothelial cells (e.g. IL-8), inducing intracellular pathways via G-protein-coupled receptors (primarily CXCR2-receptors) [Smith et al., 2004; Zarbock et al., 2007]. As a rapid consequence, integrins are fully activated and show a high-affinity conformation state. Accordingly, strength of the receptor-ligand interaction is more powerful which increases ligand binding and decreases ligand dissociation, enabling cells to arrest under flow conditions [Salas et al., 2002; Salas et al., 2004].

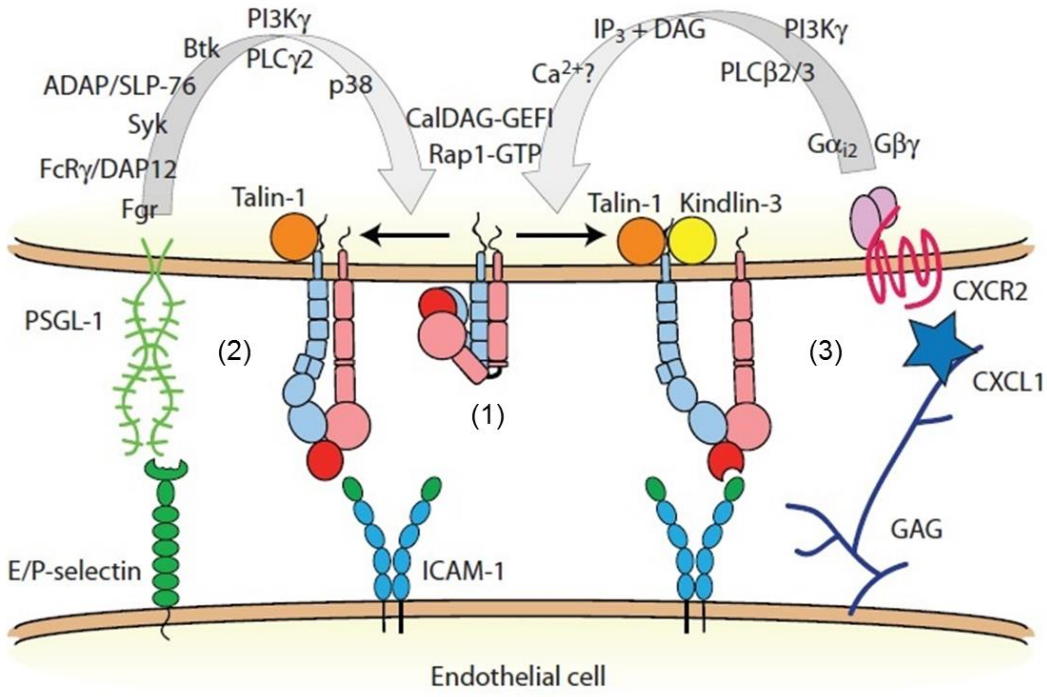

Figure 4: Leukocyte inside-out activation of LFA-1. Inactivated LFA-1 is formed in a bent V-shape with a closed headpiece (1). Engagement of E-/P-selectin to PSGL-1 triggers activation of kinases and adaptors, recruiting Talin-1. The intermediate affinity status of LFA-1 mediates slow rolling on ICAM-1 (2). Upon stimulation of CXCR2 by endothelial chemokines the conformation stage of LFA-1 merges to a high-affinity binding affinity (3), allowing leukocytes to arrest. CXCR2 is a G-protein-coupled receptor which activates a PI3Kγ-dependent, intracellular signalling cascade, enabling the cytoskeletal proteins Talin-1 and Kindlin-3 to bind to the β-subunit of the cytoplasmic tail of LFA-1. (Modified according to [Lefort; Ley, 2012])

However, the literature is rather complex and partly contradictory with respect to the role of β_2-integrins in leukocyte adhesion and the relative importance of specific β_2-integrins appears to vary depending on the type of inflammatory stimulus and experimental model [Argenbright et al., 1991; Dold et al., 2008; Issekutz, 1992; Rutter et al., 1994; Thorlacius et al., 2000]. However, it was confirmed that LFA-1 is an important adhesion molecule in leukocyte recruitment in postcapillary venules [Thorlacius et al., 2000]. Also, further intravital studies proved a key role of β_2-integrins in leukocyte adhesion [Argenbright et al., 1991; Rutter et al., 1994] in which LFA-1 has a more powerful influence on leukocyte arrest than Mac-1 does [Argenbright et al., 1991].

Intercellular adhesion molecules (ICAMs) are major counterreceptors for activated integrins. They are part of the immunoglobulin superfamily which includes five members (ICAM 1-5). Especially ICAM-1 (CD54) is of great interest in leukocyte recruitment as it is the major ligand of LFA-1 and Mac-1 [Chesnutt et al., 2006; Marlin; Springer, 1987;

Phillipson et al., 2006; Yang et al., 2005]. ICAM-1, a transmembrane glycoprotein, is constitutively expressed on endothelial cells [Jung; Ley, 1997]. However, concentrations increase upon stimulation [Jung; Ley, 1997].

1.3 Histones

1.3.1 Types and Function

Histones are a group of structural proteins first discovered by Albrecht Kossel in 1884 [Kossel, 1884]. These positively charged molecules, which allows them to link to negatively charged structures like DNA, are categorized into core histones (H2A, H2B, H3 and H4) and linker histones (H1 and H5) [Allam et al., 2014; Chen et al., 2014].

Intranuclear histones are necessary for chromatin formation by constructing nucleosomes which are built by a histone octamer and wrapping DNA, forming the basic subunit of chromatin. Each octameric core particle is structured by a H3-H4 tetramer and two H2A-H2B dimers. H1 interacts with the linker DNA which extends between nucleosomal core proteins, facilitating higher order chromatin structures. Accordingly, histones are from great importance in DNA packaging and compression. Posttranslational modification of histones, including acetylation, phosphorylation and methylation amongst others, modify gene expression, replication and regulation [Allam et al., 2014; Chen et al., 2014].

1.3.2 Extracellular Histones

1.3.2.1 Modes of histone release into the extracellular space

In contrast to their nuclear function, histones trigger numerous proinflammatory and toxic effects when translocated to the extracellular space upon cell damage or death [Saffarzadeh et al., 2012; Xu et al., 2009].

A major mechanism of histone release is “NETosis”, a pathogen-induced, new form of cell death, characterized by the release of neutrophil extracellular traps (NETs) [Blomgran et al., 2007; Fuchs et al., 2007]. NETs are DNA forming extracellular web-like structures decorated with nuclear histones as well as cytoplasmic and granule proteins [Brinkmann et al., 2004; Urban et al., 2009]. These network-like formations are expelled by activated neutrophils to trap and kill various microorganisms such as bacteria [Brinkmann et al., 2004; Ramos-Kichik et al., 2009], protozoans [Baker et al., 2008; Guimarães-Costa et al., 2009] and fungi [Fuchs et al., 2007; Remijsen et al., 2011; Urban

et al., 2009]. However, it is commonly recorded that the formation of extracellular traps ("ETosis" [Wartha; Henriques-Normark, 2008]) is not restricted to neutrophils, but occurs on eosinophils [Yousefi et al., 2008] and mast cells also [Köckritz-Blickwede et al., 2008]. As part of the innate, first line immune system NETs are built upon pathogen contact or proinflammatory stimuli [Remijsen et al., 2011]. Histones are an essential part of extracellular traps, as the antimicrobial effect of NETs is significantly reduced upon anti-histone blocking [Brinkmann et al., 2004]. Furthermore, histones mediate the prothrombotic activity of NETs and play an regulatory role during NETosis [Remijsen et al., 2011; Semeraro et al., 2011].

In addition to NETosis-mediated histone release, histones can also be set free from apoptotic or necrotic cells [Chen et al., 2014]. Necrosis, a pathological form of cell and tissue death caused by external irreparable cell damage (e.g. trauma, infections, toxins, hypoxia amongst others), involves an uncontrolled action of cell lysis. Due to the disruption of the nuclear and the outer plasma membrane it results in a dramatic discharge of intracellular components, including histones.

Besides, it was reported that histones can be concentrated in and leak from apoptotic cell blebs [Wickman et al., 2013; Wu et al., 2002]. Apoptosis is a controlled form of cell death that appears to be intrinsically programmed. It is characterized by distinctive morphological cell changes including nuclear and DNA fragmentation, chromatin condensation, cell shrinking and blebbing.

1.3.2.2 Pathogenic effects

Extracellular histones, primarily released upon a potential threat for the organism, combine a number of different mechanisms to restore homeostasis and protect the individual.

First and foremost, histones have a potent bactericidal effect on various microorganism. The high antimicrobial impact of histones in vitro was already proved more than 50 years ago [Hirsch, 1958]. However, the basic principles of the lethal effect are not completely understood yet [Allam et al., 2014]. Histones can kill Gram(+)-bacteria and Gram(-)-bacteria [Brinkmann et al., 2004; Hirsch, 1958; Ramos-Kichik et al., 2009] as well as parasites [Wang et al., 2011] in submicromolar concentrations [Brinkmann et al., 2004; Parseghian; Luhrs, 2006]. Considering a local concentration of histones in NETs, histone-induced pathogen defense is from great importance for the immune reaction.

Besides a pathogen-specific bactericidal effect, extracellular histones feature an unspecific host cell cytotoxicity, first reported by Xu et al. in 2009 [Xu et al., 2009]. It was demonstrated that extracellular histones are cytotoxic towards the endothelium in vitro and intravenous injection of histones causes death of tested mice, demonstrating cytotoxicity of histones in vivo. It could be further shown that toxic effects are mainly caused by histone 3 and histone 4 [Xu et al., 2009]. Histone-induced cytotoxicity towards alveolar epithelial cells and endothelial cells, leading to tissue damage and inflammation, was confirmed by several research groups [Bosmann et al., 2013; Saffarzadeh et al., 2012]. Direct cytotoxic effects of histones could also be demonstrated on renal endothelial cells, tubular epithelial cells and podozytes in vitro [Allam et al., 2012; Kumar et al., 2015]. Moreover, injection of histones in the renal artery led to a powerful necrosis of the kidney [Allam et al., 2012]. However, the mode of histone-induced cytotoxicity remained partly elusive. A non-regulated, charge-dependent form of cell-death was reported [Gillrie et al., 2012].

As extracellular histones have the ability to trigger thrombosis, histones may combine microbicidal and prothrombotic properties to fight invading pathogens and maintain homeostasis after injury [Fuchs et al., 2010; Semeraro et al., 2011]. However, on the other hand, platelet aggregation – which is based upon TLR2/4 activation on platelets, inducing the activation of NF-κB [Carestia et al., 2013; Semeraro et al., 2011] – is a major mediator of histone-induced death because platelet-deficient mice (in contrast to non-deficient animals) do not die from intravenously histone injection [Fuchs et al., 2011]. Indeed, histones might be the linker between inflammation and prothrombotic activity as extracellular histones increase thrombin generation, inducing a procoagulant phenotype causing dysfunctional coagulation in systemic inflammation [Semeraro et al., 2011].

Besides histone-induced cytotoxicity and histone-triggered thrombin generation, extracellular histones have the ability to cause a proinflammatory environment by various signal pathways. Once released, histones can selectively bind to Toll-like receptor TLR2 and TLR4 [Allam et al., 2012; Semeraro et al., 2011; Xu et al., 2011]. Activation of TLR2/4 triggers an inflammatory response by inducing an upregulation of proinflammatory mediators via activation of NF-κB [Kawai; Akira, 2007]. Activation of TLR9 by extracellular histones is discussed controversial [Allam et al., 2012; Huang et al., 2011]. Toll-like receptors are membrane-spanning proteins, primarily expressed at the

surface of sentinel cells which play an important role in the immune system by recognizing various PAMPs. However, TLRs can also be activated via DAMPs including extracellular histones.

Also, extracellular histones may trigger a proinflammatory environment by activating NLRP3 inflammasomes [Allam et al., 2013]. Inflammasomes are multiprotein complexes, consisting of a NOD-like receptor (NLR), an intracellular PRR recognizing PAMPs and DAMPs. They mediate activation of caspase-1, controlling maturation and secretion of proinflammatory mediators such as IL-1β and IL-18 [Schroder; Tschopp, 2010]. The best characterized inflammasome is NLRP3, activated by endogenous indicators of cellular danger or stress, such as histones [Schroder; Tschopp, 2010].

To sum up, histones can be released into the extracellular space by activated immune cells (ETosis) and dying cells (necrosis and apoptosis) (cf. Figure 5). Once released, histones show significant toxic and proinflammatory effects in vitro and in vivo. Their cytotoxicity can be directed against invading microorganisms and eukaryotic host-cells. Extracellular histones can moreover accelerate powerful inflammatory responses by binding to PRRs, e.g. TLR-activation and inflammasome mobilisation. Besides, TLR-activation increases thrombin generation (cf. Figure 5).

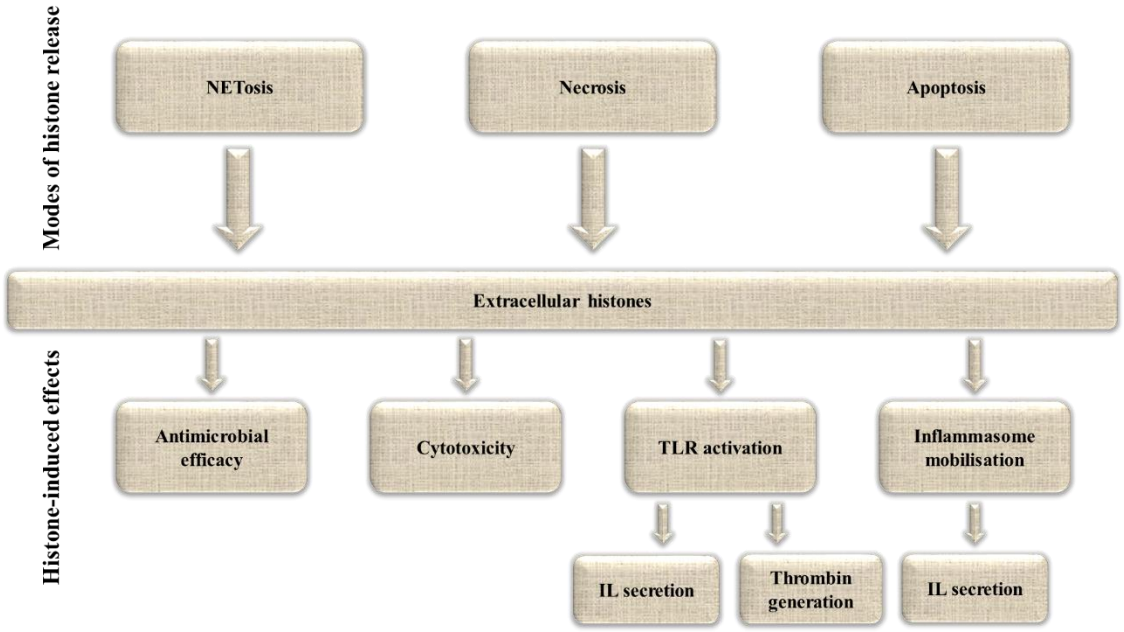

Figure 5: Schema of histone release and histone-induced effects in the extracellular space.

1.3.2.3 Pathogenic role of histones in diseases

As described, histones can act as endogenous DAMPs, playing an important role in the innate immune response. However, extracellular histones are said to be a double-edged sword because they also act as major mediators of injury in inflammatory diseases, causing fatal organ dysfunction and death. This is based upon histone-induced cytotoxicity towards host cells and an increased release of proinflammatory cytokines (e.g. supporting a progressive inflammation), accelerated by positive feedback mechanisms of histone release (cf. Figure 6).

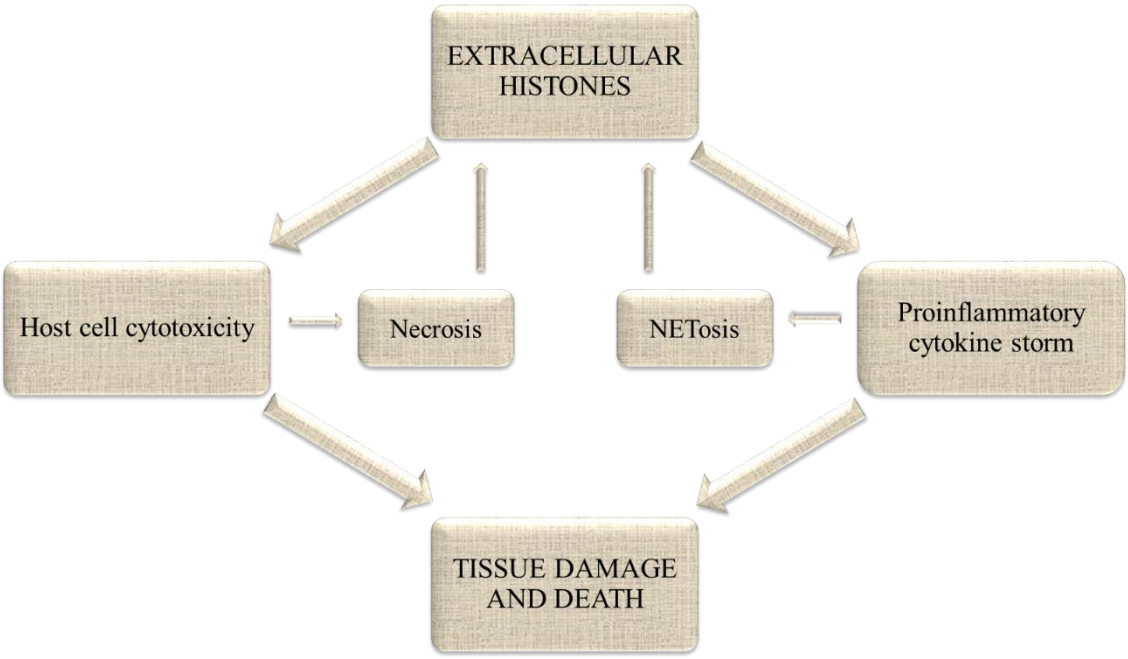

Figure 6: Circle of histone-induced tissue damage and death. Extracellular histones, released by NETosis or dying cells, trigger - on the one hand - a powerful proinflammatory response by TLR-activation and inflammasome mobilisation. Thus, PMNL and macrophages get activated, resulting in an increased generation of extracellular traps. On the other hand, extracellular histones induce necrosis of host cells due to their direct cytotoxicity. Both cases illustrate a positive feedback-mechanism, causing an augmented release of histones themselves which results in progressive severe tissue damage and death.

Indeed, several studies showed that extracellular histones play an important pathophysiological role in several acute conditions involving tissue trauma and inflammation. It could be demonstrated that extracellular histones accumulate in the plasma of patients suffering from traumatic injury, correlating with severe complications and prognosis [Chen et al., 2014; Kutcher et al., 2012]. Likewise, the bronchoalveolar lavage (BALF) of acute lung injury (ALI)-patients contains increased concentrations of

histone 3 and histone 4 [Bosmann et al., 2013] and Meyer et al. observed an increased level of nucleosomes after an ischemic stroke in mice [Meyer et al., 2012].

Moreover, it could be observed that histones are major mediators of death in both sterile inflammation and sepsis (e.g. infection) [Huang et al., 2011; Xu et al., 2009; Xu et al., 2011]. In particular, histone-induced maintenance of sepsis is caused by their ability to trigger thrombosis and inflammation as well as endothelial cytotoxicity of extracellular histones [Xu et al., 2009]. Likewise, histone-induced direct lung damaging effects were reported to lead to severe respiratory disturbances, intense tissue inflammation and occasional death in vivo [Bosmann et al., 2013]. Besides, extracellular histones are involved in the pathogenesis of severe glomerulonephritis by vascular injury in glomeruli [Kumar et al., 2015].

Interestingly, inhibition of histone function by neutrophil-depletion, neutralization of histones with specific IgG antibodies, activated protein C (APC) or heparin was associated with reduced levels of tissue accumulation of leukocytes and effectively improved histone-induced organ failure and death [Allam et al., 2012; Bosmann et al., 2013; Huang et al., 2011; Kumar et al., 2015; Meyer et al., 2012; Xu et al., 2009; Xu et al., 2011].

AIMS OF THE STUDY

Based on these consideration, this study aimed to examine the effect of histone 3 on the leukocyte extravasation process in detail. Further, the adhesive mechanisms mediating leukocyte-endothelium interactions and recruitment in response to extracellular histone challenge were determined.

To answer these scientific issues, intravital microscopic investigations on the mouse cremaster muscle were conducted. In-vivo observations were completed by molecular biological, biochemical and histological analyses.

The following questions were investigated:

1. How do extravascular histones 3 influence leukocyte-endothelium interaction and leukocyte recruitment?

2. Which are the dominating molecules mediating histone-induced effects?

3. Which is the dominating leukocyte subtype responding to histone challenge?

MATERIAL AND METHODS

3.1 Animals

Experiments were performed on male C57/BL6 mice at the age of 10-12 weeks. Mice weighed 22-26g and were maintained on 12-hours dark and 12-hours light cycles. The animals had unlimited access to water and food.

The animal experiments were approved by the Regional Ethical Committee for Animal Experimentation at Lund University, Sweden.

3.2 Experimental model

This study focused on intravital microscopy of the microcirculation of the open mouse cremaster muscle, first described in 1973 [Baez, 1973]. The M. cremaster is a thin layer of muscle fibers of the M. obliquus internus and the M. transversus abdominis that surround the testicle. It encloses the Funiculus spermaticus and descends through the inguinal canal. If specific stimuli (e.g. low temperature) occur the testis will be drawn back towards the abdominal cavity by muscle contraction. It therefore plays an important role in the heat regulation of the testicle. The cremaster muscle is supplied by an extensive system of small blood vessels. A central artery is clearly visible in the medial region of the dissected muscle which is derived from a distal segment of the A. spermatica externa, a branch of the pubic-epigastric artery [Meiniger et al., 1987]. The artery goes along with a paired vein and divides itself in the course of the vessel. From arterioles beyond metarterioles, a complex endothelial capillary system is built which continues to postcapillary venules [Baez, 1973].

3.2.1 Anesthetics and Preparation

Prior to the microsurgical preparation of the mouse cremaster muscle, animals were anesthetized by intraperitoneal administration of 75mg ketamine hydrochloride (Hoffmann-La Roche, Basel, Switzerland) and 25mg xylazine (Janssen Pharmaceutical, Beerse, Belgium) per kilogram body weight.

The preparation was conducted as follows:

Three hours before preparation and immediately prior to the injection of mentioned substances, the hair of the lower abdomen and scrotum was carefully removed by an electrical shaver. The cremaster muscle preparation was performed on a transparent

pedestal to allow transillumination, a heating pad provided conductive heat to maintain the body temperature at 37°C. The mice were placed on their back and fixed with tapes. Before starting the preparation, slight pressure was put about the lower abdomen to push the testis down into the scrotum. A midline incision of the skin and fascia was made along the ventral aspect of the right scrotum then (Figure 7A). The preparation required great attention not to touch the surrounding tissues. The incised skin was fixed by pins made of 30G needles (B/Braun, Melsungen, Germany) and shaped in "L" form. Afterwards, the apex of the scrotum was pierced by a hook attached to a thread which was secured at the pedestal by putting slight tension at the cremaster muscle sack (Figure 7B). Pins were released and the scrotal skin was pushed back gently towards the site of preparation. The surrounding tissue was carefully removed to expose the cremaster muscle then, avoiding to touch the cremaster. Afterwards, the ventral surface of the cremaster muscle was opened by a longitudinal incision (Figure 7C). Great care was taken to minimize interruptions of the vessels. The edges of the cremaster muscle were secured at the pedestal by pins and the testis was gently retracted towards the abdomen (Figure 7D). In order to do so, connective ligaments between muscle, epididymis and vessels going from the apex of the cremaster muscle to the epididymis were cut through by a thermal cautery. Finally, the dissected cremaster muscle was covered with PBS and a thin film. The preparation took about 15 minutes in total. Microscopic observations were made after an equilibration time of ten minutes.

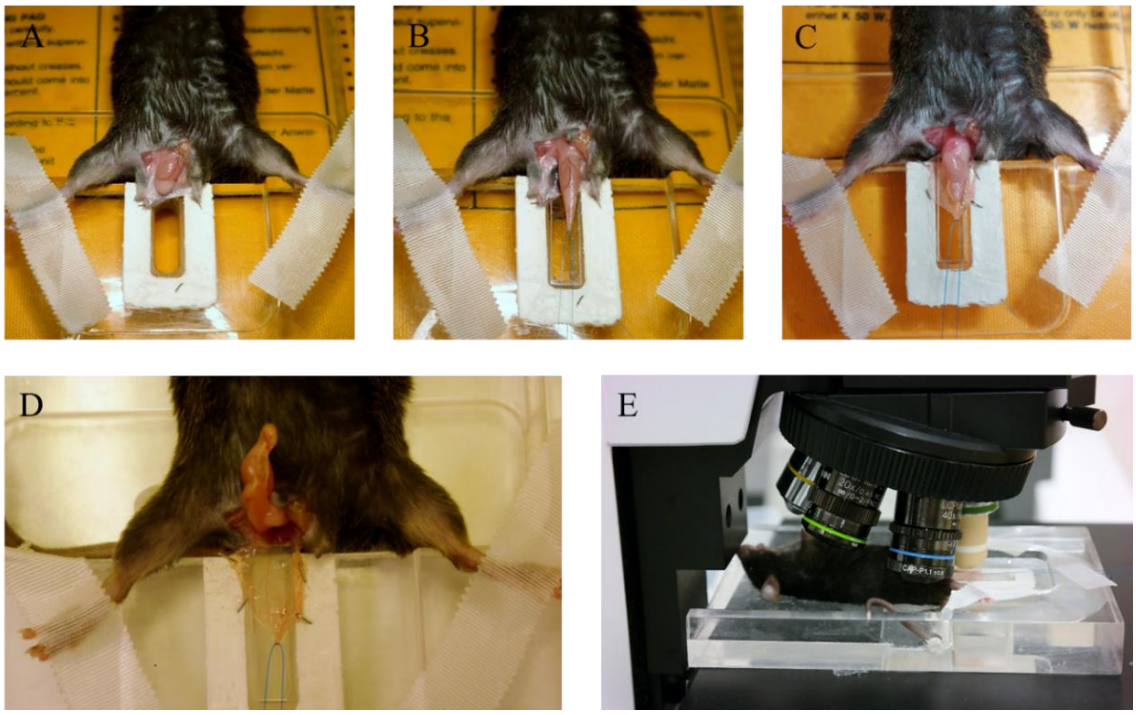

Figure 7: Microsurgical preparation of the mouse cremaster muscle (A-D). Demonstration of the IVM (E).

3.2.2 Intravital Microscopy

Observations of the cremaster microcirculation were made using a transmitted light microscope from Olympus (BX50WI, Olympus Optical Co. GmbH, Hamburg, Germany) equipped with water immersion lenses (20/NA 0.5 and 40/NA 0.75) and light illuminator attachments with fluorescence filters. The microscopic images were recorded using a charge-coupled device video camera (FK 6990 Cohu, Pieper GmbH, Berlin, Germany) for subsequent off-line analysis.

Postcapillary venules with an inner diameter between 20 and 40µm, located near the center of the preparation and with stable resting blood flow, were considered to be representative and were used for analysis of leukocyte flux and leukocyte-endothelium interactions. Five representative venules were examined per animal.

3.2.2.1 Fluorescence microscopy: microhemodynamic parameters

An incident-light fluorescence microscope was used for analysis of microvascular hemodynamics. Fluorescence is a type of luminescence which is characterized by the phenomenon that the fluorescence dye can be activated and shine by the light of a specific

wavelength. Due to a specific choice of the dye as well as excitation and emission filters several parameters can be analyzed.

In this study 0.1ml of 5% fluorescein isothiocyanate (FITC)-dextran (MW 150 000, Sigma-Aldrich, Stockholm, Sweden) were used for analysis of microvascular hemodynamics. FITC is characterized by an excitation wavelength of 490nm and an emission peak of 515nm [Schröder et al., 1976]. It is covalently labelled with a high-molecular-weight dextran (molecular weight (MW) 150 000D) by a thiocarbamoyl binding. Dextran, a biological polysaccharide, is a water-soluble, stable and non-toxic polymer, preventing an extravasation of the dye. FITC-dextran was applied by a retro-orbital injection which is a simple, effective and economical way to inject substances [Nanni et al., 2007; Li et al., 2011]. The rich capillary microcirculation of the mouse orbital venous sinus enables a fast absorption of the substances. The cremaster muscle microvasculature was visualized by a 100-W mercury lamp and a filter set for blue light epi-illumination (450–490nm excitation and >520nm emission wavelength).

3.2.3 Blood sampling

At the end of the experimental protocol blood was sampled from the tail vein and diluted 1:20 in Turks solution (0.2mg gentian violet in 1ml glacial acetic acid, 6.25% vol/vol) for quantification of systemic leukocyte subsets.

3.2.4 Flow cytometry

Flow cytometric measurements were conducted for analysis of antibody-induced effects of anti-Ly6G on blood cell subpopulations.

Flow cytometry is a laser-based cell sorting technique in which every cell of the examined fluid passes a flow cell separately and gets beamed by a laser. Thus, a specific light scatter and fluorescence signal is generated for each cell type. The level of the forward scattered light (FSC) depends on the volume of the cell. The intensity of the fluorescence signal is reliant on the used fluorescence dye and channel, assuming that the examined cells sustain the antigen to which the fluorescence-marked antibody is supposed to bind.

Two groups were compared:

1. Control group: sham (i.e. no inj. of histones) + control AB (n=5)
2. Treatment group: sham + anti-Ly6G AB (n=5)

The antibodies were given intraperitoneally 24 hours prior to blood sampling. The blood was harvested from the inferior vena cava and stained with phycoerythrin(PE)-conjugated anti-mouse Ly-6G (clone 1A8, BD Pharmingen), PE/Cy7-conjugated anti-mouse Ly6C (clone AL-21, BD Pharmingen), FITC-conjugated anti-mouse CD19 (clone MB19-1, eBioscience) and FITC-conjugated anti-mouse CD3 (clone 145-2C11, BD Pharmingen) antibodies. The cells were further conserved and erythrocytes were lysed by a lysing solution (BD Bioscience) to prevent covering of the target population.

Accordingly, the following antigens of blood cells were targeted by antibodies: lymphocyte antigen 6G (Ly6G), lymphocyte antigen 6C (Ly6C), CD19 and CD3. Ly6G is a specific antigen of neutrophils which function is left unknown [Wang et al., 2012]. It is tied to the surface of neutrophils through a GPI linker and belongs to the Ly6G/urokinase plasminogen activator receptor family. In contrast, Ly6C (lymphocyte antigen 6C), which has a similar structure as Ly6G, is found on several hematopoietic cells such as some monocyte/macrophage populations, granulocytes, endothelial cells, plasma cells, thymocytes, NK-cells and T-subsets. CD3 is a T-cell coreceptor and found on T-lymphocytes only, whereas CD19 is a B-lymphocyte antigen and only found on B-lymphocytes. The antibodies were linked to fluorescence dyes which differ in their excitation and emission range: PE (excitation range: 486-580nm, emission range: 568-590nm), PE-CY7 (excitation range: 486-575nm, emission range: 750-810nm) and FITC (excitation range: 468-509nm, emission range: 504-541nm). This enables the use of different channels for the measurement of fluorescence signals. Channel FL1-H was used for FITC-conjugated anti-CD3/anti-CD19 antibody, FL2-H for PE-conjugated anti-Ly6G antibody and FL3H for PE/CY7-conjugated anti-Ly6C antibody.

Flow cytometric analyses were performed using standard settings of FACS Calibur flow cytometer (Becton Dickinson, Mountain View, CA, USA) and Cell-Quest Pro software (BD Biosciences). A viable gate was used to exclude dead cells and fragmental components.

3.3 Experimental Methods

3.3.1 Determination of the vessel diameter

Vessel diameters (d) were measured in micrometer (µm) perpendicularly to the inner vessel wall. Representative venules with a diameter between 20 and 40µm were observed.

3.3.2 Determination of leukocyte-endothelium interactions

The leukocyte rolling flux is the number of rolling cells passing a reference point in the microvessel in one minute. Accordingly, the flux was determined by counting the number of rolling cells per 20s passing a line, placed vertically to the vessel rim, and multiplied by three. The leukocyte rolling flux was expressed as cells per minute.

Cells were considered as adherent when they were stationary for at least 20s. The number of adherent leukocytes was counted in 100µm long vascular segments and expressed as number of adherent cells per mm^2.

Leukocyte emigration was quantified by counting the number of extravascular cells within an extravascular area of 100 x 70µm immediately adjacent to each side of the venule. Leukocyte emigration was expressed as number of extravascular cells per mm^2.

Methods of determining the number of rolling, adherent and emigrated cells are illustrated in Figure 8 and 9.

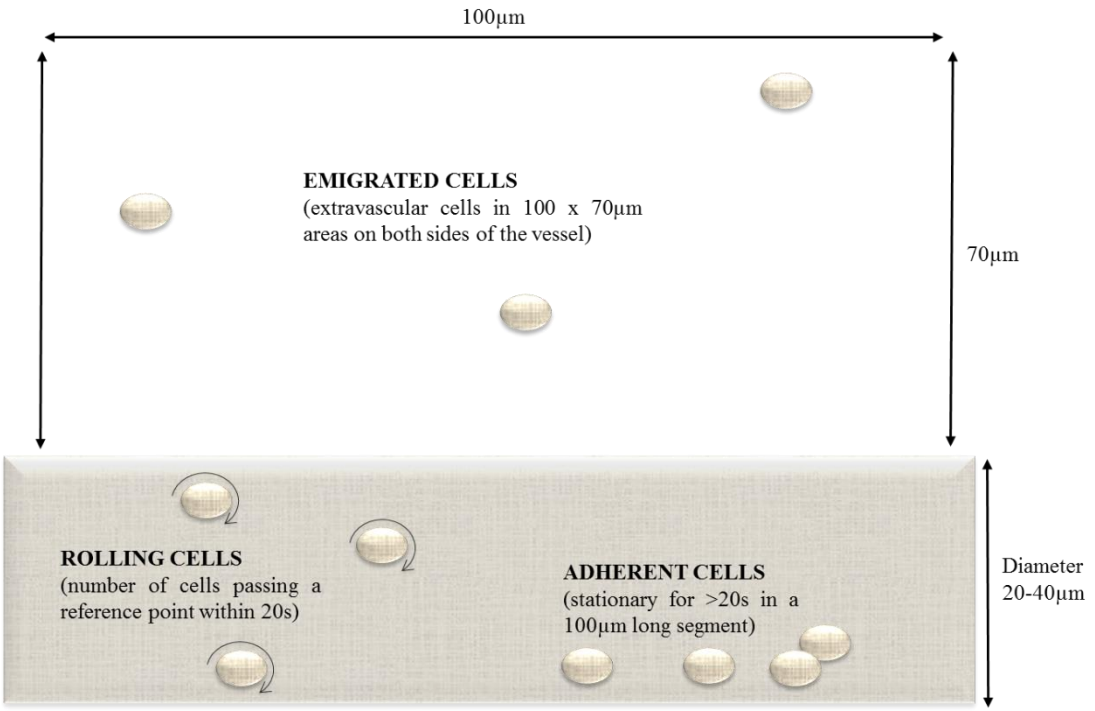

Figure 8: Illustration of measurements made in postcapillary venules. (Modified according to [Gavins; Chatterjee, 2004])

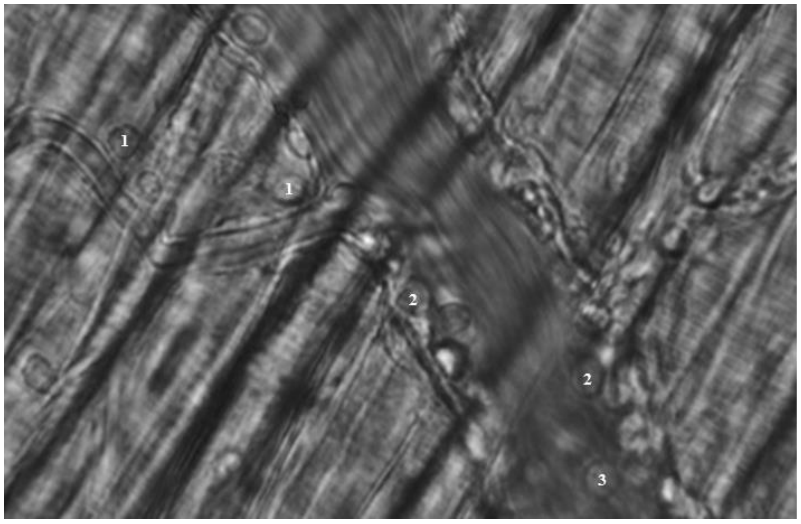

Figure 9: Illustrated image of intravital measurements of the microcirculation. Distinction between rolling cells as well as adherent and emigrated leukocytes.

3.3.3 Microhemodynamic parameters

Hemodynamics is the science dedicated to blood flow dynamics. Blood flow (VQ) describes the mass transport in a microvessel and is commonly determined as red blood cell (RBC) velocity [Slaaf et al., 1986].

The centerline RBC velocity (v_{RBC}) was analyzed by the computer-assisted image analysis system CapImage (Zeitl, Heidelberg, Germany) using the line shift method. At first, a measuring line was defined. The line was placed in the center of the vessel lumen in flow direction (Figure 10A). During the measurement period of ten seconds the gray values along the line were quantified and visualized in a line-shift-diagram (Figure 10B). The diagram indicates light and dark oblique lines which demonstrate cell movements and plasma gaps. According to the gradient of the lines the centerline RBC velocities (mm/s) were determined by the program.

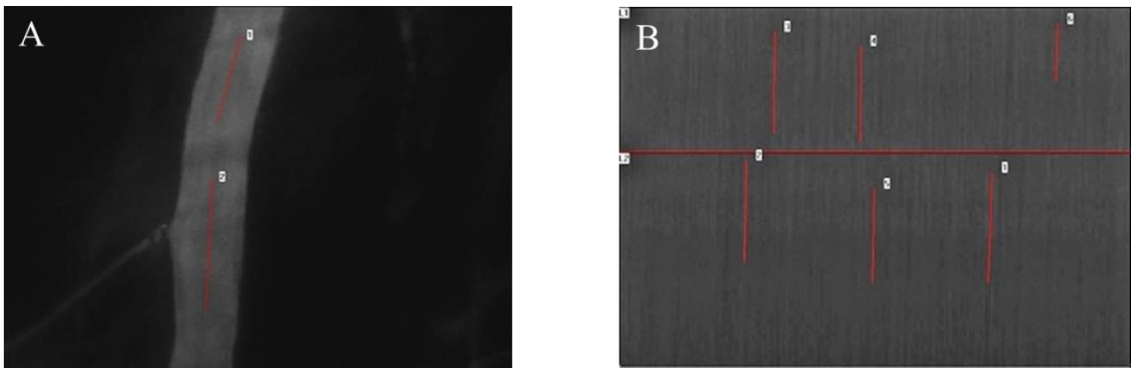

Figure 10: Determination of microhemodynamic parameters by use of the line shift method. Intravital recording of a representative venule of the mouse cremaster muscle stained with FITC-dextran. Two measurement lines for velocity reading are illustrated (A). Corresponding line shift diagram (B).

The mean blood flow velocity (v_{mean}) was calculated by multiplying the centerline RBC velocity with an empirical factor of 0.625 [Lipowsky; Zweifach, 1978]. The blood flow is the result of v_{mean} multiplied by the area. To sum up, the blood flow was calculated by:

$$VQ\ (nl/s) = \pi(d/2)^2\,(0.625\ v_{RBC})$$

3.3.4 Systemic leukocyte counts

Quantification of leukocyte subsets was done by the help of a Burker chamber using a light-optical microscope.

In general leukocytes are classified in mononuclear leukocytes (MNL) and polymorphonuclear leukocytes (PMNL) according to the morphology of the nuclei. Lymphocytes and monocytes are mononuclear leukocytes; their nuclei are not lobated. In contrast granulocytes, including neutrophil, basophil and eosinophil granulocytes, are polymorphonuclear cells. Their nuclei consist of two and more segments, connected by fine cytoplasm bridges.

Accordingly, leukocytes were considered mononuclear if a lobated nucleus wasn´t noticeable. Whereas, if several segments of the nucleus were recognized, cells were counted as polymorphonuclear leukocytes.

3.4 Experimental Protocol

3.4.1 Antibodies and biochemical substances

According to the experimental protocol the following biochemical substances and antibodies were used:

Histone 3 (1-100µg, Hoffmann-La Roche, Basel, Switzerland) and TNFα (0.5µg, R&D Systems Europe, Ltd., Abingdon, Oxon, U.K.) were diluted in 0.15ml phosphonate-buffered saline (PBS) and injected intrascrotally three hours prior to the cremaster muscle preparation.

In order to analyze the role of selectins and integrins in leukocyte-endothelium interactions, monoclonal antibodies directed against PSGL-1 (1.6mg/kg, clone 2PH1, rat IgG, BD Biosciences, San Diego, CA, USA), P-selectin (1.6mg/kg, clone RB40.34, rat IgG, BD Biosciences), L-selectin (3mg/kg, clone MEL-14, rat IgG, BD Biosciences), Mac-1 (4mg/kg, clone M1/7, rat IgG, BD Biosciences) and LFA-1 (4mg/kg, clone M17/4, rat IgG, BD Biosciences) as well as a control antibody (clone R3-34, rat IgG1, BD Biosciences) were given intravenously into the jugular vein immediately prior to histone injection.

To deplete neutrophils, an antibody against anti-Ly6G (20mg/kg, clone 1A8, rat IgG, BioXcell, West Lebanon, NH, USA) was administered intraperitoneally 24 hours before intrascrotal challenge with histone 3.

3.4.2 General effects of extracellular histones 3

At first, a dose-response relationship of histone 3 was conducted. Accordingly, histone 3-induced effects on leukocyte rolling, adhesion and emigration were analyzed for 1µg (n=5), 10µg (n=7) and 100µg (n=5) of histone 3. Results were compared to sham animals, receiving 100µl PBS intrascrotally. Animals were treated three hours prior to surgical preparation.

To rate the effect of extracellular histones 3 on leukocyte recruitment, the influence of histone 3 was compared to the one of TNFα (n=5). TNFα is a well explored, multifunctional cytokine which is part of the acute phase protein family. It is mainly released by macrophages. TNFα is known to cause a powerful leukocyte response in the cremaster muscle [Wan et al., 2002].

3.4.3 Molecular mechanisms of histone 3-induced effects

In order to determine the role of selectins and integrins in histone-induced leukocyte-endothelium interactions, animals were treated systemically with functional blocking antibodies.

The following seven groups were analyzed:

(1) Sham (n=7)
(no inj. of an AB, PBS)

(2) Control group (n=7)
(control AB, H3 10µg)

(3) Anti-PSGL-1 AB group (n=5)
(anti-PSGL-1 AB, H3 10µg)

(4) Anti-P-selectin AB group (n=5)
(anti-P-sel AB, H3 10µg)

(5) Anti-L-selectin AB group (n=5)
(anti-L-sel AB, H3 10µg)

(6) Anti-Mac-1 AB group (n=4)
(anti-Mac-1 AB, H3 10µg)

(7) Anti-LFA-1 AB group (n=5)
(anti-LFA-1 AB, H3 10µg)

The antibodies were given intravenously ten minutes prior to intrascrotal injection of histone 3. If not discussed differently, preparation of the cremaster muscle was carried out three hours after injection of histone 3 and PBS, respectively. Microscopic observations were performed after an equilibration time of ten minutes. The experimental protocol is illustrated in Figure 11.

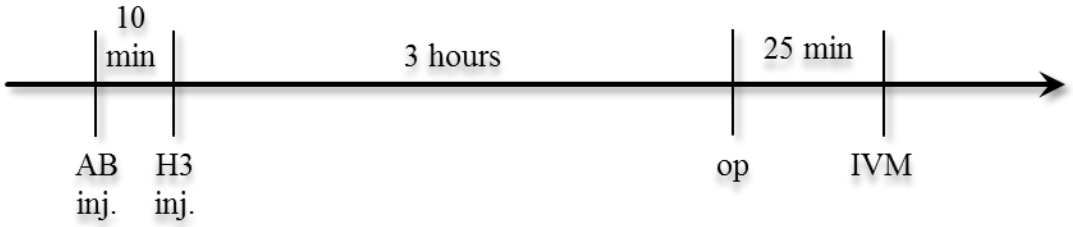

Figure 11: Illustration of the experimental protocol for analysis of molecular mechanisms of histone-induced leukocyte-endothelium interactions.

In a separate experiment leukocyte-endothelium interactions and emigration were quantified before and after administration of monoclonal antibodies directed against P-selectin, PSGL-1 and a control antibody (n=5). The microscopic observations before and after antibody injection were performed at exactly the same segment of the observed venule. The experimental procedure is demonstrated in Figure 12.

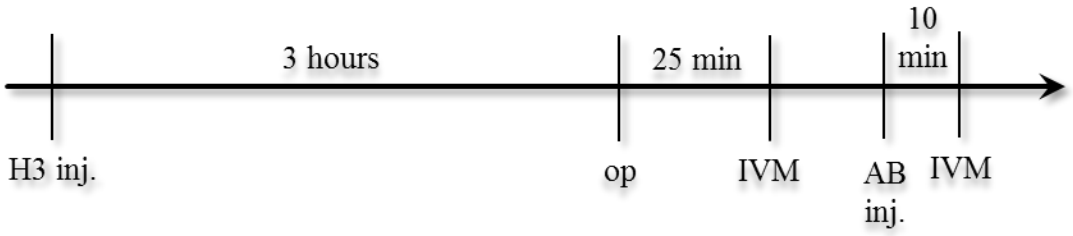

Figure 12: Illustration of the experimental protocol of molecular analysis before and after antibody treatment.

3.4.4 Responding leukocyte subpopulation to histone 3 treatment

To identify which leukocyte subpopulation is the one responding to challenge with histone 3, an antibody against Ly6G was used.

The anti-Ly6G antibody was given intraperitoneally 24 hours prior to the intrascrotal injection of histone 3 (Figure 13). Anti-Ly6G antibody is known to effectively deplete neutrophils in mice [Daley et al., 2008].

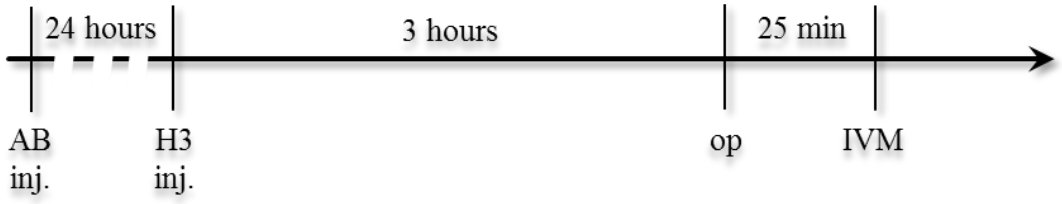

Figure 13: Illustration of the experimental protocol of neutrophil depletion.

3.5 Statistics

Five representative venules of every mouse cremaster muscle were analyzed. Excel was used to determine the mean of the diameter as well as the mean of the number of rolling, adherent and emigrated cells of every animal. These data were transferred to GraphPad PRISM (Version 6, GraphPad Software Inc., La Jolla, CA, U.S.) where statistical analyses were done.

Data are presented as mean value ± standard error of the mean (SEM) and "n" indicates the number of animals per group.

Due to a small data set (n=5 in most cases), data were considered non-parametric. Appropriate to unmatched groups and non-parametric data, statistical evaluations were performed using Mann-Whitney rank sum test for comparing two groups. $p<0.05$ was considered significant.

If more than two groups were tested, a Kruskal-Wallis test was conducted. To find out which groups deviate significantly, Mann-Whitney tests were used for pair-wise comparison. To avoid multiple comparison problems, an α-adjustment by means of Bonferroni-Holm-method was conducted by hand. The global P-value (α_g) was determined as 0.05. According to Bonferroni-Holm local α-standards (α_i) were specified by:

$$\alpha_i = \frac{\alpha_g}{(k-i+1)}$$

i is the number of tests determined, whereby i=1,…,k is valid. The P-values (P_i) of the conducted Mann-Whitney tests, arranged from the smallest to the biggest, were compared to the corresponding α_i. Data were considered significant if $P_i < \alpha_i$.

For statistical analysis of paired groups, i.e. if the same animals were observed before and after treatment, a Wilcoxon rank test for matched pairs was performed and $p<0.05$ was considered to be significant.

RESULTS

4.1 Histone 3-induced leukocyte-endothelium interactions

4.1.1 General effects of extravascular histones 3

In order to analyze the general influence of extravascular histones 3 on leukocyte endothelium interactions, histone-induced effects on leukocyte rolling, adhesion and emigration were examined.

First, a dose-response relation for histone 3-exposed tissues (1µg, 10 µg and 100µg) was conducted (Figure 14). Sham animals, which received an intrascrotal injection of PBS, showed an increased number of rolling cells per minute in this study. 40.2±4.3 rolling leukocytes per minute were observed (Figure 14A). However, the number of adherent and emigrated leukocytes remained low in unstimulated tissue. 540.7±96.4 cells per mm^2 were adherent to the inner vessel wall and 35.0±10.4 cells per mm^2 emigrated into the interstitium (Figure 14B-C). Local challenge with histone 3 did not lead to an increase of rolling leukocytes (Figure 14A). In contrast, intrascrotal injection of histone 3 caused a dose-dependent increase in firm leukocyte adhesion. Whereas 1µg of histone 3 did not lead to a clear increase of leukocyte rolling, 10µg and 100µg of histone 3 increased the number of adherent cells by 3.5- and 4.5-fold to 2009.0±166 cells per mm^2 (10µg) and 2553.0±347.7 cells per mm^2 (100µg), respectively ($p<0.01$) (Figure 14B). A similar effect of histone 3 could be seen at the emigration level of cells: histone 3 had a positive, dose-dependent effect on the number of extravascular cells. 10µg as well as 100µg of histone 3 challenge resulted in a significant increase of emigrated cells ($p<0.01$). Both concentrations enhanced emigration of cells by five-fold to about 170 cells per mm^2 compared to sham animals (Figure 14C). On the contrary, low-dose histone 3 challenge did not alter the number of emigrated leukocytes significantly.

Based on these results, 10µg of histone 3 was selected for further studies.

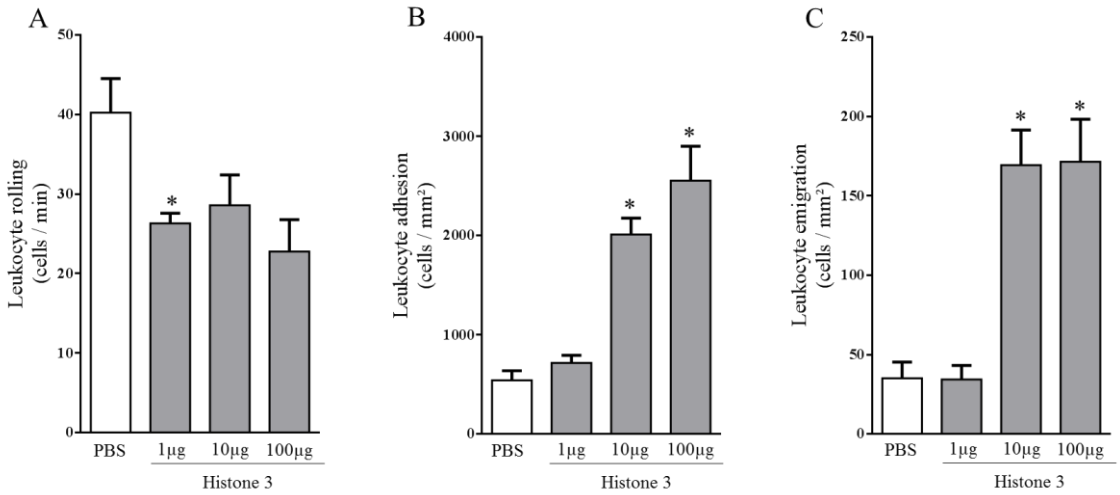

Figure 14: Dose-response relationship of histone 3. Leukocyte rolling (A), adhesion (B) and emigration (C) in mouse cremaster muscle 3 hours after intrascrotal challenge with histone 3 (1-100µg). Sham animals received intrascrotal injection of PBS. Data are shown as mean ±SEM and n=5. *p_g<0.05 vs. sham.

Observations of the histone 3 challenge (10µg) were compared to the one of TNFα to verify the proinflammatory effects of histone 3 (Figure 15). Sham animals were considered as negative control; TNFα-treated animals constituted positive control. Data of sham animals were analog to Figure 14, variations are described at this point.

As already observed in animals treated with histone 3, TNFα-challenge did not alter the number of rolling leukocytes. Accordingly, TNFα-challenge and histone 3-treatment of the cremaster muscle microcirculation evoked comparable numbers of rolling leukocytes (Figure 15A). Interestingly, it was found that 10µg of histone 3 and 0.5µg of TNFα caused a similar significant increase in leukocyte adhesion and emigration ($p<0.05$) (Figure 15B and C). The number of adherent cells was significantly increased by local injection of TNFα by more than four-fold to 1917.0±482.6 cells per mm^2. For direct comparison, histone-induced leukocyte recruitment led to an increase by almost the five-fold compared to sham animals (Figure 15B). Similar effects could be seen at leukocyte emigration. Both TNFα and histone 3 (10µg) increased the number of extravascular cells significantly ($p<0.05$). An increase by approximately the five-fold to 160.7±23.3 cells per mm^2 (TNFα) and 152.1±29.8 cells per mm^2 (H3) could be observed in these groups compared to sham animals.

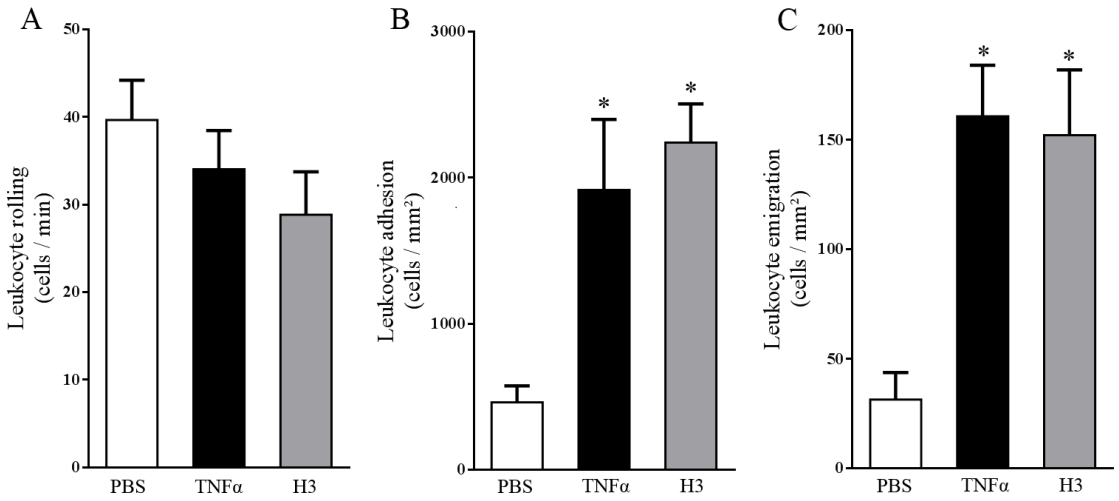

Figure 15: Comparison of histone-induced effects to TNFα. Leukocyte rolling (A), adhesion (B), emigration (C) in mouse cremaster muscle 3 hours after intrascrotal challenge with 10µg of histone 3 or 0.5µg of TNFα. Sham animals received intrascrotal injections of PBS. Data are shown as mean ±SEM and n=5. *p_g<0.05 vs. sham.

4.1.2 Responding leukocyte subpopulation to histone 3 treatment

To identify which leukocyte subpopulation mainly responds to histone 3 challenge, neutrophils were depleted by an antibody against Ly6G on neutrophils. A flow cytometry was performed to verify the specificity of the anti-Ly6G antibody towards neutrophils. Results of the treatment group were compared qualitatively and quantitatively to control animals which received a control antibody.

As illustrated in Figure 16, dot plots of control and treatment group stayed equal for $CD3^+$ (T-cells) and $CD19^+$ (B-cells) as well as $Ly6C^+/Ly6G^-$ cells (monocytes). However, $Ly6C^+/Ly6G^+$ cells (neutrophils) were depleted effectively by anti-Ly6G antibody treatment. This could further be clarified by results of quantitative measurements: Circulating $Ly6C^+/Ly6G^+$ cells were reduced by 95% in the anti-Ly6G antibody group compared to control animals ($p<0.05$). In contrast, the number of circulating $CD3^+$, $CD19^+$ and $Ly6C^+/Ly6G^-$ cells, respectively

, was not affected by anti-Ly6G treatment.

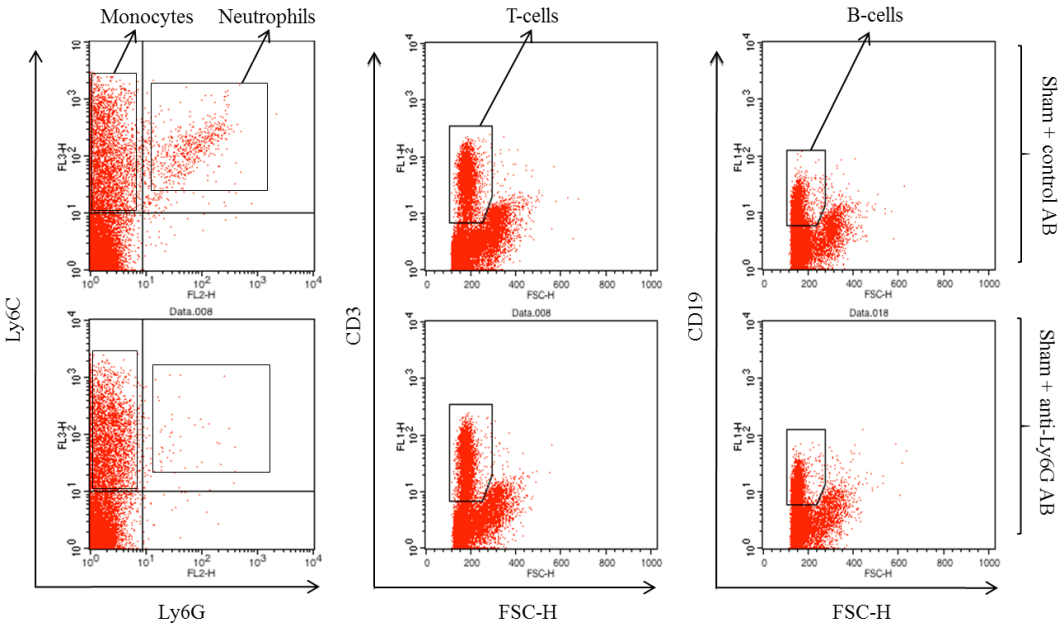

Figure 16: Neutrophil depletion. Circulating neutrophils (Ly6C+/Ly6G+), monocytes (Ly6C+/Ly6G-), T-cells (CD3+) and B-cells (CD19+) were determined by FACS analysis in animals treated intraperitoneally with an AB directed against Ly6G (clone 1A8) or a control AB 24h. These are representative of five other experiments.

Further, the effect of anti-Ly6G antibody (injected intraperitoneally 24 hours prior to histone 3 inj.) on leukocyte rolling, adhesion and emigration was investigated (Figure 17). Results were compared to a positive control treated with a control antibody. Sham data were used as negative control analogy to Figure 14.

Due to the influence of histone 3, data of the control group resembled data of animals treated with histone 3 (10µg) (cf. Figure 14). Accordingly, the control group did not show a powerful difference in leukocyte rolling, whereas leukocyte adhesion and emigration were significantly increased compared to sham animals. In comparison, injection of an antibody towards Ly6G on neutrophils decreased the number of rolling, adherent and emigrated leukocytes significantly ($p<0.05$). Histone-induced leukocyte rolling was decreased by 81% (Figure 17A) and the number of adherent cells was reduced by 94% (Figure 17B). Furthermore, anti-Ly6G antibody treatment resulted in a depression of leukocyte emigration by 81% (Figure 17C).

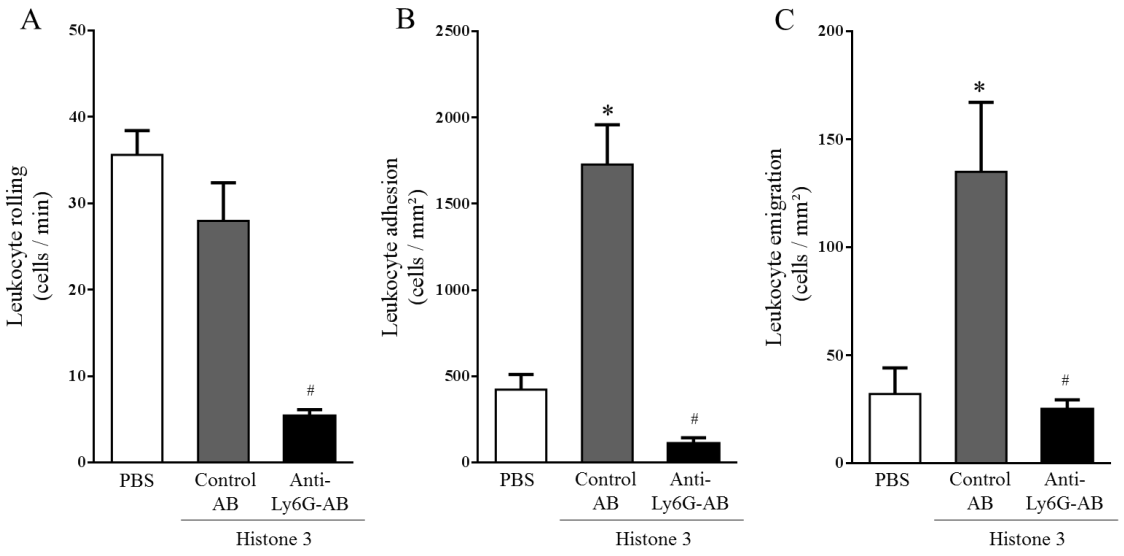

Figure 17: Neutrophil depletion. Leukocyte rolling (A), adhesion (B) and emigration (C) in mouse cremaster muscle 3 hours after intrascrotal challenge with 10µg of histone 3. Animals were pretreated with an anti-Ly6G or a control AB. Sham animals received intrascrotal injection of PBS. Data are shown as mean ±SEM and n=5. *p<0.05 vs. sham. #p<0.05 vs. control AB.

4.1.3 Molecular mechanisms of histone 3-induced effects

4.1.3.1 Selectins

To study the molecular mechanisms of histone-induced neutrophil accumulation, functional blocking antibodies were administered intravenously before intrascrotal injection of histone 3 (10µg). Results were compared to a positive control group treated intravenously with a control antibody and 10µg of histone 3 intrascrotally. Sham data were used as negative control analogy to Figure 14.

Positive control data were equal to data of animals treated with 10µg of histone 3 only (Figure 15). Accordingly, a significant ($p<0.05$ to sham) increase on the number of adherent and emigrated leukocytes could be observed, whereas leukocyte rolling did not alter significantly.

Administration of antibodies directed against PSGL-1 and P-selectin reduced histone-induced neutrophil rolling significantly ($p<0.01$ towards positive control) (Figure 18A). The number of rolling cells could be decreased by 99% by both anti-PSGL-1 and anti-P-selectin antibody. Leukocyte rolling was almost abolished to 0.1±0.1 cells per minute by anti-PSGL-1 antibody treatment and 0.2±0.2 cells per minute by anti-P-selectin antibody administration, respectively. Moreover, immunoneutralization of PSGL-1 reduced both leukocyte adhesion and emigration by 89% and 91%, respectively ($p<0.01$) (Figure 18B

and C). Likewise, functional blocking of P-selectin decreased the number of adherent and emigrated leukocytes by 93% and 95%, respectively ($p<0.01$) (Figure 18B and C).

In contrast to anti-PSGL-1 and anti-P-selectin antibody treatment, injection of an antibody directed against L-selectin did not have a significant effect on leukocyte rolling. The number of rolling cells was slightly reduced by 28% from 27.5±4.6 (control AB) to 19.9±2.2 (anti-L-sel AB) cells per minute. Significant alterations could not be demonstrated (Figure 18A). Anti-L-selectin antibody treatment led to a significant reduction of leukocyte adhesion and emigration ($p<0.05$). The number of adherent cells was reduced by 71% from 1805±214.7 (control AB) to 519.5±164.8 (anti-L-sel AB) cells per mm^2 (Figure 18B). Leukocyte emigration was decreased by 60% compared to positive control. 56.0±11.2 extravascular cells were observed for the anti-L-selectin antibody group (Figure 18C).

Representative images from intravital microscopy are shown in Figure 18D-F. Observations of control animals showed a high number of rolling leukocytes just as an increased number of adherent and emigrated cells. Intravascular and extravascular leukocytes could clearly be seen (Figure 18D). In contrast, injection of antibodies directed against PSGL-1 and P-selectin almost abolished leukocyte recruitment. Therefore, only very few intra- and extravascular cells could be observed (Figure 18E and F).

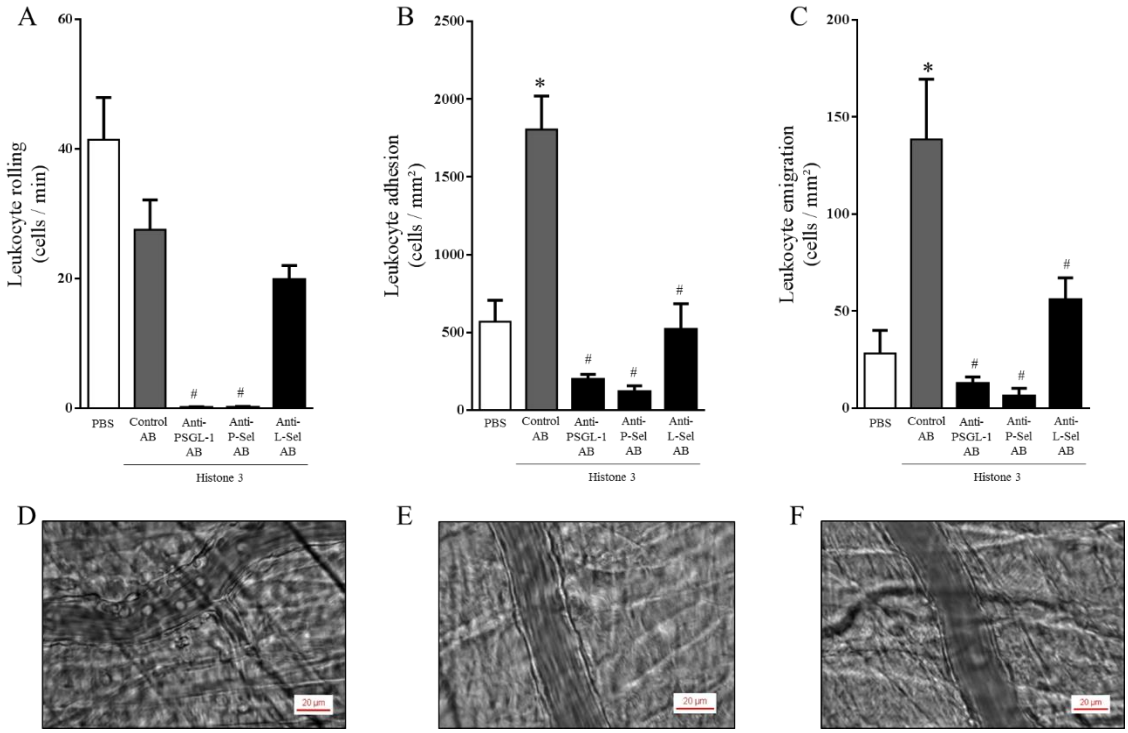

Figure 18: Selectin-dependent molecular mechanisms. Leukocyte rolling (A), adhesion (B) and emigration (C) in mouse cremaster muscle 3 hours after challenge with 10µg of histone 3. Animals were pretreated with anti-PSGL-1, anti-P-selectin, anti-L-selectin and a control AB. Sham animals received intrascrotal injection of PBS. Data are shown as mean ±SEM and n=5. *$p<0.05$ vs sham. $^{\#}p_g<0.05$ vs. control AB. Representative images of venules in the cremaster muscle 3 hours after local stimulation with 10µg of histone 3 in animals pretreated with control AB (D), anti-PSGL-1 AB (E) and anti-P-selectin AB (F).

To clarify if the observed effects of PSGL-1 and P-selectin antibodies were due to direct influence on leukocyte rolling and direct influence on leukocyte adhesion or if the described reduction of adherent leukocytes was a consequence of the reduced number of rolling cells, additional experiments were designed. Neutrophil-endothelial interactions were quantified before and after administration of anti-PSGL-1 and anti-P-selectin antibodies. Results before and after antibody treatment were related to each other and the treatment groups were verified by comparison to a positive control group treated with a control antibody.

All animals received an intrascrotal injection of histone 3 before antibody treatment, that means all groups were treated equally at this point of the experimental protocol. Therefore, no significant alterations regarding the number of cells observed before antibody treatment could be seen between the groups.

Also, no variation could be observed in the control group with regard to leukocyte rolling, adhesion and emigration before and after antibody application. However, injection of a

P-selectin antibody reduced neutrophil rolling by 100% but had no effect on neutrophil adhesion in tissues stimulated with histone 3 (Figure 19A and B). Also, immunoneutralization of PSGL-1 after histone 3 challenge attenuated neutrophil rolling by 100%, whereas it did not impact the number of adherent neutrophils (Figure 19A and B).

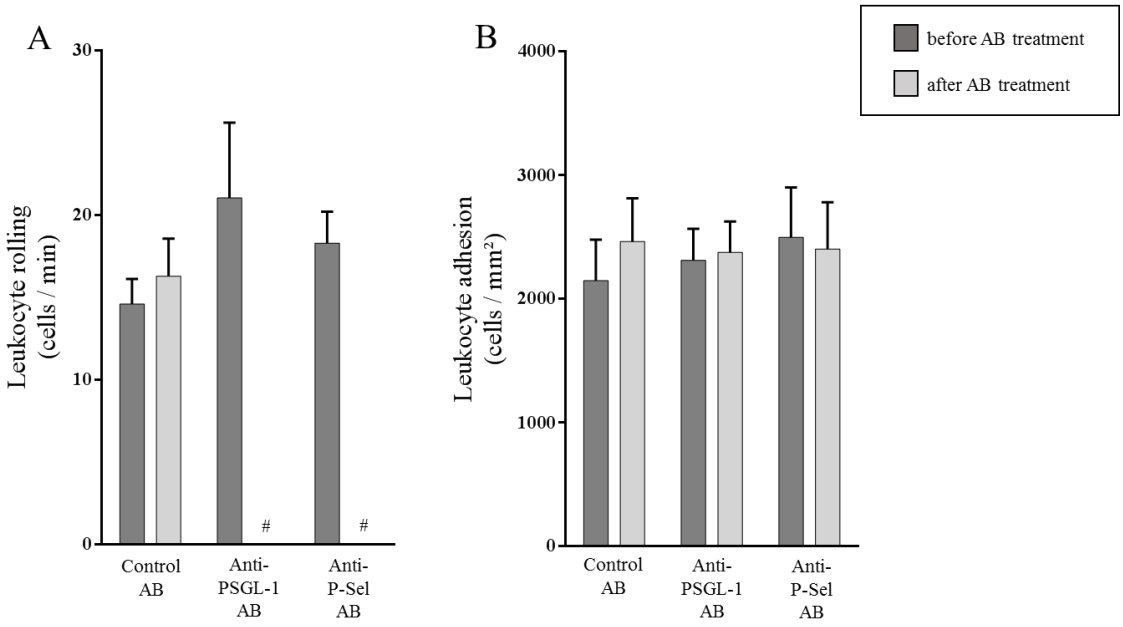

Figure 19: Selectin-dependent molecular mechanisms. Leukocyte rolling (A) and adhesion (B) in mouse cremaster muscle 3 hours after intrascrotal challenge with 10µg of histone 3. Leukocyte endothelium interactions were quantified before and after administration of an anti-P-selectin, an anti-PSGL-1 and a control AB. Data are shown as mean ±SEM and n=5. $^{\#}p<0.05$ vs. before administration.

4.1.3.2 Integrins

Further, the role of β_2-integrins in histone-induced leukocyte recruitment was examined. Antibodies directed against Mac-1 and LFA-1 were administered intravenously immediately prior to intrascrotal challenge with histone 3. Results were compared to positive control animals treated with a control antibody. Observations of the positive control group were analog to Figure 18. Data from sham animals was considered as negative control equally to Figure 14.

Immunoneutralization of Mac-1 and LFA-1 did not cause a significant alteration of leukocyte rolling (Figure 20A). In contrast, following steps of the leukocyte recruitment cascade were strongly influenced by antibody treatment against β_2-integrins. Administration of an antibody directed against Mac-1 reduced the number of firmly adherent cells significantly by 87% ($p<0.01$) from 1897±302.4 cells per mm^2 (control

AB) to 251.2±44.4 cells per mm^2 (anti-Mac-1 AB) in histone 3 exposed tissue. Leukocyte emigration was clearly decreased by 70% from 1897.0±302.4 cells per mm^2 (control AB) to 32.7±7.9 cells per mm^2 (anti-Mac-1 AB) ($p<0.05$). Inhibition of LFA-1 decreased histone-induced neutrophil adhesion by 84% to 312.3±57.5 cells per mm^2 ($p<0.01$). Leukocyte emigration was reduced by 77% to 25.0±4.4 cells per mm^2 ($p<0.01$) (Figure 20B and C).

Representative images from intravital microscopy are shown in Figure 20D-F. Observations of control animals showed a high number of rolling leukocytes just as an increased number of adherent and emigrated cells. The intravascular and extravascular leukocytes could clearly be seen (Figure 20D). In contrast, injection of antibodies directed against Mac-1 and LFA-1 almost abolished leukocyte adhesion and emigration whereas leukocyte rolling was not influenced by the antibody treatment. Accordingly, a few intravascular cells, which are representatives of rolling leukocytes, could be seen. Extravascular cells were very rare though (Figure 20E and F).

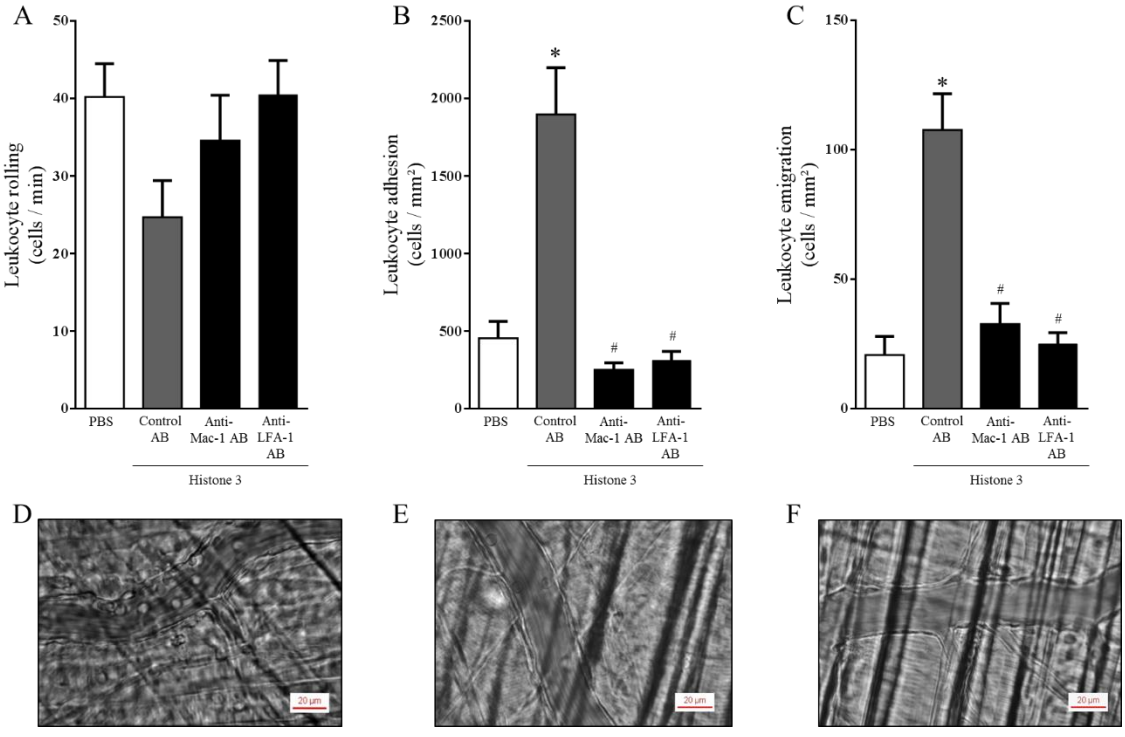

Figure 20: Integrin-dependent molecular mechanisms. Leukocyte rolling (A), adhesion (B), emigration (C) in mouse cremaster muscle 3 hours after intrascrotal challenge with 10µg of histone 3. Animals were pretreated with an anti-Mac-1, an anti-LFA-1 and a control AB. Sham animals received intrascrotal injection of PBS. Data are shown as mean ±SEM and n=5. *$p<0.05$ vs. sham. $^{\#}p_g<0.05$ vs. control AB. Representative images of venules in the cremaster muscle 3 hours after local stimulation with 10µg of histone 3 in animals pretreated with control AB (D), anti-Mac-1 AB (E) and anti-LFA-1 AB (F).

4.2 Microhemodynamic parameters

To examine whether the local injection of histone 3 and TNFα, respectively, or the systemic antibody administration had an impact on the microvascular hemodynamic values, fluorescence microscopic investigations were performed and analyzed. Results of TNFα- and histone 3-treated groups were compared to sham animals; results of animals treated with functional antibodies were compared to positive control animals which received a control antibody. Data is presented in Table 2.

According to the experimental setting representative venules with a diameter between 20 and 40µm were observed. As a result, the average diameter was about 30µm in all groups. The red blood cell, centerline velocity was measured in millimeter per second (mm/s) by use of the line-shift method. No significant alterations could be observed between the particular groups. The centerline velocity was constant between 1.2 and 1.3mm/s.

Based on the red blood cell velocity, the blood flow was calculated and described in nanoliter per second. No specific differences could be observed between the groups.

To sum up, microvascular hemodynamics were not influenced by the treatment of the animals. The red blood cell velocity as well as the blood flow of the treated groups were equal to sham animals.

Table 2: Microhemodynamic parameters. Diameter, flow velocity and blood flow in cremaster muscle venules were quantified in sham animals and animals treated with 10µg of histone 3 intrascrotally. Animals were pretreated with anti-P-selectin, anti-PSGL-1, anti-Mac-1, anti-LFA-1 and a control AB. Data are shown as mean ±SEM and n=5.

Group	Diameter (µm)	RBC velocity (mm/s)	Blood flow (nl/s)
Sham	30.4 ± 1.6	1.2 ± 0.1	0.53 ± 0.1
TNF-α	31.7 ± 1.9	1.3 ± 0.1	0.64 ± 0.1
H3, 10µg	30.7 ± 1.9	1.2 ± 0.0	0.55 ± 0.1
Control	30.5 ± 1.6	1.2 ± 0.0	0.55 ± 0.1
Anti-PSGL-1	27.6 ± 1.1	1.3 ± 0.0	0.49 ± 0.0
Anti-P-Selectin	28.0 ± 1.3	1.3 ± 0.1	0.49 ± 0.1
Anti-L-Selectin	29.2 ± 1.7	1.3 ± 0.1	0.53 ± 0.1
Anti-Mac-1	32.1 ± 1.1	1.2 ± 0.0	0.60 ± 0.0
Anti-LFA-1	30.8 ± 0.5	1.2 ± 0.0	0.56 ± 0.0
Anti-Ly6G	29.6 ± 1.8	1.2 ± 0.0	0.50 ± 0.1

4.3 Systemic leukocyte count

To determine whether the injection of histone 3 or the antibody administration influenced the systemic number of leukocytes, blood was harvested from the tail vein and a systemic leukocyte count was conducted. Results of TNFα- and histone 3-treated groups were compared to sham animals; results of groups treated with functional blocking antibodies were compared to positive control animals.

As seen in Table 3 local injection of TNFα and histone 3 did not affect the number of systemic leukocytes. Neither the number of mononuclear leukocytes nor the number of polymorphonuclear cells was influenced clearly.

Systemic injection of antibodies directed against PSGL-1, P-selectin and Mac-1 did not impact the number of mononuclear and polymorphonuclear cells substantially. The same result could be observed for the number of mononuclear cells in the anti-LFA-1 antibody group. In contrast, the number of polymorphonuclear cells was increased significantly by systemic injection of an antibody directed against LFA-1. However, the number of total leukocytes stayed equally to the other groups. Injection of an anti-L-selectin antibody decreased the number of mononuclear leukocytes slightly. A significant reduction could not be observed though. The number of polymorphonuclear cells was not affected by injection of an anti-L-selectin antibody.

Summarizing, local injection of histone 3 and TNFα as well as systemically injection of the named antibodies had no effect on the number of circulating leukocytes.

Table 3: Systemic leukocyte counts. Systemic mononuclear (MNL) and polymorphonuclear (PMNL) leukocyte counts were quantified in the named groups. 10^6 cells/ml, data are shown as mean ±SEM and n=5. *p_g<0.05 vs. sham. $^{\#}p_g$<0.05 vs. control AB.

Group	MNL	PMNL	Total leukocytes
Sham	4.0 ± 0.5	0.6 ± 0.1	4.6 ± 0.6
TNFα	4.5 ± 0.7	0.6 ± 0.1	5.1 ± 0.8
H3, 10µg	4.3 ± 0.4	0.4 ± 0.1	4.7 ± 0.5
Control	4.0 ± 0.2	0.5 ± 0.1	4.5 ± 0.3
Anti-PSGL-1	4.8 ± 0.5	0.4 ± 0.1	5.2 ± 0.6
Anti-P-Selectin	4.4 ± 0.2	0.6 ± 0.0	5.0 ± 0.3
Anti-L-Selectin	2.5 ± 0.2	0.5 ± 0.1	3.0 ± 0.8
Anti-Mac-1	4.1 ± 0.2	0.6 ± 0.1	4.7 ± 0.3
Anti-LFA-1	4.0 ± 0.2	0.9 ± 0.1 $^{\#}$	4.9 ± 0.3

4.4 Summary of results

This experimental study used the intravital microscopy of the cremaster muscle of C57/BL6 mice to determine the effect of extravascular histone 3 towards leukocyte-endothelium interactions. Functional blocking antibodies were administered systemically to analyze molecular mechanisms of histone-induced neutrophil-endothelium interactions in vivo. Hemodynamic parameters were observed by fluorescence microscopy and a systemic leukocyte count was conducted.

Results can be summarized as follows:

1. Extravascular histones 3 induce leukocyte-endothelium interactions and leukocyte recruitment.

2. Neutrophils are the dominating leukocyte subtype responding to histone challenge.

3. Histone-induced leukocyte recruitment is mediated by P-selectin- and PSGL-1-dependent rolling which is a precondition for subsequent leukocyte adhesion.

4. Histone-induced leukocyte adhesion is inter alia mediated by Mac-1 and LFA-1.

The findings show that histone-induced neutrophil rolling is mediated by P-selectin and PSGL-1. In fact, P-selectin/PSGL-1-dependent neutrophil rolling was found to be a precondition for neutrophil adhesion and emigration in histone-dependent tissue accumulation of neutrophils. Moreover, it was found that histone 3-induced neutrophil adhesion is supported by Mac-1 and LFA-1. Thus, these results increase the understanding of the adhesive mechanisms regulating histone-dependent neutrophil accumulation at sites of inflammation.

DISCUSSION

5.1 Discussion of material & methods

5.1.1 Intravital microscopy of the muscle microcirculation

Intravital microscopy is a well-known and established method for in vivo observations in the field of inflammatory research. It dates back to the 19th century when Julius Cohnheim observed frogs` tongues and mesenteries under a light microscope [Taibott, 1968]. Intravital microscopy can be conducted at a number of different tissues: rodent mesentery [Gavins; Chatterjee, 2004], dorsal skinfold chambers of rodents [Lehr et al., 1993], hamster cheek pouch [Svensjö et al., 1978], bat wings [Wiedeman, 1973], rabbit ear [Dansker, 1958] and the cremaster muscle [Baez, 1973] – just to name a few of the big variety of tissue preparations. Each tissue comprises several advantages and disadvantages, however, all of which are inter alia used to examine and quantify leukocyte endothelial interaction in vivo.

The basis for this is the unique setting of microscopic observations combined with in vivo preparations, allowing to analyze dynamic processes [Gavins; Chatterjee, 2004]. Accordingly, intravital microscopy is a well-suited method for analysis of dynamic interactions between leukocytes and endothelial cells in the microcirculation. It allows observations of the multistep recruitment cascade involving the three main, sequential but overlapping steps: leukocyte rolling, leukocyte adhesion and leukocyte emigration on postcapillary venules. Accordingly, several previous studies used intravital microscopy to observe leukocyte-endothelium interaction signalling in organs and tissues such as the liver [Vollmar et al., 1995], skin [Nolte et al., 1994], mesentery [Mayadas et al., 1993] and cremaster muscle [Thorlacius et al., 1997].

This study is based on the intravital microscopy of the cremaster muscle of mice, a tissue which comprises several advantages. First and foremost the cremaster muscle is particularly suitable as it is thin and transparent, enabling an outstanding optical resolution and a direct observation under a conventional light microscope [Gavins; Chatterjee, 2004]. Preparation of the open cremaster muscle, first carried out in 1973 [Baez, 1973], is a well standardized procedure [Donndorf et al., 2013]. As the whole microvasculature can be observed at one time, Baez´ findings marked a dramatic improvement in contrast to the examination of the non-dissected cremaster muscle which was carried out before [Grant, 1964]. A main advantage of the cremaster muscle, e.g. in

comparison to the mesentery, is the absence of peristaltic movement artefacts [Donndorf et al., 2013]. Moreover, the anatomical structure of the mice´ microcirculation assures a normal, stable blood flow in by far the most venules [Gavins; Chatterjee, 2004]. Indeed, it is possible to minimize interruption of peripheral vessels during ventral incision of the muscle because the A. cremasterica splits at the proximal end of the scrotum muscle sack and the vessels, which connect the muscle and the epididymis, are at the dorsal end of the muscle pouch. Due to the convincing advantages of the cremaster muscle for intravital microscopic observations, several studies concerning dynamic processes of leukocyte-endothelium interactions are based upon this tissue [Thorlacius et al., 1997; Thorlacius et al., 2000; Wan et al., 2003].

However, a main disadvantage of intravital microscopy in general and in vivo observations of the cremaster muscle in particular is the potential activation of the leukocyte recruitment cascade by surgical manipulation, resulting in a mild inflammatory response [Fiebig et al., 1991; Ley, 1994]. A trauma-induced upregulation of rolling leukocytes in postcapillary venules - termed as spontaneous rolling - is well-documented for mesenteric venules [Doré et al., 1993; Fiebig et al., 1991; Kubes; Kanwar, 1994; Ley, 1994] and studies revealed similar observations for the cremaster muscle [Guo et al., 2000]. In contrast, leukocyte rolling is said to be absent in undisturbed tissues [Yamaki et al., 1998a; Yamaki et al., 1998b]. Molecular bases of spontaneous rolling are discussed controversially [Doré et al., 1993; Mayadas et al., 1993; Kubes; Kanwar, 1994; Ley, 1994; Guo et al., 2000]. However, latest studies suggest a main influence of P-selectin [Doré et al., 1993; Guo et al., 2000; Mayadas et al., 1993].

Spontaneous rolling needs to be taken into serious account when interpreting scientific results. As preparation-induced trauma results in a release of endogenous proinflammatory mediators, leukocyte rolling might be increased close to maximum in a given venule. Accordingly, exogenous mediators may not evoke a further increase as their potential effect may be overlaid by irritation-mediated endogenous stimuli [Yamaki et al., 1998a]. That is why it is difficult to determine whether the tested exogenous stimulus triggers rolling mechanisms per se. Hence, it is necessary to perform the preparation protocol in exactly the same way at all animals. Moreover, great care has to be taken not to touch the surrounding tissue in order to avoid superfluous irritation of the microcirculation.

5.1.2 Examination techniques

Intravital light microscopy investigations were complemented by the use of the fluorescence dye FITC-dextran and subsequent fluorescence microscopic observations, facilitating analysis of microhemodynamic parameters such as the blood flow. FITC is, due to its molecular structure, reactive to plasma proteins. It therefore strains the intravascular plasma, whereas it does not bind to blood or endothelial cells. Because of its named characteristics, its good availability and sustainability FITC-dextran is often used as a fluorescence dye in microcirculatory research [Reeves et al., 2012]. It is an optimal contrast agent to measure the blood flow. However, it was reported recently that extracellular histones enhance microvascular permeability, resulting in an increased transmembrane leakage of FITC-dextran [Allam et al., 2012].

As already described elsewhere, hemodynamic analyses of this study were based upon measurement of the red blood cell velocity, allowing to define the blood flow by determining the mean blood flow velocity. The red blood cell velocity needs to be differentiated from the white blood cell velocity which describes the velocity of the rolling leukocytes. The white blood cell velocity was not analyzed in this study. However, it is generally accepted that the red blood cell velocity and the white blood cell velocity are in linear relation to each other, saying that as the red blood cell velocity decreases there is a decline in the rolling velocity of white cells [Ley; Gaehtgens, 1991; Perry; Granger, 1991].

As hemodynamic parameters depend on the diameter of the vessels, venules of about the same size were analyzed to avoid distortion of the results. Venules between 20µm and 40µm were considered representative. Concerning an average size of leukocytes between seven and 20µm, depending on the subtype, this measuring range constitutes a vessel diameter just above the leukocyte diameter, promoting margination and tethering of leukocytes [Gavins; Chatterjee, 2004].

The current study focused on observations of venules because transmigration of cells usually takes place in postcapillary venules [Atherton; Born, 1973; Ley; Gaehtgens, 1991; Perry; Granger, 1991]. Several reasons are discussed to explain why leukocyte rolling and adhesion are much less common in arterioles: Schmid-Schönbein et al. discussed the increased probability of leukocyte margination in venules in comparison to arterioles based on rheological reasons [Schmid-Schönbein et al., 1980]. As leukocyte margination enhances the probability of leukocyte-endothelium interactions it may promote leukocyte

recruitment. Further, Ley and Gaehtgens as well as Perry and Granger revealed that restriction of leukocyte rolling to venules is not caused by existing hemodynamic differences but endothelial differences between the two vessel sections [Ley; Gaehtgens, 1991; Perry; Granger, 1991]. The venular endothelium seems to be prone for leukocyte recruitment as it expresses various adhesion molecules [Ley; Gaehtgens, 1991].

This study analyzed the impact of specific endothelial molecules and molecules on leukocytes towards histone-induced leukocyte recruitment. As a big variety of ligands and receptors were found to mediate leukocyte recruitment it becomes obvious that only a limited number of molecules could be tested. That is why it was focused on the main molecular structures mediating the three predominant steps of the leukocyte recruitment cascade which are: endothelial P-selectin and its counterreceptor PSGL-1, as well as the β_2-integrins LFA-1 and Mac-1. Additionally, the neutrophilial molecule L-selectin was examined as its function is discussed controversially in the literature [Arbonés et al., 1994; Eriksson et al., 2001; Jung; Ley, 1999; Ley et al., 1993; Sperandio et al., 2003; Tu et al., 1996].

Adhesion molecules were targeted by antibodies. When using monoclonal antibodies to target specific proteins, one should be aware that individual antibodies recognize different epitopes. Consequently, differences between several studies can be caused by the use of different antibodies. Also, different antibodies can have additional effects such as immune-mediated damage or elimination of circulating cells on which the target antigen is expressed. Last-mentioned manipulation of results was prevented by systemic leukocyte counts in the current study. Besides functional blocking by monoclonal antibodies, the use of knock-out mice is an another established method to examine specific protein function. However, in this case it is necessary to consider the possibility that elimination of one gene may cause upregulation in expression or function of another gene product that compensates for the function of the lost gene product.

It should be mentioned at this point that no blinding of the examiner was carried out. Hence, an unconscious impact of the examiner cannot be excluded.

5.2 Discussion of results

5.2.1 Proinflammatory effects of extracellular histones

Leukocyte recruitment, which stands for the infiltration of blood-borne leukocytes towards the place of an initial damaging insult, is a major player of an appropriate inflammatory response, enabling recovery of homeostasis. The extravasation of blood cells is a multistep cascade, occurring upon pathological stimuli such as cell damage caused by mechanical, chemical, thermal as well as radiation-mediated trauma. On the other hand, leukocytes start to infiltrate towards the site of infection in order to defend the organism against the invading pathogen.

Indeed, several exogenous and endogenous stimuli are known to cause leukocyte extravasation such as LPS [Yipp et al., 2002], IL-1 [Bevilacqua et al., 1985a; Bevilacqua et al., 1985b] and C5a [Argenbright et al., 1991]. Moreover, TNFα, which is secreted by activated macrophages and other mononuclear leukocytes, is a well characterized inducer of leukocyte infiltration [Gamble et al., 1985; Kunkel et al., 1996; Månsson et al., 2000; Wan et al., 2002].

Further, recently published data showed that nuclear cell components, such as DNA, HMGB1 and histones, have the capacity to trigger activation of the innate immune system when released to the extracellular space [Chen et al., 2014; Fischer et al., 2012; Scaffidi et al., 2002]. A special interest was put on histone-induced effects. Histones, commonly known as intranuclear proteins necessary for chromatin formation, are released into the extracellular space upon cell damage and death. It was recently found that activated neutrophils can expel nuclear components to form web-like structures (NETs) in order to defend the organism against an invading pathogen [Brinkmann et al., 2004]. Indeed, histones were found to play a major role in NETs [Brinkmann et al., 2004]. However, histones can also be set free from damaged, necrotic cells. Released histones were reported to bind to and activate TLR2 and TLR4 on immune cells, leading to a proinflammatory cytokine storm [Allam et al., 2012; Semeraro et al., 2011; Xu et al., 2011]. However, other studies showed that histones mainly cause toxic effects on endothelial and epithelial cells, representing a potential source of inflammation [Bosmann et al., 2013; Saffarzadeh et al., 2012; Xu et al., 2009]. Accordingly, histone-dependent inflammatory reactions are frequently associated with increased tissue accumulation of leukocytes.

Consistently, this study showed that local challenge of histone 3 is a potent stimulator of neutrophil recruitment in vivo. It was demonstrated that histone 3 challenge causes a similar leukocyte response as TNFα which is a well-known inducer of leukocyte infiltration [Gamble et al., 1985; Kunkel et al., 1996; Månsson et al., 2000; Wan et al., 2002]. In fact, both TNFα and histone 3 increased the number of adherent and emigrated leukocytes significantly in comparison to the negative control group which underlines their potential proinflammatory impact. However, the number of rolling cells did not alter between the treated and the negative control groups (cf. Figure 15). This is due to surgical trauma-induced stimulation of leukocyte rolling. It is known that surgical manipulation of tissues induces spontaneous rolling of leukocytes, resulting in a unphysiological high number of rolling cells in the negative control group [Doré et al., 1993; Fiebig et al., 1991; Guo et al., 2000; Kubes; Kanwar, 1994; Ley, 1994]. Accordingly, TNFα challenge and histone 3 treatment could not lead to a further increase in leukocyte rolling. However, the proinflammatory effect of these mediators could be proven by an increased level of leukocyte adhesion and transmigration which are not affected by surgical manipulation. These findings suggest that histones act as proinflammatory compounds in multicellular tissues. The observations are in accordance with a study by Allam et al., claiming the proinflammatory impact of extracellular histones [Allam et al., 2012]. Allam et al. examined the impact of local extracellular histone challenge on the microcirculation of the cremaster muscle in vivo similar to this study. However, they used high concentrations (500µg per animal) of a mixture containing all histone types. In contrast, this study focused on histone 3-induced effects. Moreover, the present study could convincingly show that extracellular histones act as proinflammatory stimulators even in low concentrations (10µg per animal) what emphasizes the potent proinflammatory effects of extracellular histone 3.

One could assume that the observed histone-induced increase in leukocyte recruitment is triggered by TLR-mediated release of proinflammatory cytokines. Indeed, several studies showed convincingly that histones are able to activate TLR2 and TLR4, resulting in an upregulation of proinflammatory mediators via activation of NF-κB [Allam et al., 2012; Semeraro et al., 2011; Xu et al., 2011]. Also, Allam et al. observed that histone 4 induces expression of proinflammatory cytokines (in particular IL-6 and TNFα) in bone marrow-derived dendritic cells directly by TLR2/4-signalling [Allam et al., 2012]. However,

histone-triggered activation of inflammasomes may contribute to the proinflammatory potential of histones also.

In addition, Allam et al. observed that around 90% of the transmigrated cells are neutrophils [Allam et al., 2012]. This observation could be confirmed by the present study. Neutrophils were depleted by use of an anti-Ly6G antibody which effectively inhibited neutrophils while having no impact on monocytes, T-cells and B-cells. Regarding this, immunodepletion of circulating neutrophils not only decreased the number of circulating neutrophils by more than 95% but also abolished histone 3-induced leukocyte-endothelium interactions and tissue recruitment, showing that neutrophils are the major cell type reacting to histone challenge. Accordingly, histones preferentially stimulate leukocyte recruitment in acute inflammation. This becomes obvious if one is aware of neutrophils´ characteristics and function: Neutrophils are a major mediator of acute inflammation and are recruited rapidly to the site of injury [Kolaczkowska; Kubes, 2013]. In contrast, chronic inflammation is mainly supported by monocytes and lymphocytes. The notion that histone-induced leukocyte recruitment plays a more important role in acute inflammation than in chronical inflammatory alterations is supported by a study showing that histone 4 triggers neutrophil infiltration in the peritoneal cavity [Allam et al., 2013] and another study reporting that a mixture of different histones induces neutrophil accumulation in the lung [Bosmann et al., 2013].

5.2.2 Molecular mechanisms of histone 3-dependent leukocyte recruitment

5.2.2.1 Selectins

Tissue accumulation of leukocytes is generally considered to be a multistep process in which a reversible, selectin-mediated rolling process along the vessel wall in the direction of blood flow is followed by a shear-resistant, irreversible, integrin-dependent adhesion step and transmigration of cells into the surrounding tissue.

Many different studies proved that leukocyte rolling in vivo mainly depends on P-selectin/PSGL-1-interactions [Månsson et al., 2000; Moore et al., 1995; Norman et al., 2000; Norman et al., 1995; Wan et al., 2002]. However, molecular mechanisms controlling histone-triggered leukocyte infiltration remained elusive. Herein, it was found that histone 3-evoked leukocyte recruitment is mediated by P-selectin/PSGL-1-signalling also. In fact, immunoneutralization of P-selectin and its major counterreceptor PSGL-1

not only inhibited histone 3-provoked neutrophil rolling by more than 99 % but also concomitantly abolished neutrophil adhesion and extravasation, suggesting that P-selectin/PSGL-1 interactions play a crucial role in histone-dependent tissue infiltration of neutrophils. However, when anti-P-selectin or anti-PSGL-1 antibodies were administered after stimulation with histone 3, leukocyte rolling was again abolished whereas no effect on histone 3-evoked firm adhesion and transmigration could be observed. One could conclude that the decrease of firm leukocyte adhesion and recruitment after anti-P-selectin or anti-PSGL-1 antibody treatment was due to the reduction of the number of rolling leukocytes along the endothelium. That is, P-selectin and PSGL-1 are major mediators of histone-induced leukocyte rolling while having no direct effect on leukocyte adhesion and emigration. Corresponding, neutrophil rolling must be blocked prior to histone challenge in order to decrease subsequent firm adhesion and extravasation of neutrophils. Considered together, these results indicate that P-selectin/PSGL-1-mediated rolling is a precondition for histone-induced adhesion and tissue accumulation of neutrophils in vivo.

Indeed, these data extend on previous studies showing that P-selectin and PSGL-1 play a critical role in supporting leukocyte rolling in TNFα-induced inflammation [Månsson et al., 2000; Norman et al., 2000; Wan et al., 2002] as well as in models of septic lung injury [Asaduzzaman et al., 2009], reperfusion injury [Riaz et al., 2002] and cholestatic liver damage [Dold et al., 2010].

Also, data of the present study indicate that histone-induced leukocyte rolling is not triggered by E-selectin/leukocyte interactions because immunoneutralization of P-selectin decreased the number of rolling leukocytes to an absolute minimum. These findings are in accordance with several studies showing that E-selectin has no or little effect on rolling adhesive interactions [Frenette et al., 1996; Johnston et al., 1997; Månsson et al., 2000; Milstone et al., 1998; Norman et al., 2000; Wan et al., 2002].

The impact of L-selectin on leukocyte rolling is discussed controversially in the literature. It is widely accepted that L-selectin mediates secondary capture by interaction with PSGL-1 on rolling leukocytes [Eriksson et al., 2001; Sperandio et al., 2003; Tu et al., 1996]. However, several studies revealed different data concerning early L-selectin-mediated leukocyte rolling. Whereas some studies reported a major influence of L-selectin in initializing leukocyte rolling [Andrian et al., 1992; Arbonés et al., 1994; Ley

et al., 1993], others did not observe a decrease in leukocyte rolling after inhibition of L-selectin function [Jung; Ley, 1999; Weninger et al., 2000].

In the present study no significant decrease in histone-induced leukocyte rolling could be observed after functional L-selectin blocking. That was to be expected as anti-P-selectin treatment already abolished histone-induced leukocyte rolling. However, a significant decrease in leukocyte adhesion and emigration could be seen.

Observations made for L-selectin-treated mice may be due to inhibition of L-selectin/PSGL-1 interactions by antibody treatment. L-selectin/PSGL-1 interactions are known to trigger secondary capture of free-flowing leukocytes [Eriksson et al., 2001; Sperandio et al., 2003; Tu et al., 1996]. The phenomena of primary, i.e. direct attachment of leukocytes to the endothelium, and secondary capture, i.e. transient interaction of free-flowing leukocytes with rolling leukocytes and subsequent attachment to the endothelium, were not addressed in this study though. Hence, no statements can be given regarding the question if the observed effects were due to inhibition of secondary capture. However, one would expect a decrease in the number of rolling cells in this case. It is questionable if the observed, slight decrease of histone-induced leukocyte rolling after functional blocking of L-selectin is due to inhibition of secondary capture. Moreover, both primary and secondary capture play a tangential role in small venules (<45µm) [Eriksson et al., 2001]. Leukocyte rolling in venules of this vessel size is mainly initiated by direct passage from the capillaries, i.e. leukocytes start rolling as soon as they reach the activated endothelium as they are already in contact with the vessel wall [Eriksson et al., 2001].

On the other hand, some studies revealed that binding of L-selectin and PSGL-1 triggers activation of β_2-integrins and hence influences leukocyte adhesion [Sikorski et al., 1996; Stadtmann et al., 2013]. These observations may explain why histone-induced leukocyte adhesion was reduced whereas the number of rolling leukocytes did not alter strongly: If L-selectin/PSGL-1-mediated activation of β_2-integrins is blocked by inhibition of L-selectin, leukocyte arrest, which is highly dependent on activated LFA-1, will be reduced. As a consequence, the number of emigrated cells will be decreased too.

Summing up, these data indicate an influence of L-selectin on histone-triggered leukocyte adhesion whereas it does not impact histone-induced leukocyte rolling. Nevertheless, it is obvious that PSGL-1 and P-selectin have a greater impact on histone-induced leukocyte

recruitment because elimination of these antigens resulted in a more powerful reduction of leukocyte rolling, adhesion and emigration than L-selectin inhibition did.

Importantly, immunoneutralization of P-selectin, L-selectin and PSGL-1 had no effect on the number of circulating leukocytes, validating that the observed decrease in the number of rolling, adherent and transmigrated cells is not caused by systemic alterations. Likewise, animal treatment did not change the red blood cell velocity nor the blood flow significantly. Hence, observed effects are not influenced by hemodynamic changes.

5.2.2.2 Integrins

Leukocyte adhesion is a major step in the extravasation of leukocytes, following initial rolling of the cells. It is a precondition for cells to transmigrate from the vascular lumen [Lawrence; Springer, 1991; Lindbom et al., 1992; Månsson et al., 2000]. It is generally accepted that the β_2-integrins LFA-1 and Mac-1 are crucial to allow cells to arrest on the endothelium. However, literature is rather complex and partly contradictory regarding the role of the specific integrins [Argenbright et al., 1991; Dold et al., 2008; Issekutz, 1992; Rutter et al., 1994; Thorlacius et al., 2000]. Thorlacius et al. observed that LFA-1 is a major adhesion molecule mediating rapid, TNFα-induced leukocyte arrest in postcapillary venules of the muscle microcirculation in mice, whereas late cytokine-induced leukocyte adhesion is not primarily dependent on LFA-1 [Thorlacius et al., 2000]. It was also reported that early leukocyte adhesion in C5a-induced leukocyte recruitment in rabbits is significantly dependent on both LFA-1 and Mac-1, in which LFA-1 has a more powerful influence on leukocyte adhesion [Argenbright et al., 1991].
The present study examined mechanisms regulating histone-induced firm adhesion and accumulation of leukocytes. It could be shown for the first time that inhibition of β_2-integrins decreased histone 3-evoked neutrophil adhesion on the venular endothelium in vivo. Surprisingly, functional inhibition of LFA-1 and Mac-1, respectively, decreased leukocyte adhesion and emigration equally three hours after histone challenge, suggesting that both LFA-1 and Mac-1 support adhesive interactions between circulating neutrophils and endothelial cells in response to histone challenge. Importantly, observed effects were neither caused by systemic alterations in the number of circulating leukocytes nor by changes of hemodynamic parameters.

These data are partly contradictory to observations made by Thorlacius et al. and Argenbright et al, whose studies supported a crucial role of LFA-1 in the leukocyte recruitment cascade [Argenbright et al., 1991; Thorlacius et al., 2000]. However, a major difference in the experimental setting needs to be considered: The current study observed leukocyte adhesion and emigration three hours after histone challenge, whereas previous named data sets were made shortly after cytokine stimulation. However, Thorlacius et al. augmented their data by observations made four hours after TNFα-challenge, known to induce de novo synthesis, in which they found that LFA-1 plays a weaker role compared to early TNFα-induced leukocyte recruitment [Thorlacius et al., 2000]. This is in accordance with a previous study reporting that LFA-1 initiates first stable contact and that Mac-1 establishes more sustainable adhesion onto the endothelium of inflamed organs [Ding et al., 1999]. Thus, it may be suggested that early histone-induced leukocyte adhesion is predominately mediated by LFA-1, whereas other adhesion leukocytic molecules such as Mac-1 mediate firm adhesion at later times. However, the current study cannot make a clear statement on this consideration because histone-induced leukocyte recruitment was observed at one time point only. Nevertheless, it is comprehensible that both LFA-1 and Mac-1 may cooperate for optimal recruitment of inflammatory cells.

It is interesting to note that one previous study reported a role of integrins in facilitating rolling adhesive interactions of leukocytes in the cremaster muscle [Dunne et al., 2002]. However, the current study proved that immunoneutralization of LFA-1 and Mac-1 had no effect on histone 3-induced neutrophil rolling. These findings are supported by several investigations demonstrating that inhibition of LFA-1 and Mac-1 decreases leukocyte adhesion while having no impact on leukocyte rolling [Andrian et al., 1991; Arfors et al., 1987; Becker et al., 2001; Mihaescu et al., 2007; Nolte et al., 1994]. Also, considering the critical role of P-selectin and PSGL-1, it is perhaps not surprising that β_2-integrins appear to play no role in histone 3-provoked neutrophil rolling.

5.3 Conclusion and Prospects

In conclusion, this study documents that histone 3 is a potent stimulator of leukocyte recruitment in vivo, provoking a powerful inflammatory tissue response. It was found that neutrophils are the major leukocyte subset responding to histone-induced tissue inflammation. Moreover, the current study defines adhesive mechanisms regulating histone-induced accumulation in the extravascular space: Histone-triggered leukocyte

rolling, which is a precondition for subsequent stable arrest, is mainly dependent on P-selectin and PSGL-1, whereas Mac-1 and LFA-1 support histone-evoked neutrophil adhesion on endothelial cells in vivo.

Indeed, extracellular histones are crucial for an organism as they are part of the innate immune system. Histone release by NETosis should be mentioned here. Indeed, histone-mediated microbe defense and histone-caused inflammation are the major histone-induced steps towards homeostasis. Also, extracellular histones from dying cells are of great importance for the organisms because the evoked inflammatory response enables tissue repair.

However, extracellular histones were also reported to cause major injuries in inflammatory diseases, leading to fatal organ dysfunction and death [Bosmann et al., 2013; Chen et al., 2014; Huang et al., 2011; Kumar et al., 2015; Kutcher et al., 2012; Xu et al., 2011; Xu et al., 2009]. In order to comprehend the influence of extracellular histones on the pathogenesis of various diseases, it is important to note that leukocyte recruitment is a rate-limiting step in mediating tissue damage in many inflammatory diseases, including sepsis, acute pancreatitis [Abdulla et al., 2011], colitis [Zhang et al., 2001], as well as liver injury [Dold et al., 2008] and ischemia reperfusion [Riaz et al., 2002]. Here, the current study could clarify adhesive mechanisms regulating histone-induced neutrophil-endothelium interactions. The findings indicate that targeting P-selectin and PSGL-1 as well as LFA-1 and Mac1 might be of beneficial value in histone-dependent tissue inflammation in conditions such as sepsis [Xu et al., 2009], liver injury [Xu et al., 2011], acute lung injury [Bosmann et al., 2013], severe glomerulonephritis [Kumar et al., 2015], ischemic stroke [Meyer et al., 2012] and hepatic ischemia / reperfusion injury [Huang et al., 2011]. In this context, it should be mentioned that inhibition of leukocyte rolling, e.g. targeting P-selectin and PSGL-1, might be a theoretically more attractive step of pharmacological targeting knowing that the rolling interaction is due to endothelial cell activation, whereas rolling leukocytes remain inactivated until stimulation by a chemoattractant provoking firm adhesion to the endothelium. Thus, prevention of leukocyte rolling results in the dislodgement of non-activated leukocytes back into the circulation. In contrast, inhibition of firm adhesion by targeting LFA-1 or Mac-1 releases activated leukocytes back into the circulation where they can cause remote tissue damage in the liver or lung.

Indeed, several studies revealed that inhibition of histone function might be a useful tool to limit histone-induced leukocyte accumulation and organ failure [Allam et al., 2012; Bosmann et al., 2013; Huang et al., 2011; Kumar et al., 2015; Xu et al., 2009]. In this regard, Bosmann et al. observed that neutralization of histone 4 by a monoclonal antibody reduces the intensity of ALI [Bosmann et al., 2013] and Kumar et al. reported that anti-histone antibody treatment abrogate severe glomerulonephritis [Kumar et al., 2015]. Accordingly, with an extended knowledge about histone-triggered molecular pathways and specific adhesion molecules activated by extracellular histones, a more precise pharmacological targeting of infectious and inflammatory disorders might be possible, reducing negative side effects and improving patients' survival rate.

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THESES

1. Leukocyte recruitment is a central step of the inflammatory tissue response and hereby a major mediator of homeostasis. Extravasation of leukocytes is based upon a multistep cascade, including selectin-mediated leukocyte rolling, integrin-triggered, shear-resistant adhesion and subsequent transmigration of leukocytes across the endothelial cell layer.

2. Extracellular histones, released upon NETosis or cell damage, show a potent bactericidal effect and are known to trigger host cell cytotoxicity. Moreover, these proteins have the capacity to cause tissue injury associated with increased leukocyte accumulation.
   However, molecular mechanisms regulating histone-induced leukocyte recruitment remain elusive.

3. Intravital microscopy of the mouse cremaster microcirculation was used to examine the role of specific adhesion molecules in histone-dependent leukocyte recruitment. Histones type 3 were given locally to the cremaster muscle sack and specific adhesion molecules were blocked by systemic administration of monoclonal antibodies.

4. Leukocyte-endothelium interactions in representative venules were analyzed with regard to the number of rolling, adherent and emigrated leukocytes.

5. Immunofluorescence observations were conducted to determine hemodynamic data which were used for verification of the results.
   Systemic leukocyte counts were performed for every experimental group to exclude systemic effects of the injected antibodies.

6. Extracellular histones 3 induced leukocyte recruitment in a dose-dependent manner. A strong proinflammatory impact of histones was recorded, comparable with that of TNFα. That is, histone 3 is a potent proinflammatory compound in vivo and neutrophils are the major leukocyte subtype reacting to histone challenge.

7. Inhibition of P-selectin and PSGL-1 almost abolished leukocyte rolling, adhesion and transmigration. However, if the antibodies were given after histone challenge, leukocyte rolling was again attenuated. In contrast, it did not impact the number of adherent neutrophils.

   It could be concluded that histone-induced neutrophil rolling, which is a precondition for subsequent adhesion of leukocytes, is predominately mediated by P-selectin/PSGL-1 interactions.

8. Immunoneutralization of LFA-1 and Mac-1 did not cause significant alterations of leukocyte rolling, while neutrophil adhesion and emigration were almost abolished. Accordingly, histone-induced leukocyte adhesion is inter alia mediated by Mac-1 and LFA-1.

9. Definition of specific selectins and integrins as potential targets may help to improve pharmacological intervention in histone-dependent inflammatory diseases.

THESES IN GERMAN

1. Die Extravasation von Leukozyten im Rahmen von entzündlichen Prozessen ist von entscheidender Bedeutung für eine adäquate Gewebereaktion und nimmt eine wichtige Rolle bei der Wiederherstellung der Homöostase ein. Es handelt sich um einen mehrstufigen Prozess. Ein Selektin-vermitteltes Rollen von Leukozyten entlang des aktivierten Endothels ist dabei die Voraussetzung für die nachfolgende Integrin-assoziierte feste Adhärenz. Abschließend transmigrieren die Zellen durch die Endothelschicht.

2. Histone werden unter anderem durch NETosis und Zellzerstörung in den Extrazellularraum freigesetzt. Mehrfach konnte die bakterizide Wirkung von extrazellulären Histonen nachgewiesen werden. Jedoch ist auch bekannt, dass extrazelluläre Histone zelleigene Strukturen angreifen können. Dies hat eine nicht unbeträchtliche Toxizität gegenüber den umliegenden Zellen zur Folge. Des Weiteren werden extrazelluläre Histone mit der Pathogenese von zahlreichen Krankheiten, die mit einer erhöhten Rekrutierung von Leukozyten in den Extravasalraum einhergehen, in Verbindung gebracht.

3. In der vorliegenden Arbeit sollten erstmalig die molekularen Grundlagen von Histon-getriggerten Wirkungen untersucht werden. Dafür wurde insbesondere die Rolle von spezifischen Adhäsionsmolekülen in der Histon-induzierten Extravasation der Leukozyten analysiert.

4. Die Studie basiert auf der Intravitalmikroskopie der Mikrozirkulation des M. cremasteris der Maus. Die Intravitalmikroskopie ist eine weit verbreitete Methode zur Untersuchung der dynamischen Leukozyten-Endothel-Interaktionen.
   Während Histone Typ 3, TNFα (positive Kontrolle) bzw. PBS (negative Kontrolle) lokal in das Skrotum injiziert wurden, wurden die spezifischen Adhäsionsmoleküle durch systemische Antikörpergabe inhibiert.

5. Entscheidend für die Analyse der Leukozyten-Endothel-Interaktionen war die Quantifizierung der rollenden, der adhärenten und der transmigrierten Leukozyten in repräsentativen Venolen.

6. Die Daten wurden durch hämodynamische Analysen und Betrachtungen der systemischen Leukozytenzahl erweitert.

7. Extrazelluläre Histone Typ 3 führten zu einem signifikanten Anstieg von Leukozytenadhärenz und -transmigration. Es konnte eine Wirkung vergleichbar mit der von TNFα festgestellt werden. Extrazelluläre Histone zeigen folglich eine starke proinflammatorische Wirkung, insbesondere auf neutrophile Granulozyten.

8. Die Immunoneutralisierung von P-Selektin und PSGL-1 führte zu einer deutlichen Reduktion in der Anzahl von rollenden, adhärenten und emigrierten Zellen. Wurden die Antikörper hingegen nach Histongabe verabreicht, wurde ausschließlich das Leukozytenrollen gehemmt. Adhärenz und Transmigration waren unverändert.
   Folglich ist das Rollen der Neutrophilen, das durch die Histone provoziert wurde, vor allem durch P-Selektin/PSGL-1-Interaktionen bedingt. P-Selektin und PSGL-1 haben jedoch keinen direkten Einfluss auf den Histon-induzierten Vorgang der Adhärenz und Transmigration.

9. Die Immunoneutralisierung von LFA-1 und Mac-1 führte zu einer Aufhebung der Leukozytenadhärenz und -transmigration. Das Rollen der Leukozyten blieb durch die Antikörperbehandlung jedoch unverändert.
   Es wurde geschlussfolgert, dass LFA-1 und Mac-1 eine entscheidende Rolle in der Adhärenz und Transmigration von Leukozyten in Histon-getriggerten Prozessen spielen.

10. Die Definition von spezifischen Histon-induzierten Selektinen und Integrinen kann eine Verbesserung der pharmakologischen Therapie von Histon-getriggerten entzündlichen Krankheiten ermöglichen.

AFFIDAVIT

EIDESSTATTLICHE ERKLÄRUNG

Ich erkläre an Eides statt, dass ich die vorliegende Arbeit mit dem Titel „Adhesive mechanisms of histone-induced neutrophil-endothelium interactions in the muscle microcirculation“ selbstständig und nur unter Benutzung der angegebenen Quellen und Hilfsmittel angefertigt habe. Die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen sind als solche kenntlich gemacht.

Ich versichere weiterhin, dass diese Arbeit nicht vorher und auch nicht gleichzeitig bei einer anderen als der Medizinischen Fakultät der Universität Rostock zu Eröffnung eines Promotionsverfahrens eingereicht worden ist.

Rostock, den 25.05.2016

Johanna Pügge