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The role of extracellular MRP8/14 in leukocyte recruitment *in vivo*

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List of abbreviations

APC	Antigen presenting cell
AE	Adhesion efficiency
CalDAG-GEFI-I	Calcium and diacylglycerol-regulated guanine nucleotide exchange factor-I
CAM	Cellular adhesion molecule
CXCL1	CXC Chemokine ligand 1
CXCR2	CXC Chemokine receptor 2
DAMP	Damage-associated molecular pattern
E-selectin	Endothelial-selectin
EC	Endothelial cell
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbent assay
ESL-1	E-selectin ligand-1
FACS	Fluorescence-activated cell sorting
FcRγ	Fragment (crystallisable) receptor γ
fMLP	Formyl-methionyl-leucyl-phenylalanin
GPCR	G-protein coupled receptor
HMEC-1	Human microvascular endothelial cells-1
HUVEC	Human umbilical vein endothelial cell
ICAM-1	Intercellular cell adhesion molecule-1
IFNγ	Interferon gamma
IL-1	Interleukin-1
IL-8	Interleukin-8
IP-10	Interferon gamma inducible protein 10 (CXCL10)
ITAMs	Immunoreceptor tyrosine-based activation motifs
IVM	Intravital microscopy
KC	Keratinocyte-derived chemokine
kDa	Kilodalton
L-selectin	Leukocyte-selectin
LAD 1	Leukocyte adhesion deficiency type 1
LFA-1	Lymphocyte function associated antigen 1
LPS	Lipopolysaccharids
mAbp1	Mammalian actin-binding protein 1
Mac-1	Macrophage-1 antigen
MMP	Matrix metalloproteases
Mo-MDSC	Monocytic myeloid-derived suppressor cells
MRP8/14	Myeloid related protein 8/14
MSU	Monosodium urate monohydrate crystals
NADPH	Nicotinamide adenine dinucleotide phosphate

NET	Neutrophil extracellular traps
NFκB	Nuclear factor kappa light chain enhancer of activated B-cells
P-selectin	Platelet-selectin
p38MAPK	p38 mitogen activated protein kinase
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PMA	Phorbol-12-myristat-13-acetate
PMN	Polymorphonuclear leukocytes
PRR	Pattern recognition receptor
PSGL-1	P-selectin glycoprotein ligand-1
RAGE	Receptor for advanced glycation endproducts
Rap1	Ras-related protein 1
RF	Rolling flux
RFF	Rolling flux fraction
RNA	Ribonucleic acid
rm	Recombinant murine
ROS	Reactive oxygen species
SCR	Short consensus repeats
sLe^x	Sialyl Lewis X
Syk	Spleen non-receptor tyrosine kinase
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor α
Vav1	Vav Guanine nucleotide exchange factor 1
VCAM-1	Vascular cell adhesion molecule-1
VLA-4	Very late antigen-4
WBC	White bood cell

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Abstract

Severe inflammation as reaction against invading pathogens as well as against endogenous danger signals is mediated by complex interactions of both, the innate and adaptive immune system. In the context of clinical studies, the functional relevance of the heterodimeric protein MRP8/14 as biomarker of chronic inflammation activity and autoimmune diseases has been emerged recently. MRP8/14, also known as S100A8/A9 or calprotectin, plays an important immune-modulating role. As ‘damage-associated molecular pattern molecule’ (DAMP) it is secreted by neutrophils and monocytes at sites of inflammation. The serum and fecal levels of MRP8/14 correlate with phagocyte activity in diseases such as arthritis, dermatitis, vasculitis and chronic inflammatory bowel disease and serve as prognostic marker to monitor the severity of the disease and its response to medical treatment.

Recent findings suggest that MRP8/14, secreted by activated polymorphonuclear cells, plays an important and probably underestimated functional role during inflammatory responses. In this experimental study, we wanted to prove that extracellular MRP8/14 contributes to leukocyte recruitment, underlining its pro-inflammatory character and amplification of inflammation.

Making use of intravital microscopy and the murine cremaster muscle model, we could show that E-selectin dependent secretion and autocrine binding of MRP8/14 to Toll-like receptor 4 (TLR4) induces β_2-integrin dependent leukocyte slow rolling *in vivo.* Further, we could demonstrate that this mechanism also promotes β_2-integrin dependent neutrophil adhesion and transmigration.

These insights could contribute to the future development of new anti-inflammatory therapies targeting MRP8/14 in diseases with elevated and unwanted leukocyte recruitment.

Zusammenfassung

Schwere Entzündungsreaktionen gegen eingedrungene Pathogene wie auch auf körpereigene Strukturen sind Folgeerscheinungen eines komplexen Zusammenspiels des angeborenen und des adaptiven Immunsystems.

Im Rahmen klinischer Studien konnte bereits wiederholt die Relevanz des heterodimeren Proteins MRP8/14 als Biomarker der Entzündungsaktivität bei chronischen Infektionen und Autoimmunerkrankungen belegt werden. Das Protein MRP8/14, auch bekannt unter dem Namen S100A8/A9 oder Calprotectin, spielt dabei eine wichtige immun-modulatorische Rolle. Als „damage-associated molecular pattern molecule“ (DAMP) wird es an Orten der Entzündung vermehrt aus neutrophilen Granulozyten und Monozyten freigesetzt.

Die Serum- bzw. Fäkalkonzentration von MRP8/14 kann eine Aussage über die Aktivität von Phagozyten in Erkrankungen wie Arthritiden, Dermatitiden, Vaskulitiden und chronisch entzündlichen Darmerkrankungen zulassen. Dabei korreliert die Höhe der Serumkonzentration mit der Schwere der Erkrankung und kann als prognostischer Marker auch das Ansprechen dieser auf eine medikamentöse Therapie anzeigen.

MRP8/14 spielt als körpereigenes Protein, ausgeschüttet aus aktivierten polymorphonukleären Granulozyten, eine wesentliche und wahrscheinlich unterschätzte Rolle im Rahmen von Entzündungsprozessen. Mit dieser experimentellen Arbeit soll gezeigt werden, dass extrazelluläres MRP8/14 eine wesentliche Rolle in der Leukozytenrekrutierung spielt und als pro-inflammatorisches Protein die Entzündungsreaktion verstärken kann.

Mit Hilfe der Intravitalmikroskopie am murinen Cremastermuskel konnten wir zeigen, dass die E-Selektin abhängige Ausschüttung von MRP8/14 und dessen autokrine Bindung an den Toll-like-Rezeptor (TLR) 4 zu einer Reduktion der Rollgeschwindigkeit der Leukozyten *in* vivo führt. Darüber hinaus wurde nachgewiesen, dass die MRP8/14 – TLR4 abhängige Aktivierung von β_2-Integrinen nicht nur das langsame Rollen, sondern auch die Adhäsion und Transmigration der Leukozyten fördert.

Die in diesem Projekt gewonnenen Kenntnisse über das Protein MRP8/14 können dazu beitragen, zukünftige immunmodulatorische Therapien für Erkrankungen mit unerwünschter Neutrophilenrekrutierung zu entwickeln.

1 Introduction

1.1 Innate immune response in acute inflammation

The mammalian immune system consists of two components, the innate and adaptive system, that both have humoral and cellular parts to handle infections or prevent autoimmune reactions. Both components have gained more interest in the field of translational research over the last fifty years and the understanding of their complex function and underlying mechanisms is fundamental for developing new and exciting therapies in the future.

The innate system has an immediate reacting defence component which can react against its hostile environment. It contains physical barriers such as skin and mucosal membranes as well as chemical barriers such as antimicrobial peptides or the complement system. Once the pathogen has trespassed given barriers, the cell-mediated innate immunity serves as the first line of host defence. The group of myeloid phagocytic cells includes monocytes/macrophages, neutrophils as well as basophils and eosinophils. They recognize pathogen-associated molecular patterns (PAMPs) by evolutionary highly conserved pattern recognizing receptors (PRR) such as Toll-like receptors (TLRs), C-type lectin receptors or NOD-like receptors. By detection and recognition of foreign chemical structures (for example lipopolysaccharides, LPS, glycans or nucleic acids from other organisms) these receptors are able to induce signalling pathways within myeloid phagocytic cells. Pro-inflammatory cytokines and mediators such as histamine, bradykinin, serotonin, prostaglandin and reactive oxygen species (ROS) are actively released in order to promote pathogen destruction and to further facilitate leukocyte recruitment from post-capillary venules to the site of infection. These mediators also sensitize the microenvironment, leading to acute inflammation.

In 2004, Seong and Matzinger described an endogenous danger-signal, damage-associated molecular pattern (DAMP) that appeared to be recognised by the PRRs. Living a double life, DAMPs not only exert important intracellular functions which might not be related to immune processes, they also play a major role as extracellular mediator and activator of inflammation. DAMPS are released during injury of cell death (for example during necrosis) (Seong *et al.*, 2004). One major

DAMP molecule is MPR8/14, also known as S100A8/A9 or calprotectin. It belongs to the S100 protein family and can be found in neutrophils as well as monocytes that will be recruited to the site of inflammation. Recent studies increased the evidence of this protein's importance in leukocyte recruitment.

1.2 Leukocyte recruitment cascade

In order to fight invading pathogens or sterile inflammatory processes, innate immune cells have to enter sites of inflammation and to leave the microvascular system. Exiting the vascular compartment follows a well-described cascade of adhesion and activation steps consisting of five main steps which usually occur in postcapillary venules: capturing and rolling, slow rolling leading to arrest and adhesion strengthening, intraluminal crawling and finally transendothelial migration (Fig. 1) (Ley *et al.*, 2007; Mayadas *et al.*, 2014). Within each step, a complex interplay occurs between white blood cells and endothelial cells, that will be described in more detail below.

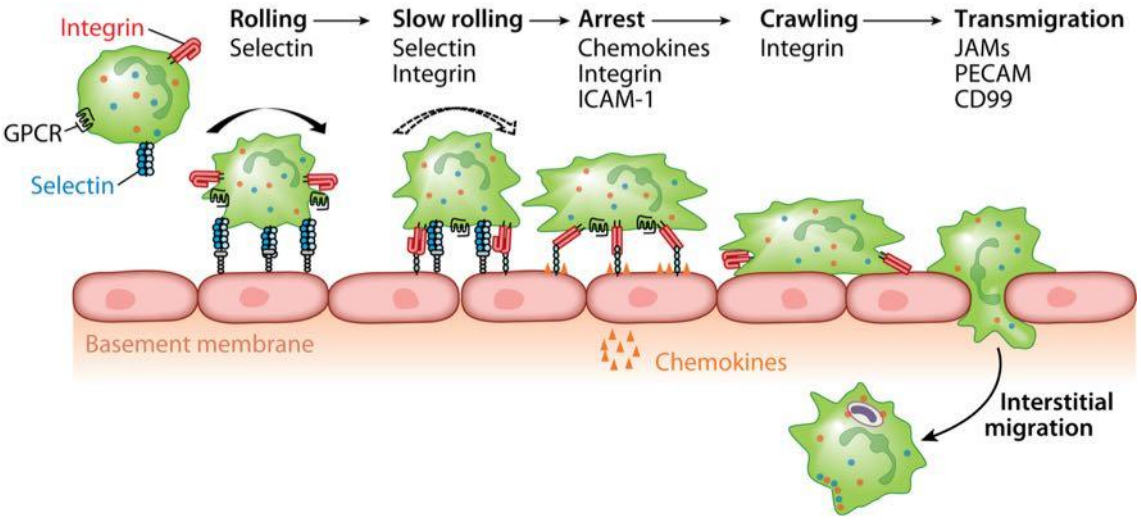

Fig. 1: Leukocyte recruitment cascade (modified from Mayadas *et al.*, 2014).
The multistep recruitment cascade consists of five main steps, (slow) rolling, arrest and adhesion strengthening, intraluminal crawling and finally transendothelial migration. Each step is characterized by complex leukocyte-endothelial interactions. Rolling is dependent on selectin selectin-ligand interactions. This results not only in slow stable rolling but is accompanied by complex inside-out signalling events with gradual integrin activation eventually triggering firm adhesion. Leukocyte crawling is characterized by slow movement along the endothelial surface. Finally, leukocytes transmigrate into the interstitial space *via* the trans- or paracellular route.

1.2.1 Leukocyte capture and rolling

The first step of the leukocyte recruitment cascade consists in selectin-mediated capture and rolling. In inflamed tissue leukocytes get into contact with inflamed endothelial cells that present selectins. Selectins bind to selectin-ligands on leukocytes leading to rolling. Rolling can be considered as dynamic and shear stress dependent equilibrium between bond formation and breakage (Begandt *et al.*, 2017; Sperandio, 2006).

1.2.1.1 Selectins

Selectins are type I transmembrane glycoproteins that have a similar structure to and share properties of C-type lectins: at the luminal site (N-terminal site) a calcium-dependent-lectin-domain recognizes carbohydrate moieties on selectin ligands. The lectin domain is followed by an epidermal growth factor (EGF)-like module and a domain of short consensus repeats (SCR). The latest varies among the different selectins and across the various species. Attached to the transmembrane domain, the short C-terminal cytoplasmatic tail follows with putative intracellular signalling function.

Surface expression of endothelial selectins mainly depends on the context: P- (platelet-) selectin represents the molecule that appears minutes upon inflammatory stimulation of the endothelial surface, for example by histamine, thrombin or surgical intervention. This is achieved by mobilisation of Weibel-Palade bodies to the endothelial cell surface that contain P-selectin (Mcever, 2015; Schmidt *et al.*, 2013).

Other mediators such as tumour necrosis factor α (TNF-α), interleukin-1 (IL-1) or lipopolysaccharides (LPS) exhibit an additional effect on selectin expression by inducing synthesis of P- and E- (endothelial-) selectin that will be presented on endothelial cells (ECs) several hours after stimulation (Murphy *et al.*, 2009). Since E-selectin is known to be less efficient than P-selectin in primary capture of leukocyte from free flow, P- and E-selectin work synergistically on initial rolling. In *ex vivo* flow chamber experiments, co-immobilisation of P- and E-selectin exhibited an increasing number of rolling PMNs in comparison to single selectins (Smith *et al.*, 2004b). Finally, E-selectin has been shown to dramatically reduce rolling velocity.

L-selectin is expressed on leukocytes and is a key player in lymphocyte homing into secondary lymphatic tissue (Arbones *et al.*, 1994). Upon stimulation with formyl-methionyl-leucyl-phenylalanin (fMLP) or interleukin-8 (IL-8), L-selectin is shed from the leukocyte surface (Arbones *et al.*, 1994). It also functions as mediator of secondary tethering of leukocytes during inflammation (Sperandio *et al.*, 2003).

1.2.1.2 Selectin ligands

Selectin ligands are characterised by a specific carbohydrate determinant, namely the tetrasaccharide sialyl Lewis X (sLe^x). It has an essential role in binding to all three selectins. On leukocytes, selectin ligands are important for recruitment to the site of inflammation. In contrast, during lymphocyte homing selectin ligands are expressed on the endothelial surface interacting with L-selectin on lymphocytes (Sperandio *et al.*, 2009). In the context of inflammation in peripheral tissues, relevant selectin ligands on leukocytes are: P-selectin glycoprotein ligand-1 (PSGL-1), E-selectin ligand-1 (ESL-1) and CD44 (Hidalgo *et al.*, 2007; Sperandio *et al.*, 2009).

PSGL-1 binds to P-, E- and L-selectin under static and dynamic conditions. It is the most important selectin ligand, mediating leukocyte rolling during inflammation.

1.2.2 Leukocyte slow rolling and adhesion

Leukocyte rolling on inflamed endothelium triggers diverse signalling pathways on both, rolling cells and endothelial cells that are important for further steps leading *via* slow rolling towards adhesion and finally to extravasation. After initial rolling of PMNs on either P- or E-selectin, PSGL-1 dependent signalling induces an intermediate β_2-integrin activation. β_2-integrins are transmembrane heterodimeric proteins that are expressed on hematopoetic cells and consist of one characteristic β_2-chain that is combined with one out of four different α-chains, subclassifying different heterodimers: p150.95 ($\alpha_X\beta_2$; CD11x/CD18), $\alpha_D\beta_2$ (CD11d/CD18), lymphocyte function-associated antigen-1 (LFA-1; CD11a/CD18; $\alpha_L\beta_2$) and macrophage-1 antigen (Mac-1; CD11b/CD18; $\alpha_M\beta_2$) (Pick *et al.*, 2013). The last two

are constitutively expressed on most leukocytes and are key players with distinct roles in slow rolling, adhesion and transmigration of leukocytes (Springer *et al.*, 2012).

Integrins exist in three major conformations, a bent, inactive conformation with low affinity to the ligand, an extended confirmation with intermediate affinity and finally the fully extended, high affinity conformation with separated subunits at the transmembraneous region (Fig. 2). These different activity states of β_2-integrins on human neutrophils can be detected by various monoclonal antibodies. KIM-127 binds to the second EGF-like domain of the β_2-subunit, recognizing the intermediate and high activity conformation whereas mAb24 only binds to the I domain of the β_2-chain of the fully extended β_2-integrin (Fig. 2) (Johansson *et al.*, 2013).

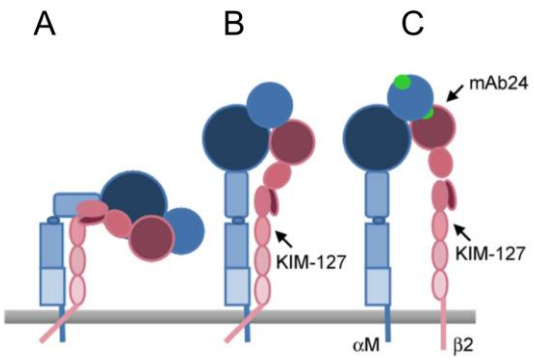

Fig. 2: Conformations of β_2-integrins and recognition by conformation-specific monoclonal antibodies (modified from Johansson *et al.*, 2013).
β_2-integrins exist in three major conformational status that reflect their ligand binding affinity and activity. Bent, inactive status with low affinity (A), partially extended with intermediate affinity (B), fully extended with high affinity (C) to ligand. Monoclonal antibodies such as KIM127 and mAB24 help to differentiate these conformations.

In humans, absence of the β_2-chain (CD18) is the cause of leukocyte adhesion deficiency type 1 (LAD 1) leading to recurrent severe bacterial infections due to inefficient leukocyte recruitment.

Upon selectin-selectin ligand interactions, inside-out signals lead to β_2-integrin activation. These signals relay on intracellular signalling events that start with PSGL-1-dependent phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) in fragment (crystallisable) receptor γ (FcRγ) or DNAX activa-

tion protein of 12kDa (DAP-12). This is followed by downstream signalling including spleen non-receptor tyrosine kinase (Syk) that phosphorylates SLP76 and ADAP. Further, phospholipase C (PLC)-γ2 and calcium diacylglycerol-regulated guanine nucleotide exchange factor-I (CalDAG-GEF-I) as well as Ras-related protein 1 (Rap1) lead to activation of β2-integrin by recruitment of talin1 that interacts with the cytosolic tail of the integrins (Zarbock *et al.*, 2011).

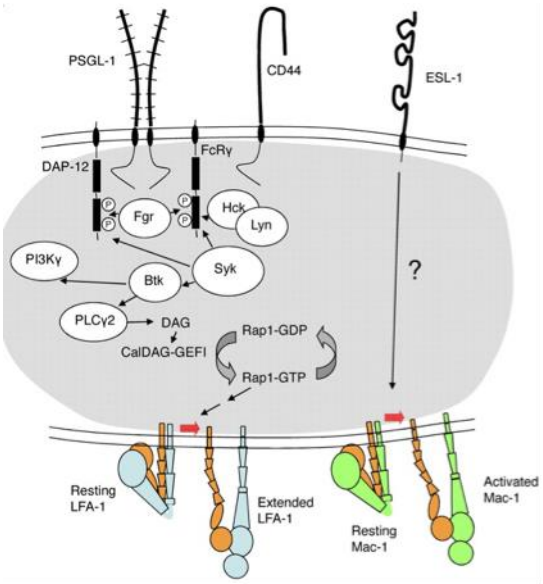

Fig. 3: Signalling pathways triggered by selectin ligands in neutrophils (Zarbock *et al.*, 2011).
P-selectin or E-selectin binding to PSGL-1 triggers a complex intracellular signalling cascade, involving Src-family-kinases, Syk and activation of CalDAG-GEFI in order to activate LFA-1. The ESL-1 dependent pathway remains still unknown.

This results in a continuous deceleration of leukocyte rolling velocity from approximately 5 to 3 µm/s *in vivo* which is termed “slow rolling” (Kunkel *et al.*, 2000). This is mainly attributed to the β2-integrin LFA-1, since blocking Mac-1 had no effect on rolling velocity in auto-perfused flow chambers (Zarbock *et al.*, 2007). Rolling also enables neutrophils to sense endothelium expressed chemokines, such as CXCL1 (also known as keratinocyte-derived chemokine, KC), which binds to G-protein coupled chemokine receptors (GPCR) such as CXCR2 and further activates rolling neutrophils. Hereby, β2-integrins shift into an extended high affinity state leading to firm arrest (Begandt *et al.*, 2017; Smith *et al.*, 2004a).

1.2.3 Leukocyte post-arrest modifications and extravasation

The final steps of the leukocyte recruitment cascade are characterized by intraluminal crawling and transendothelial migration, also known as diapedesis. After firm adhesion, neutrophils scan the endothelial surface for suitable migration spots along the endothelial layer. The previously mentioned outside-in signalling is a crucial step towards crawling. Upon ICAM-1 binding to β_2-integrins, signals from outside are transduced, leading to activation of Src tyrosine kinases that in turn activate non-receptor spleen tyrosine kinase Syk to the lamellipodium (Pick *et al.*, 2013). Additional downstream signalling factors such as Vav-1 then induce flattening and spreading of leukocytes by reorganization of the cytoskeleton. Active perpendicular crawling (a very slow movement of leukocytes with a velocity of <10µm/min) of chemokine-stimulated leukocytes highly depends on macrophage antigen (MAC)-1 and is aimed to find the right location for transmigration (Begandt *et al.*, 2017).

Diapedesis can be performed in two different ways. Once PMNs have found their right extravasation spot, they can migrate either by taking the paracellular route through the junctions between endothelial cells or by using the transcellular route, directly through an endothelial cell's body (Carman *et al.*, 2008). For neutrophils, paracellular diapedesis is the preferred route (Nourshargh *et al.*, 2014; Pick *et al.*, 2013).

1.3 The heterodimeric protein MRP8/14

MRP8/14 is a calcium-binding heterodimer which is constitutively expressed by polymorphonuclear cells (PMN) (Pruenster *et al.*, 2016). In neutrophils, this dimer constitutes roughly 40% and in monocytes 5-10% of the cytosolic content. Especially in monocytes, its expression depends on the stage of differentiation (Lagasse *et al.*, 1992).

In osteoclasts, keratinocytes and epithelial cells, MRP8/14 expression can be induced during prolonged inflammation and wound healing (Thorey *et al.*, 2001; Yen *et al.*, 1997). However, its function in these cells is still not completely understood. During embryogenesis, MRP8 reveals an important role in the vascular system since mice lacking MRP8 undergo early resorption by day 9.5 (Passey *et al.*, 1999a). In contrast, mice with absent MRP14 are viable but show severe functional deficiencies in inflammatory responses (Manitz *et al.*, 2003). Interestingly, the genetic knock out of MRP14 (MRP14 $^{-/-}$) presents as functional double knock out due loss of MRP8 in absence of MRP14 (Manitz *et al.*, 2003).

Besides its important role during inflammation, several other functions for MRP8/14 are discussed (Pruenster *et al.*, 2016). This includes antimicrobial effects due to chelation of nutrient metals such as the divalent cations like Zn^{2+} and Mn^{2+} and the prevention of inadequate adaptive immune response by blocking the development of pro-inflammatory dendritic cells. Furthermore, there is increasing evidence of a pro-atherosclerotic effect of MRP8/14 (Liang *et al.*, 2018).

1.3.1 Nomenclature

At the beginning of its discovery in the early 80ties of last century, the protein MRP8/14 was known under several synonyms. Initially, the protein complex MRP8/14 was discovered by Dale *et al.* in 1983 who described a novel protein extracted from *in vitro* cultured human granulocytes and provisionally named it L1-Antigen (Leukocyte-1 Antigen). This was also the first publication that mentioned very high protein concentrations in the plasma from patients that suffered from various inflammatory and malignant conditions (Dale *et al.*, 1983).

At about the same time, an association between rheumatoid arthritis and *migration-inhibitory factor related protein*, MRP was described (Odink *et al.*, 1987). Following the atomic mass in kilodalton (kDa), both components received the suffixes 8 and 14.

Henceforth, origin, structure and function of the heterodimer have been investigated intensively and the protein was known under several names depending on clinical and functional associations: synonyms like cystic fibrosis antigen, calgranulin A and B, p8 and p14 are mentioned in several publications. In addition, the structural homology to calcium-binding proteins of the S100 family and its physical co-localization at the locus of the S100 protein family on human chromosome 1q21 led to the names S100A8 and S100A9 (Lagasse *et al.*, 1988). Today, MRP8/14, S100A8/A9 and calprotectin are used in the literature.

1.3.2 Structure of MRP8/14 (S100A8/A9)

One main property of S100 family proteins is their Ca^{2+}- binding capacity. This is achieved by two EF-hand calcium-binding sites. Binding of a calcium ion to these acidic sites results in a conformational change (Calissano *et al.*, 1976).

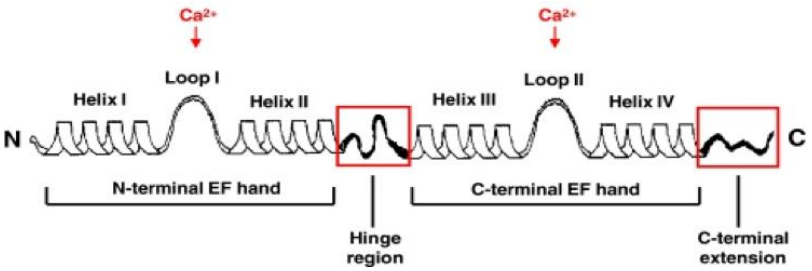

Fig. 4: Schematic illustration of the secondary structure of a S100 protein (Donato R., 2001).
Two distinct EF-hands are flanked by hydrophobic regions and are separated by one central hinge region. The canonical, carboxy- terminal EF-hand Ca 2+-binding site is described as more calcium-affine than the amino-terminal loop.

Most of the S100 proteins exert their biological activity as non-covalent homo- or heterodimer. In regard to the MRP8/14 (S100A8/A9), the heterodimer is the main active form *in vivo*. Interestingly, upon calcium binding, extracellular MRP8/14 (S100A8/A9) can convert into a heterotetramer complex $(S100A8/A9)_2$ that is biological inactive due to the hidden TLR-4/MD2-binding site. Vogl *et al.* could show, that this conformational change leads to an autoinhibition and thereby restricted activity of the heterodimeric S100A8/A9 (Vogl *et al.*, 2018). This is of critical clinical significance as high levels of S100A8/A9 in serum as observed in many chronic inflammatory disorders (see section 1.4) consist exclusively of the inactive S100A8/A9 heterotetramer (Vogl *et al.*, 2018).

1.3.3 Intracellular function of MRP8/14

MRP8/14 influences gene expression of myeloid stem cells by inhibiting specifically casein kinases I and II that are important for the regulation of topoisomerase and RNA-polymerase (Hsu *et al.*, 2009). Also, Schonthaler *et al.* postulated that MRP8/14 can relocate to the nucleus of keratinocytes and interact with histones influencing complement factor C3 gene expression (Schonthaler *et al.*, 2013).

Furthermore, Kerkhoff *et al.* (2005) showed that MRP8/14 functions as activator of phagocytic NADPH-oxidase and thereby facilitates the uptake of fatty acids in endothelial cells (Kerkhoff *et al.*, 2005).

MRP8/14 interacts with cytoskeletal components such as intermediate filaments and myosin as well as keratin and vimentin. Especially the phosphorylation dependent interaction between the tetrameric protein and microtubules is of functional significance: Polymerisation of tubulin subunits is promoted by $(MRP8/14)_2$ (Leukert *et al.*, 2006). Once the MRP14 component is phosphorylated via p38 MAPK in a calcium-dependent manner, the stabilisation effect on microtubule is abrogated (Vogl *et al.*, 2004). Thus, the protein has a direct influence on the cell's mobility, which might have relevance in the context of phagocytes' migratory capacity during inflammation.

1.3.4 Release and secretion of MRP8/14

Since MRP8/14 is assigned to the group of DAMPs, its cellular release is of highest importance for danger signalling in immune defence. MRP8/14 can be actively secreted by myeloid cells or passively released due to tissue damage or necrosis (Pruenster *et al.*, 2016).

In general, secretion is a multi-step process that is used by cells to unload proteins into their extracellular environment. Classically, the proteins destined for secretion are shuttled from the endoplasmic reticulum to the Golgi apparatus where the cargo is packed into vesicles and then eventually shipped to the cell surface for exocytosis. Alternatively, there is a non-conventional, Golgi-independent pathway of secretion, which has recently been described for interleukin-1β (IL-1β). Neither the classical nor the alternative pathway have been proven for

the secretion of MRP8/14. Rammes *et al.* postulated a protein kinase C- and tubulin-dependent secretion pathway (Rammes *et al.*, 1997). In 2009, Urban *et al.* reported another mechanism of MRP8/14 release during neutrophil extracellular trap (NET) formation that is required as an antifungal response to *Candida albicans* infection (Urban *et al.*, 2009). Similar to NET formation, MRP8/14 release is linked to NADPH-oxidase dependent ROS production (Tardif *et al.*, 2015; Urban *et al.*, 2009). Nevertheless, the exact intracellular mechanisms leading to MRP8/14 secretion are still unknown.

1.3.5 Extracellular function of MRP8/14 and its role in innate immunity

Since MRP8/14 is released by PMNs in contact with inflammatory activated endothelial cells and extracellular matrix (Mahnke *et al.*, 1995), the extracellular function of this protein is closely related to pathomechanisms of a local inflammation. The soluble MRP8/14 complex binds to various surface receptors, on neutrophils, macrophages, endothelial cells as well as thrombocytes. Depending on the receptor interaction, the protein exerts multiple, partly diverse extracellular effects.

The receptor for advanced glycation end products (RAGE) as well as Toll-like receptor 4 (TLR4) have been shown to bind to the subunit MRP8 of soluble MRP8/14 (Bjork *et al.*, 2009; Vogl *et al.*, 2007). Recently, CD36 was discussed as putative receptor for the subunit MRP14 of soluble MRP8/14, facilitating the uptake of arachidonic acids into endothelial cells and facilitating thrombosis (Kerkhoff *et al.*, 2001; Wang *et al.*, 2014b).

1.3.5.1 MRP8/14 on neutrophils

Released into the extracellular space, MRP14 is able to increase neutrophil phagocytosis activity *via* induction of NADPH-oxidase, followed by intracellular ROS production (Simard *et al.*, 2011). In 2007, Vogl *et al.* revealed that MRP8/14 interacts directly with the TLR4-MD2-receptor complex on PMNs followed by activation of MyD88 and NF_KB (nuclear transcription factor), resulting in increased transcription and release of TNF-α that explains the above described extracellular effects and intensifies inflammation (Vogl *et al.*, 2007).

In vitro, human MRP8/14 is a very strong stimulus for neutrophil adhesion to the extracellular matrix protein fibronectin (Anceriz *et al.*, 2007) as well as to fibrinogen (Ryckman *et al.*, 2003b). The latter is driven by up-regulation of β_2-integrin Macrophage-1 antigen (Mac-1) on the surface of neutrophils.

1.3.5.2 MRP8/14 on monocytes/macrophages

Initially, Odink *et al.* described a role of MRP8/14 on monocytes in the clinical context of arthritis revealing the protein's specific origin in myeloid cell lines that primarily accumulated in the joint's synovial membranes and destructed cartilage (Odink *et al.*, 1987). Further, Zwadlo *et al.* concluded from the different presentation of MRP8 and MRP14 in acute or chronic inflammation sites that the protein might play an important role in monocyte differentiation (Zwadlo *et al.*, 1988). In addition, a regulatory role on number and function of myeloid-derived suppressor cells (MDSCs) in human adults has been contributed to MRP8/14. Surprisingly, Heinemann *et al.* reported, that MPR8/14 controls birth associated expansion of immunosuppressive monocytic myeloid-derived suppressor cells (Mo-MDSCs) in newborns, thereby improving the clinical outcome in neonatal septic shock (Heinemann *et al.*, 2017).

In line with these findings the epitope 27E10 was used to identify human immature monocytes with membrane associated MRP8/14 complexes that dominate in acute inflammatory disorders (Bhardwaj *et al.*, 1992). This epitope is found on monocytes that do not only secrete MRP8/14 but also migrate through the endothelial cell line HMEC-1 stimulated with TNF-α. As Eue *et al.* showed in 2000, the secretion of the heterodimer by $27E10^+$ monocytes leads to an upregulation of CD11b and promotes firm adhesion to endothelial cells presenting ICAM-1 (Eue *et al.*, 2000). Thereby MRP8/14 exerts rather a self-inducing effect on monocytes, probably *via* G_α-protein coupled receptors, than interfering directly with the adhesion-ligand interaction. Human monocytes that lack this epitope either migrate later or can be induced to do so in early stages by addition of MRP8/14 *in vitro* to monocytes of $MRP14^{-/-}$ mice (Van Lent *et al.*, 2008).

Similar to the protein's effect on neutrophils, the heterodimer complex boosts the production of cytokines by monocytes and macrophages, including TNF-α, IL-1 and INFγ, especially in arthritis (Sunahori *et al.*, 2006).

Further, Wang *et al.* showed an increased expression of interferon gamma inducible protein 10 (IP-10) upon MRP8/14 stimulation on the human monocyte cell line THP-1, that was dependent on TLR4 receptor signalling including downstream effector TRIF but not MyD88 (Wang *et al.*, 2015). Also, production of matrix metalloproteases (MMP) 3, 9 and 13 is enhanced by MRP8/14 stimulated macrophages which helps to explain the tissue destructive activity of macrophages in antigen- induced arthritis (Van Lent *et al.*, 2008).

1.3.5.3 MRP8/14 on endothelial cells

Hogg *et al.* made the initial discovery of an endothelial-associated MRP8/14 complex detected at sites of leukocyte migration in 1989 (Hogg *et al.*, 1989). It was later shown that it interferes with glycosaminoglycans (Robinson *et al.*, 2002; Srikrishna *et al.*, 2001). Furthermore, Viemann *et al.* could show a thrombogenic and pro-inflammatory response of MRP8/14 binding to human microvascular endothelial cells (HMEC-1) with upregulation of cytokines, especially interleukin-8 (IL-8), and Intercellular cell adhesion molecule 1 (ICAM-1) (Viemann *et al.*, 2005). At the same time, transcription of cadherins is downregulated and caspase-dependent cell death leads to loss of endothelial integrity, facilitating leukocyte migration (Viemann *et al.*, 2007).

According to Ehlermann *et al.,* MPR8/14 also exerts pro-atherosclerotic effects. Its binding to the receptor for advanced glycation end products (RAGE) on HUVECs induces NFkappaB mediated secretion of interleukin-6 (IL-6), ICAM-1, VCAM-1 and MCP1 in a dose dependent manner after HUVEC were pre-stimulated with advanced glycation end products (Ehlermann *et al.*, 2006).

1.4 Translational aspects of MRP8/14

Initially, the heterodimeric protein MRP8/14 was described in the clinical context of rheumatoid arthritis, indicating its important role as an amplifier of autoimmune diseases by the innate immune system. Today, serum and plasma levels of MRP8/14 are fully implemented to serve as biomarker and to monitor treatment with biologicals in autoimmune diseases such as rheumatoid arthritis, juvenile idiopathic arthritis, psoriasis, systemic lupus erythematosus and myositis (Anink *et al.*, 2015; Foell *et al.*, 2007). Both proteins are found in feces of patients with chronic inflammatory bowel disease and levels of calprotectin are used to distinguish this disease from irritable bowel syndrome.

Moreover, MRP8/14 can function as diagnostic and prognostic marker in septic patients on intensive care units, but hasn't reached any clinical evidence yet to qualify as unique marker correlating with sepsis severity (Gao *et al.*, 2015).

MRP8/14 was also found to strongly correlate with cardiovascular risk factors, such as hyperlipidemia, obesity, smoking and hyperglycemia (Cotoi *et al.*, 2014; Schiopu *et al.*, 2013). Morrow *et al.* showed in their PROVE IT-TIMI 22 trial, that MRP8/14 serum levels are higher in patients with myocardial infarction (MI) and recurrent ischemic events within 30 days post-MI than in patients without recurrent ischemic events (Morrow *et al.*, 2008). Also, in female healthy patients, higher plasma levels of MRP14 are correlated with higher cardiovascular risk (Averill *et al.*, 2012). Summing up, elevated plasma levels of MRP8/14 are correlated with an increased risk for recurrent cardiovascular events and thus can function as risk predictor for such events.

In 2004, the small molecule oxo-quinolone-3-carboxamide "Paquinimod" was firstly introduced as a potential substance for medical treatment of autoimmune disorders (Jonsson *et al.*, 2004). Bjork *et al.* could later show, that direct interaction of the compound with human MRP14 led to an inhibition of the protein's interaction with TLR4 receptor and receptor for advanced glycation end products (RAGE) and thereby reduction of pro-inflammatory properties of MRP14 (Bjork *et al.*, 2009).

Recently, a small phase II clinical trial on the use of Paquinimod as oral capsule once daily for 8 weeks in 9 systemic sclerosis patients has been completed. Biomarkers were measured in blood and skin lesion biopsies and Quality of Life (QoL) was assessed. Over all, a significant reduction of chemokine CCL2 and CCR2 in serum and a slight reduction of pro-fibrotic genes of these patients were observed. Quality of Life did not change in the short-term clinical trial (Results not published, URL: https://ClinicalTrials.gov/show/NCT01487551).

1.5 Preliminary work concerning the role of MRP8/14 at sites of inflammation

Previous findings of our group showed that E-selectin is able to trigger MRP8/14 release *in vitro* and *in vivo* during acute TNF-α induced inflammation.

In a MRP8/14 release-assay, murine bone marrow derived neutrophils were incubated with either PBS, immobilized E-selectin or P-selectin or Phorbol-12-myristat-13-acetate (PMA). Here, E-selectin led to a higher extend of MRP8/14 release than P-selectin after 10 and 30 minutes of incubation (Fig. 5).

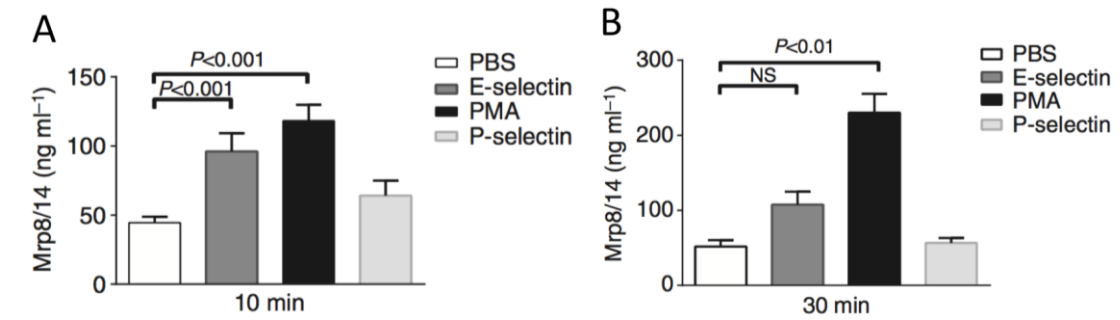

Fig. 5: Neutrophil interaction with E-selectin induces MRP8/14 release *in vitro* (modified from Pruenster *et al.*, 2015).
Neutrophils derived from bone marrow of control mice (C57BL/6 WT) were incubated with different possible MRP8/14 release stimulating factors such as E-selectin-Fc, P-selectin-Fc or PBS. E-selectin revealed to be the stronger stimulator in comparison to P-selectin concerning MRP8/14 release from neutrophils within 10 (A) and 30 minutes (B) of incubation.

Blocking E-selectin with anti E-selectin antibody (9A9) led to low MRP8/14 release after 10 minutes of incubation similar to control.

In order to assess the role of E-selectin in MRP8/14 secretion *in vivo*, serum levels of murine MRP8/14 were measured before and two hours after intrascrotal injection of rmTNF-α, using an enzyme-linked immunosorbent assay (ELISA). Upon anti E-selectin antibody 9A9 application, the amount of soluble MRP8/14 was significantly decreased in comparison to control. Neutrophil depletion 24h before rmTNF-α treatment resulted in similar levels to E-selectin inhibition, indicating that MRP8/14 circulating in serum under inflammatory conditions mainly derives from neutrophils (Fig. 6).

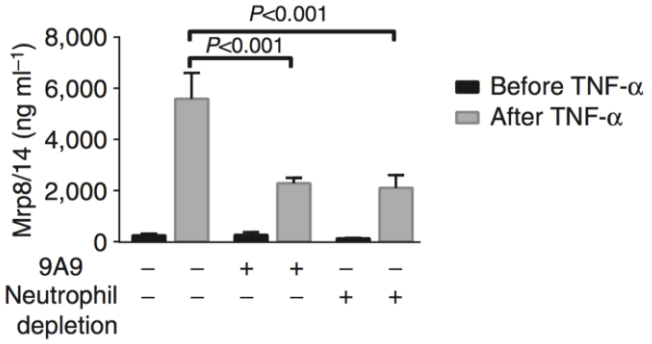

Fig. 6: MPR8/14 release from neutrophils is triggered by E-selectin after TNF-α stimulation *in vivo* (modified from Pruenster *et al.*, 2015).
Neither blocking E-selectin with anti E-selectin blocking antibody 9A9, nor neutrophil depletion 24h before intrascrotal TNF-α-injection led to an increase of MRP8/14 release in comparison to control.

Previous studies from Vogl *et al.* identified TLR4 receptor as potential receptor of extracellular MRP8/14 (Vogl *et al.*, 2007). So, our group was interested if upon E-selectin stimulation, extracullar released MRP8/14 binds to TLR4 expressed on the neutrophils in an autocrine manner. In fluorescence-activated cell sorting (FACS), the quantity of extracellular MRP8/14 bound to neutrophils was measured. Stimulation of neutrophils with E-selectin displayed 20% more of MRP14 protein bound to their surface than on unstimulated cells. If rat anti-mouse TLR4 antibody was added, the amount of MRP14 on the cellular surface of the same cells was completely diminished in comparison to IgG2b (Fig. 7).

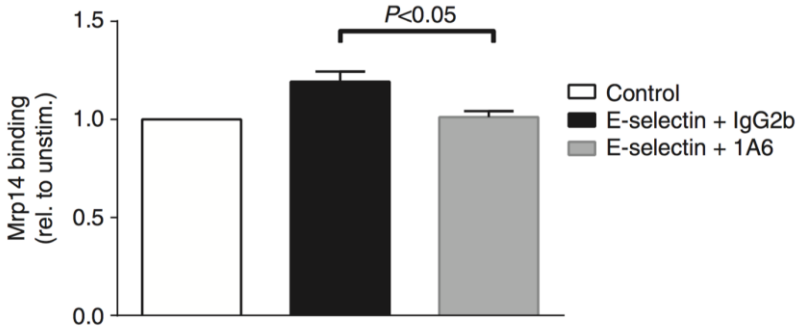

Fig. 7: Surface-bound MRP8/14 can be attributed to autocrine TRL4 binding (modified from Pruenster *et al*., 2015).
Flow cytometry was used to detect extracellular surface-bound MRP14. Upon additional rat anti-mouse TLR4 antibody (grey bar), the percentage of MRP14 protein bound on leukocytes was reduced in comparison to E-selectin stimulated cells (black bar). Unstimulated controls are displayed in white bar and set to 1.

Our group could show that upon this autocrine binding to TLR4, MRP8/14 promotes β_2-integrin activation. The activation status of β_2-integrins on human neutrophils upon MPR8/14 or LPS stimulation was analysed using KIM127 and

mAB24, two activation specific antibodies for the β_2-integrin LFA-1. The latter converted into its activated form upon both, hMRP8/14 and LPS stimulation, leading to a higher KIM127 (Fig. 8 A) and mAB24 (Fig. 8 B) surface expression. But inhibiting either TLR4 with anti TLR4 antibody 1A6 or binding of MRP8/14 to TLR4 with Paquinimod, resulted in decreased binding of KIM127 and mAb24, suggesting that β_2-integrin activation is dependent on the presence of MRP8/14-TLR4 binding.

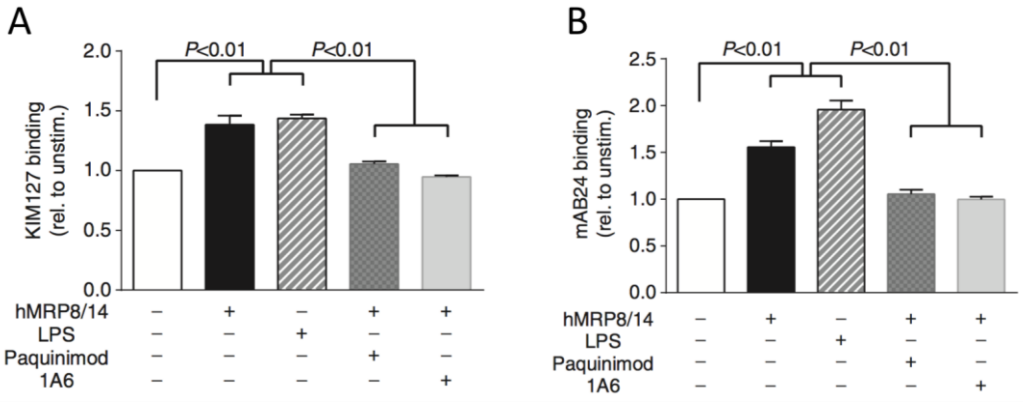

Fig. 8: β_2-integrin LFA-1 is activated by MRP8/14 *via* TLR4 binding (modified from Pruenster *et al*., 2015).

Stimulation of human neutrophils with human MRP8/14 or LPS resulted in β_2-integrin activation. Pre-incubation with rat anti-human polyclonal TLR4 antibody 1A6 or Paquinimod decreased KIM127 and mAB24 binding in flow cytometry, displaying reduced β_2-integrin activation.

2 Hypothesis and aims of this study

Earlier work from our group revealed a functional relevance of extracellular MRP8/14 in the inflammatory response. However, the exact *in vivo* function of MRP8/14, released upon E-selectin dependent neutrophil rolling, remains unclear.

This study aims to directly proof the hypothesis that MRP8/14 release upon E-selectin stimulation is a critical factor for neutrophil recruitment.

To achieve this, I would like to answer the following questions:

1) Does blocking MRP8/14 or its receptor TLR4 alter leukocyte rolling velocity in an inflammatory scenario *in vivo*?

2) Is the adhesion of leukocytes compromised if MRP8/14 or its receptor TLR4 is blocked?

3) Does the blockade or MRP8/14 or TLR4 alter transendothelial migration of leukocytes?

In order to investigate all these questions, we studied inflamed postcapillary venules of the murine cremaster muscle by intravital microscopy.

3 Material and methods

1. Substances for experimental approaches

ABR215757 (Paquinimod)	Active Biotech AB, Lund, Sweden
Anti-mouse E-selectin antibody (CD62E) (9A9)	Provided by Prof. Klaus Ley, La Jolla Institute for Immunology, USA
Isotonic Sodiumchloride 0,9%	Fresenius Kabi Deutschland GmbH, Bad Homburg, Germany
PBS/10% DMSO (carrier substance)	Invitrogen, Carlsbad, California, USA / Sigma-Aldrich, St. Louis, USA
Pertussis toxin from *Bordetella pertussis*	Sigma-Aldrich, St. Louis, USA
Rat anti-mouse TLR4 antibody (1A6)	NovImmune SA, Geneva Switzerland
Rat IgG2b isotype control	eBioscience, San Diego, USA
Recombinant murine TNF-α (rmTNF-α)	R&D Systems, MN, USA

2. Materials for surgical experiments

2.1 Reagents

Bepanthen Augen- und Nasensalbe 5g (eye-creme)	Bayer Vital GmbH, Leverkusen, Germany	
Isotone NaCl-Lösung 0,9% (isotonic Sodiumchloride)	Fresenius Kabi Deutschland GmbH, Bad Homburg, Germany	
Heparin-Natrium-25000-ratiopharm® (Heparin-sodium)	ratiopharm GmbH, Ulm, Germany	
Ketavet® 100mg/ml (Ketamine hydrochloride)	Pfizer Pharmacia GmbH, Karlsruhe, Germany	
N_2 95%/CO_2 5% gas	Linde AG, Munich, Germany	
Rompun® 2% (Xylazin hydrochloride)	Bayer HealthCare, Leverkusen, Germany	
Superfusion-buffer for intravital microscopy (modified from Klitzman *et al.*, 1979)	Buffer I 292.9 g NaCl 13.3 g KCl 11.2 g $CaCl_2$ 7.7 g $MgCl_2$ H_2O	Buffer II 57.5 g $NaHCO_3$ H_2O

2.2 Tools and materials

Dumont #7 Fine Forceps (curved, tip dimension: 0.07x0.03mm)	Fine Science Tools GmbH, Heidelberg, Germany
Gemini™ (cautery system)	Roboz, Gaithersburg, Maryland, USA
Graefe Forceps (curved serrated stainless steel 0.8mm)	Fine Science Tools GmbH, Heidelberg, Germany
Micro Serrefine (straight, 15mm) (carotid clamp)	Fine Science Tools GmbH, Heidelberg, Germany
Omnican®100 (single-use syringe)	B.Braun Melsungen AG, Melsungen, Germany
PE60 Intramedic™ (non-sterile) (tracheal tube)	Becton, Dickinson and Company, NJ, USA
Portex© (carotis catheter tube)	Smiths Medical, NH, USA
Silk braided suture (non-sterile) (silk ligament)	Pearsalls Limited, Taunton, Great Britain

2.3 Technical devices

Isopad™ (heating pad)	Thermocoax Isopad GmbH, Heidelberg, Germany
Olympus SZ2-STS	Olympus UK Ltd KeyMed House, Southend-on-Sea, Great Britain
Olympus TH4-200 (cold light source for stereo microscopy)	Olympus UK Ltd KeyMed House, Southend-on-Sea, Great Britain
Plexiglas stage	Custom-made
ProCyte Dx® (Haematology analyser)	IDEXX Laboratories Ltd, Windsor, UK

3. Materials for whole-mount staining

3.1 Reagents

4% PFA (paraformaldehyde)	AppliChem GmbH, Darmstadt, Germany
Acetic acid	Merck Chemicals GmbH, Darmstadt, Germany
Ehtyl alcohol (70%, 96% and 99,9%)	CLN GmbH Chemikalien Laborbedarf, Niederhummeldorf, Germany
Giemsa's azur eosin methylene blue solution	Merck Chemicals GmbH, Darmstadt, Germany
Xylene Eukitt ® quick-hardening mounting medium for microscopy	FLUKA, Sigma-Aldrich, St. Louis, MO, USA

Xylene Mixture of Isomers (M = 106.17g/mol)	AppliChem GmbH, Darmstadt, Germany

3.2 Tools and materials

BD Discardit™ II 5ml syringe	Becton, Dickinson and Company, NJ, USA
Microscope cover slips 24x40mm	Gerhard Menzel Glasbearbeitungswerk GmbH & Co. KG, Braunschweig, Germany
Microscope slides (Thermo Fisher Scientific Menzel)	Gerhard Menzel Glasbearbeitungswerk GmbH & Co. KG, Braunschweig, Germany
Minisart ®, single use filter system	Sartorius GmbH & Co.KG, Ratingen, Germany
Single-use Pasteur Pipette	VITLAB GmbH, Grossostheim, Germany

4. Imaging

Olympus BX51WI upright microscope for *in vivo* imaging equipped with:	Olympus GmbH, Hamburg, Germany
Water immersion objective x40, 0.80NA (Intravital Microscopy)	Olympus GmbH, Hamburg, Germany
Velocity OD-RT (Circu-Soft) Instrumentation	CircuSoft Instrumentation LLC, Hockessin, USA
VirtualDub Software (Recording)	
Zeiss Axioskope 40 microscope equipped with an oil immersion objective x100, 1.25NA (for whole mounts imaging)	Zeiss, Jena, Germany
CCD camera Kappa® (Typ CF8/1 HS)	Kappa, Gleichen, Germany
Water immersion objective x20, 0.95NA (Flow chamber experiments)	Olympus GmbH, Hamburg, Germany

3.1 Animals

Intravital microscopy experiments were performed on male wildtype mice of the colony C57BL/6 NRj, aged between 10 and 30 weeks. Mice were obtained from Janvier Labs (Saint Berthevin, France) and kept in adequate animal welfare at the Walter Brendel Center for Experimental Medicine, Ludwig-Maximilians-Universität, Munich, Germany. Within a constant day-night rhythm of 12h ± 2h all animals were housed in polycarbonate cages providing easy access to drinking water and autoclaved food pellets. All conducted experiments were approved by the local government of Oberbayern, Germany, with the reference numbers AZ 55.2-1-54-2531-76/12, -175/09 and -355/13.

3.2 Cremaster muscle model

The *Musculus cremaster* accompanies the *fascia spermatica interna* and covers the spermatic cord and the testis as it descends through the inguinal canal and thereby serves as a protection and temperature regulator of the testis.

Due to its thin layer of tissue and easy accessibility from outside, this muscle was already an object of interest in the second half of the 20th century. The model evolved originally from inflammation studies on histological specimens of the rat cremaster muscles (Majno *et al.*, 1961). In the early 1960ties this approach was expanded to *in vivo* studies of microvascular responses to vasoactive drugs and finally to the opened cremaster muscle in mice for *in vivo* observation and real-time imaging of cellular pathways and intercellular signalling (Baez, 1973; Grant, 1964).

3.2.1 Anesthesia

Mice were anaesthetized via intraperitoneal injection of 125mg/kg ketaminhydrochloride (Ketavet®; Pfizer Pharmacia GmbH, Karlsruhe) and 12.5mg/kg xylazinhydrochloride (Rompun®; Bayer HealthCare, Leverkusen) in a total volume of 1ml. It was applied as a bolus of 0.1ml/8kg of body weight. For injection, single-

use syringes were used with a total volume of 1ml (Omnican®100; B. Braun Melsungen AG, Melsungen). Anaesthesia was maintained during the whole experiment by bolus injection every 45 minutes depending on body weight.

3.2.2 Procedure

The anesthetized mouse was positioned on its back and fixed on a 35,0°C warmed heating pad (Isopad™; Heidelberg, Germany) to hold body temperature throughout the experiment. All surgery was performed on a stereomicroscope (Olympus SZ2-STS, Hamburg, Germany). For vital stabilisation and steady conditions during the whole experiment we inserted a tracheal tube (PE60, Intramedic™; Becton, Dickinson and Company, NJ, USA) into the mouse trachea after preparing a small surgical field at its neck in the mid-sagittal line. The tube was secured with two knots using silk threads (Fig. 9 A-D). Thereafter, the right or left *Arteria carotis* was exposed for cannulation with a small tube of an inner diameter of 0.28mm (Portex©; Smiths Medical, NH 03431, USA). After undermining the artery, a ligation was placed at the cranial part using a silk knot and a vessel clamp set towards the heart. Next, an incision was made close to the first ligation but without cutting the whole artery. The mentioned tube was inserted into the hole in the artery and connected to a single-use syringe filled with normal saline containing 20U/ml of sodium-heparin (20U/ml of Heparin-Natrium-25000-ratiopharm®; ratiopharm GmbH, Ulm). Two knots were added for fixation. Finally, the clip was opened and blood flow was checked (Fig. 9 E-K).

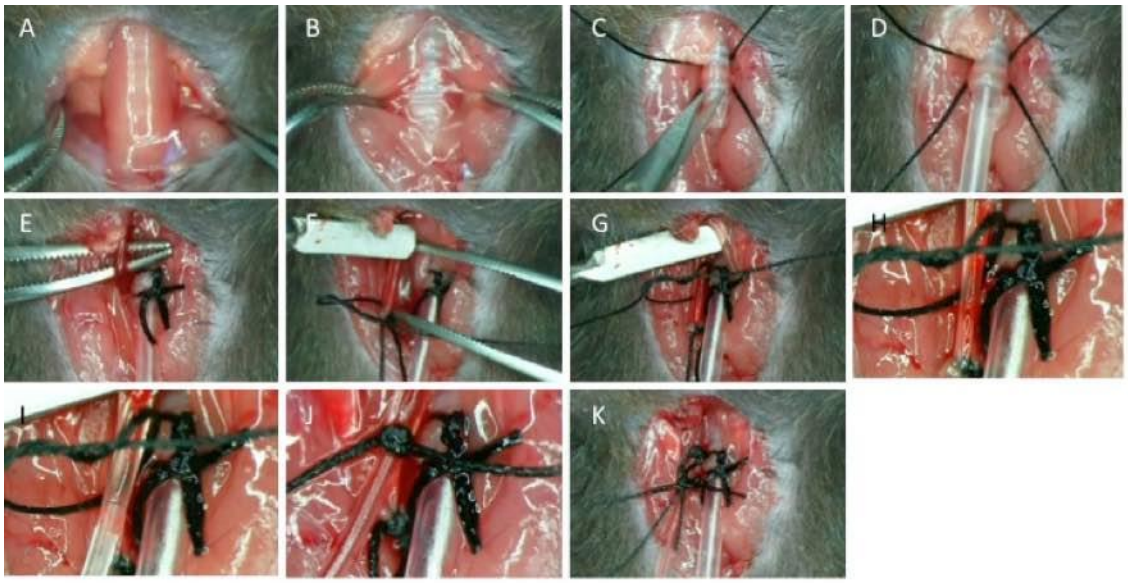

Fig. 9: Surgical placement of a tracheal tube and catheterisation of the carotid artery.
(A) Stereoscopic upright view of the neck opened at mid-sagittal line. Mouse lays on its back, bottom of the picture pointing towards its nose. Trachea enclosed by muscular sheath. (B) Muscles divided into two, laterally fixed by forceps. (C) Two black silk knots undermining the Trachea; Scissors pointing towards intracartilage tissue. (D) After incision of trachea, tube (PE60) is inserted into Trachea and fixed with the knots (not shown). (E) Left carotid artery is exposed from surrounding tissue. (F) Two black silk slings are around the artery. One is already knotted at the distal end of the artery; a clamp is added at the proximal end. (G) Placement of a safety knot that remains open during incision of carotid artery. (H) Zoom-in, incision made at the bottom of the artery. (I) Catheter-tube is inserted and fixed by safety knot (not shown). (J) Proximal blood clamp is removed and blood flows revers the catheter tube. (K) Final safety knot.

3.2.3 Surgical preparation of *Musculus Cremaster*

Following neck surgery, the anesthetized mouse was transferred to a plexiglas stage and legs were fixed with leucoplast tape to prepare microsurgery of the cremaster muscle and optimize stability under the microscope. To this end, the scrotum was grabbed with forceps and a small skin incision of 4mm was made at its lower end. With a stump forceps the cremaster muscle, wrapped in connective tissue, was exteriorized and freed from connective tissue. Then it was placed onto the stage, opened longitudinally and edges were secured with pins to spread the muscle for intravital microscopy. The testicle and epididymal tissue were pinned aside. In contrast to the preparation described by Baez in 1973, all veins connecting either muscle tissue or epididymis were left intact (Baez, 1973). Potential bleeding was diminished using a Gemini™ cautery system (Roboz, Gaithersburg, Maryland, USA). During the procedure as well as during microscopy physiological superfusion buffer (for precise composition see 3.3.1) was irrigated on to the specimen.

3.3 Intravital microscopy

3.3.1 Setup

Intravital microscopy (IVM) is a well-known and well-established method in the field of microscopic imaging techniques and it serves to observe physiological processes in the microcirculation under *in vivo* conditions.

Intravital microscopy was first described by Malpighi in 1661, who observed moving particles in the capillary network of a frog lung (Sperandio *et al.*, 2006).

Observing the inflammation's effect on microcirculatory leukocyte-endothelium interaction has been described for either murine mesenteric tissue or the cremaster muscle, as those two tissues are reasonably accessible. During the last century the cremaster muscle model was implemented by Baez to investigate microcirculatory phenomena such as leukocytes rolling and adhesion (Baez, 1973).

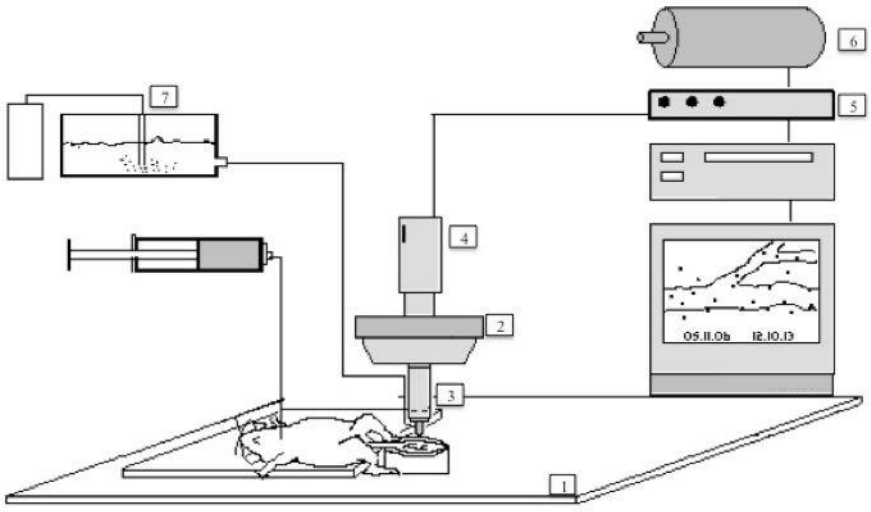

Fig. 10: Schematic illustration of the intravital microscopy setup.
(1) Optical table of the (2) upright transillumination microscope; (3) Water-immersion x40 objective 0.8NA (Olympus); (4) Recording was performed by a CCD-Camera (Kappa® CF Typ 8); (5) VHS recorder and time generator; (6) dual photodiode system; (7) heated superfusion-buffer.

During the *in vivo* observation of the *cremaster* muscle tissue, which was fixed onto a custom-made plexiglas stage and placed under the transillumination microscope, the cremaster muscle could be moved in xyz-axis. This provided an easy way to find an appropriate field of view (Fig. 10).

All experiments were performed on an Olympus BX 51WI microscope using a water-immersion x40 objective with a numerical aperture of 0.8 (Olympus Mplan FI/RI). The record and digital analysis of intravascular rolling and adhesion of leukocytes were done by a CCD-Camera (Kappa® CF 8 HS, Gleichen, Germany), which was not only connected to a VHS recorder (Panasonic, Japan), including a time generator (Video Timer, FOR.A Company limited, Japan) but also linked to a digital recording software (Virtual Dub).

Furthermore, the centerline blood flow velocity of each recorded vessel was measured by using a dual photodiode system related to a digital temporal intensity cross-correlation program (CircuSoft Instrumentation, Hockessin, USA, originally developed by Wayland & Johnson 1967) (not shown). According to Lipowsky *et al.,* the measured centerline velocity was multiplied by an empirical factor of 1.6 to convert it into the mean blood flow velocity (Lipowsky *et al.*, 1978).

Only venules with an average blood flow velocity of 800µm/s until 5000µm/s were taken for recordings.

To obtain steady in vivo conditions, the exteriorized living cremaster muscle tissue was permanently bathed in thermocontrolled (35°) superfusion buffer, whose composition was as following:

Buffer I and II were prepared as stock (Table. 1). Before the experiments, buffer I and II were mixed in a 1000ml cylinder with 100ml of superfusion buffer I, 800ml deionized water and finally another 100ml of superfusion buffer II.

Superfusion buffer I	**[g]**
NaCl	292.9
KCl	13.3
$CaCl_2$	11.2
$MgCl_2$	7.7
deionized H_2O	3.8 liters
Superfusion buffer II	**[g]**
$NaHCO_3$	57.5
deionized H_2O	3.8 liters

Table. 1: Ingredients and their amount in [g] for superfusion buffers (Ley *et al.*, 1991).

The final solution was equilibrated with N_2 95%/CO_2 5% (Linde AG, Munich, Germany) using a foam dispenser to avoid crystallisation of the salt.

During the experiment, the carotid artery catheter was used to collect small blood samples (50µl) into heparinized tubes for differential blood cell count using the haematology analyser ProCyte Dx® (IDEXX Laboratories, Windsor, UK).

3.3.2 Analysis of leukocyte rolling and adhesion

Intravital microscopy is suitable not only to provide an insight into the microcirculation but also to assess endothelial-leukocyte interactions. For recorded vessels, we derived several geometric and hemodynamic parameters such as vessel diameter (*d*) and segment length (*l*), that were both measured by using Fiji software (ImageJ) (Schindelin *et al.*, 2012).

Parameter	Definition	Unit
Leukocyte rolling flux	Number of rolling leukocytes passing a perpendicular line placed across the observed vessel in one minute	N/min
Leukocyte rolling flux fraction (RFF)	Rolling flux divided by the complete leukocyte flux deduced from total white blood cell count (N/µl) and blood flow (µm/s)	
Leukocyte rolling velocity	Movement of leukocytes in intermittent and/or continuous contact with the endothelial surface	µm/s
Vessel diameter	Average orthogonal linear distance between endothelial cells on opposite sides of vessel	µm
Center blood flow velocity	Measured blood flow velocity in the center of the vessel and converted into mean blood flow velocity by multiplying with an empirical correction factor (Lipowsky *et al.*, 1978)	µm/s
Adherent cells	Number of adherent leukocytes (not moving out of one cell's diameter within 30 sec) per surface area	N/mm^2
Adhesion efficieny	Number of adherent leukocytes per surface area divided by the complete leukocyte flux (white blood cell count)	

Table. 2: Index of geometric and hemodynamic parameters derived from postcapillary venules and important for the assessment of leukocyte rolling and adhesion by intravital microscopy (Adapted and modified from Sperandio *et al.*, 2006).

Postcapillary venules with mean diameters ranging from 25µm to 45µm were selected for further analysis.

Additionally, leukocyte rolling flux (RF) was assessed, which describes the number of rolling cells passing an imaginary line over the vessel within one minute (rolling leukocytes/min) (Ley *et al.*, 1991). The total peripheral leukocyte flux is highly influenced by either artificially evocated or intrinsic inflammation. Because rolling flux does not take into account the amount of systemically circulating leukocytes, rolling flux fraction (RFF) was introduced and calculated by dividing RF

through total white blood cell count (WBC), mean blood flow velocity (ϑ), and the cross sectional area of the vessel:

$$RFF = \frac{RF}{WBC \times \vartheta \times (\frac{d}{2})^2 \pi}$$

Firm adhesion of leukocytes is defined as being stationary for at least 30 seconds. The total number of adherent cells (per surface area) was assessed for the whole vessel for at least one minute of observation. Total white blood cell count (WBC) and vessel surface area were used to calculate leukocyte adhesion efficiency (AE):

$$AE\ (\%) = \frac{total\ amount\ of\ adherent\ cells}{WBC \times d \times l \times \pi}$$

As blood flow exerts hemodynamic forces on leukocytes, their recruitment as well as their rolling depends on shear stress. A certain shear stress is needed for stable rolling of leukocytes on either P- or E-Selectin (Sundd *et al.*, 2011; Sundd *et al.*, 2013). Therefore, we also assessed wall shear rates (γ_w) to make sure hemodynamic parameters were similar between the groups (Smith *et al.*, 2002).

$$\gamma_w = 4.9 \times \frac{8\bar{\vartheta}_{bl}}{d}$$

Leukocyte rolling velocity was determined by tracking at least 10 leukocytes per one postcapillary venule with the Fiji plug-in MTrackJ (Meijering *et al.*, 2012). With a distinct frame rate, 25 pictures were taken per second, leukocytes were tracked and the total rolling distance was measured to finally calculate rolling velocity.

Finally converted into percentage of discrete velocity groups, we could sort them into a cumulative distribution of rolling velocities.

3.4 *In vivo* model of inflammation

3.4.1 TNF-α induced inflammation

In order to enforce our previous studies on E-Selectin dependent MRP8/14 expression, in each experimental approach we injected 500ng recombinant murine (rm) TNF-α (R&D Systems, MN, USA) in 150µl sodium chloride intrascrotally two hours prior to intravital microscopy. As a consequence of TNF-α application, endothelial P- and E-Selectin as well as ICAM-1 and VCAM-1 are upregulated on the cell surface and leukocyte rolling and adhesion can be observed (Sperandio *et al.*, 2001).

3.4.2 Series of experiments

In the first set of experiments, dedicated to analysing leukocyte rolling velocity upon E-selectin triggered MRP8/14 release, C57BL/6 NRj wildtype mice were pre-treated with intraperitoneal injection of either Paquinimod (10mg/kg) (Fig. 11 B), that blocks MRP8/14 binding to TLR4 or rat anti-mouse blocking TLR4 antibody (1A6) (100µg/mouse) (Fig. 11 A). Sixty minutes later, recombinant murine TNF-α was injected intrascrotally to induce sterile inflammation.

The murine cremaster muscle was dissected two hours after injection of TNF-α and observations were made using intravital microscopy (see chapter 3.4). Controls were performed with PBS/10% DMSO (carrier substance of Paquinimod), respectively rat IgG2b isotype (100µg/mouse).

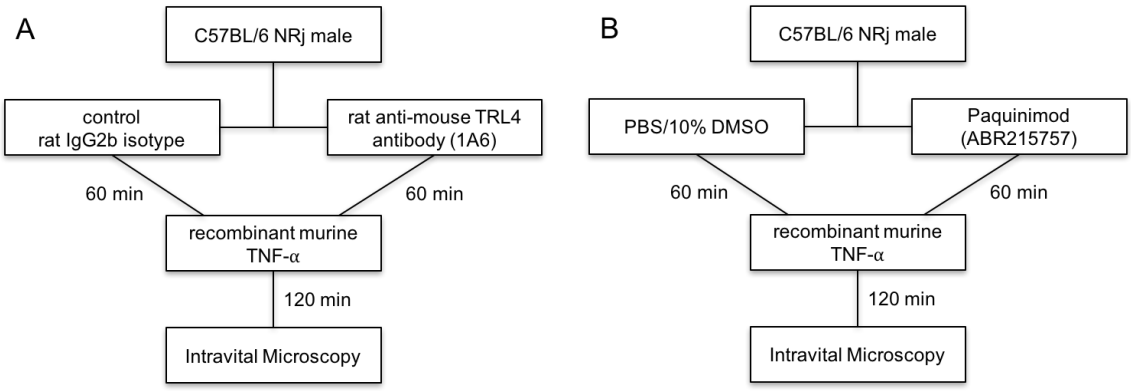

Fig. 11: Set of experiments to analyse leukocyte rolling velocity *in vivo*.
Wildtype C57BL/6 NRj male mice were pre-treated with either rat anti-mouse TLR4 antibody (A) or Paquinimod (B), respectively. In addition, the control substances rat IgG2b isotype (A) or PBS/10%DMSO (B) were

applied 60 minutes before sterile inflammation was induced by intrascrotal injection of recombinant murine TNF-α. Intravital microscopy was performed after a total of three hours after initial injection.

For analysis, at least 5 postcapillary venules per murine cremaster with a mean diameter ranging from 25µm to 45µm were recorded with a minimum of one minute recording time. Leukocyte rolling velocity was assessed as described in section 3.4.2.

In a second set of experiments, the role of MRP8/14 in leukocyte adhesion and finally transmigration was analysed.

Previous studies showed that upon inflammation, E-selectin and chemokines have synergistic effects on full β_2-integrin activation leading to leukocyte adhesion (Smith *et al.*, 2004a). So, in order to identify the role of E-selectin mediated MRP8/14 on adhesion, we minimized the existing overlapping mechanism by blocking the chemokine pathway with pertussis toxin. Pertussis toxin is an exotoxin secreted by *Bordetella pertussis*, a small gram-negative bacterium that induces human respiratory tract infections. It was previously shown that pertussis toxin can ADP-ribosylate the heterotrimeric G_i proteins, thereby inhibiting G-protein-coupled chemokine receptors on neutrophils (Brito, 1997). Therefore, the first set of experiments was extended by additional injection of 4µg pertussis toxin into the peritoneum simultaneously to the other substances (Fig. 12).

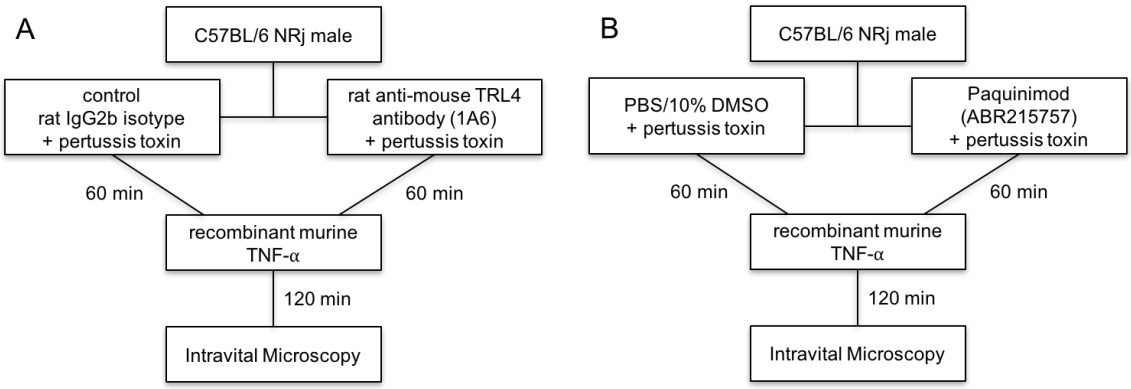

Fig. 12: Set of experiments to analyse leukocyte adhesion and transmigration *in vivo.*
Wildtype C57BL/6 NRj male mice were pre-treated with either rat anti-mouse TLR 4 antibody (A) or Paquinimod (B) or their respective control substances. Simultaneously, pertussis toxin was injected into the peritoneum 60 minutes before sterile inflammation was induced with intrascrotal injection of recombinant murine TNF-α. Intravital microscopy was performed after a total of three hours after initial injection.

In a third set, additional injection of anti E-selectin blocking antibody (9A9) was used besides pertussis toxin, Paquinimod and anti TLR4 antibody (Fig. 13) to demonstrate that our results could not be attributed to other, E-selectin independent pathways of MRP8/14 release and the protein's autocrine binding to TLR4 receptor.

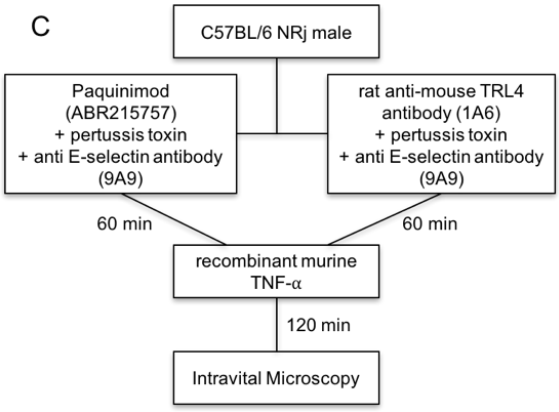

Fig. 13: Set of experiments to analyse leukocyte adhesion and transmigration *in vivo* with additional anti E-selectin blocking antibody.
Wildtype C57BL/6 NRj male mice were pre-treated with either rat anti-mouse TLR 4 antibody or Paquinimod. Simultaneously, pertussis toxin and anti E-selectin blocking antibody (9A9) were injected into the peritoneum 60 minutes before sterile inflammation was induced with intrascrotal injection of recombinant murine TNF-α. Intravital microscopy was performed after a total of three hours after initial injection.

Similar to the first set of experiments, records of at least 5 postcapillary venules per murine cremaster muscle were collected. Leukocytes that maintained their position for at least 30 seconds were considered as "adhesive" and count along the vessel's surface area.

For transmigration, all cremaster muscle tissues were collected, fixed and analysed as described in section 3.5.

3.5 Staining for whole-mount samples

To assess the number of extravasated cells by Giemsa-Staining, cremaster muscles as used for intravital microscopy were fixed on object-slides (SuperFrost®, Gerhard Menzel GmbH, Braunschweig, Germany) and conserved in an 4% paraformaldehyde-solution (PFA) (AppliChem GmbH, Darmstadt, Germany) containing 0.2M phosphate buffer (adjusted to pH 7.4) for at least 24h at 8°C.

Before Giemsa staining, cremaster muscles were washed three times five minutes in 0.1M phosphate buffer (adjusted to pH 7.4) with 5% ethanol. Then, Giemsa stain (Merck Chemicals, Darmstadt, Germany) was applied on the slices for at least 2 minutes, followed by wash out with 0.03% acetic acid solution until slices obtained a light pink appearance. The ascending alcohol series with 75%, 95%, 99% ethanol was followed by dewatering in xylol solution. Xylene Eukitt ® quick-hardening mounting medium for microscopy (FLUKA, Sigma-Aldrich, Saint-Louis, MO, USA) was dribbled to the object slides and a cover slip was used to seal the stained cremaster muscle.

Using a Zeiss Axioskop 40 microscope with an oil immersion objective x100, 1.25NA (Zeiss, Jena, Germany), the number of transmigrated cells/mm^2 was analysed using the prepared and stained cremaster muscle whole mounts.

3.6 Statistics

All data were analysed using Graph Pad Prism 7.0 Software (GraphPad Software Inc., San Diego, USA). For pairwise comparison of experimental groups, an unpaired t-test (Mann-Whitney U) was performed. For multiple comparisons, a one-way analysis of variance (ANOVA) was used with Tukey's *post-hoc* test (comparison of all experimental groups against each other). A p-value < 0.05 was considered statistically significant.

4 Results

In order to investigate whether E-selectin dependent MRP8/14 release and subsequent autocrine binding and activation of TLR4 receptor on neutrophils plays a key role in leukocyte slow rolling (section 4.1), adhesion and transmigration (section 4.2) under inflammatory conditions *in vivo*, all experiments were performed 2 hours after intrascrotal TNF-α injection, which leads to local expression of E-selectin on the inflamed endothelium of postcapillary venules of the cremaster muscle (Smith *et al.*, 2004a).

4.1 MRP8/14 reduces rolling velocity of neutrophils *in vivo*

Before analysing leukocyte rolling velocity in the first set of experiments, we collected microvascular (vascular diameter in µm) and hemodynamic parameters such as wall shear rate (s^{-1}) as well as total number of circulating white blood cells (WBC; cells/µl) to obtain comparable hemodynamic and microvascular conditions between the groups (Table. 3 + 4). Unpaired t-test (Mann-Whitney-U) was performed.

Here, neither microvascular nor hemodynamic parameters showed significant differences between blocking substances and their controls in both groups.

Group **A**	Substances	Mice *n*	Venules *n*	Diameter *µm*	Shear rate s^{-1}	WBC *cells/µl*
	IgG2b isotype	5	23	32.06 ± 1.39	1,523 ± 118	3,984 ± 587
	1A6	4	29	31.27 ± 1.43	1,842 ± 150	3,690 ± 475
Statistics				n.s.	n.s.	n.s.

Table. 3: Microvascular and hemodynamic parameters in TNF-α stimulated cremaster muscle venules pre-treated with rat anti-mouse TLR4 antibody (1A6) or IgG2b (control substance) (Group A).
All parameters are presented as mean ± SEM. There were no significant differences in comparison of all parameters listed (n.s.) in ordinary unpaired t-test.

Group **B**	Substances	Mice *n*	Venules *n*	Diameter *µm*	Shear rate s^{-1}	WBC *cells/µl*
	Control	3	16	30.31 ± 1.96	1,317 ± 204	3,787 ± 420
	Paquinimod	4	21	30.77 ± 1.21	1,538 ± 125	3,775 ± 340
Statistics				n.s.	n.s.	n.s.

Table. 4: Microvascular and hemodynamic parameters in TNF-α stimulated cremaster muscle venules pre-treated with Paquinimod or control substance (PBS/10%DMSO) (Group B).
All parameters are presented as mean ± SEM. There were no significant differences between the groups (n.s.) using an unpaired t-test.

Overall, these data show that application of Paquinimod or rat anti-mouse TLR4 antibody (1A6) did not affect the number of circulating leukocytes.

In the TNF-α induced inflammation model, slow rolling of leukocyte depends on E-selectin and the β_2-integrin LFA-1 (Dunne *et al.*, 2002). According to our hypothesis, we wanted to show, that MRP8/14 is an indispensable protein released by leukocytes that mediates E-selectin-dependent β_2-integrin activation and thereby promotes slow rolling. So, we analysed rolling velocities of leukocytes in mouse cremaster muscle venules, pre-incubated with either rat anti-mouse TLR4 antibody (1A6) or IgG2b isotype as well as in the presence or absence of Paquinimod (ABR215757) or its carrier substance (control, PBS/10% DMSO).

In group A, n= 225 cells for IgG2b isotype and n= 214 cells for rat anti-mouse TLR4 antibody (1A6) were analyzed. In group B, n= 98 cells for control and n= 71 cells for Paquinimod were analyzed.

Leukocytes of control mice in both groups displayed a mean rolling velocity of 3.41 ± 0.12 µm/s (Group A, IgG2b isotype) and 3.79 ± 0.34 µm/s (Group B, control) respectively. Pre-treatment of mice with either rat anti-mouse TLR4 antibody (1A6) or Paquinimod led to a similar significant increase in rolling velocity to 7.20 ± 0.39 µm/s (Group A, anti TLR4 antibody 1A6, Fig. 14 A) and 7.50 ± 0.65 µm/s (Group B, Paquinimod, Fig. 14 B) respectively.

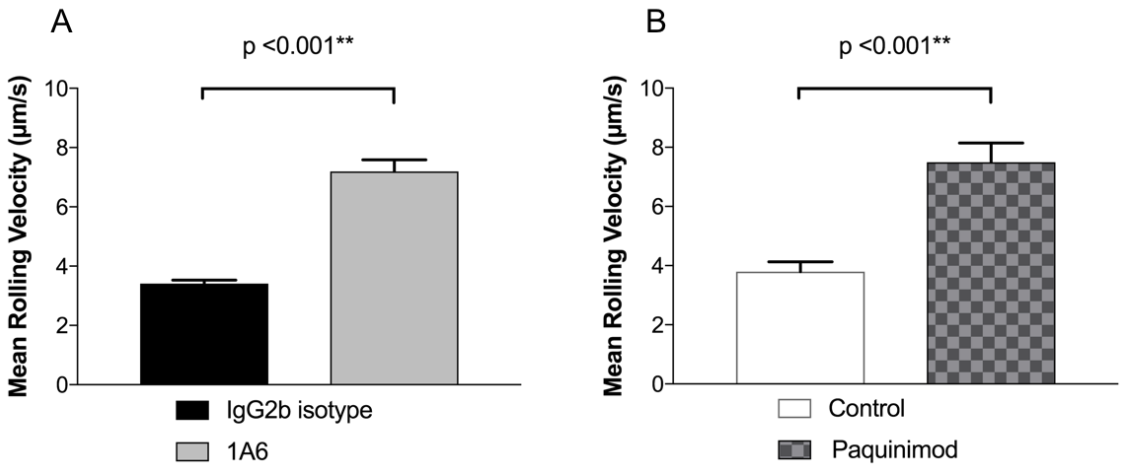

Fig. 14: Rolling velocity of neutrophils is reduced by MPR8/14 *in vivo.*

Mean rolling velocities of leukocytes in TNF-α stimulated cremaster muscle after pre-treament with either rat anti-mouse TLR4 antibody 1A6 (A) or Paquinimod (B) and their control substances. Unpaired t-test was performed. All values are presented as mean ± SEM.

These findings on leukocyte slow rolling suggest that MRP8/14, upon binding to TLR4, regulates leukocyte rolling velocities *in vivo*.

4.2 MRP8/14 increases leukocyte adhesion in TNF-α stimulated cremaster muscle venules *in vivo*

According to Smith *et al.,* leukocyte adhesion in TNF-α stimulated cremaster muscle venules depends on a synergistic effect of E-selectin and chemokines (Smith *et al.*, 2004a). So, in order to suppress the chemokine dependent signalling for activation of β_2-integrins, pertussis toxin (PTx) was added in the second set of experiments.

Similar to preceding experiments, all groups were compared for vascular diameter (in µm), wall shear rate (s^{-1}) and total white blood cell count (WBC; cells/µl) (Table. 5 + 6). Unpaired t-test (Mann-Whitney-U) was performed. No significant differences were found between parameters of controls and blocking substances in both groups besides white blood cell count in group A (Table. 5).

Group **A**	Substances	Mice *n*	Venules *n*	Diameter *µm*	Shear rate s^{-1}	WBC *cells/µl*
	IgG2b isotype + PTx	3	30	29.91 ± 1.4	1,311 ± 95	6,626 ± 1902
	1A6 + PTx	5	28	28.63 ± 0.6	1,260 ± 103	3,126 ± 200
Statistics			n.s.	n.s.	n.s.	*p<0.05

Table. 5: Microvascular and hemodynamic parameters in TNF-α stimulated cremaster muscle venules pre-treated with pertussis toxin (PTx) and either rat anti-mouse TLR4 antibody (1A6) or IgG2b (control substance) (Group A).
There was a significant difference in comparison of total white blood cell count (p<0.05). Otherwise all other groups didn't show any significant differences (n.s.) between the parameters listed. Unpaired t-test was performed. All parameters are presented as mean ± SEM.

Group **B**	Substances	Mice *n*	Venules *n*	Diameter *µm*	Shear rate s^{-1}	WBC *cells/µl*
	Control + PTx	3	16	36.02 ± 1.5	1,570 ± 93	3,566 ± 449
	Paquinimod + PTx	3	21	26.86 ± 0.6	1,355 ± 152	3,803 ± 1034
Statistics			n.s.	n.s.	n.s.	n.s.

Table. 6: Microvascular and hemodynamic parameters in TNF-α stimulated cremaster muscle venules pre-treated with pertussis toxin (PTx) and either Paquinimod or control substance (PBS/10% DMSO) (Group B).
There were no significant differences (n.s.) between all parameters listed. Unpaired t-test was performed. All parameters are presented as mean ± SEM.

In our first set of experiments in which we primarily assessed leukocyte rolling velocity, we could also show, that there was no significant reduction of the number of adherent cells per mm^2 upon single application of Paquinimod or 1A6 alone in comparison to their control substances (Fig. 15).

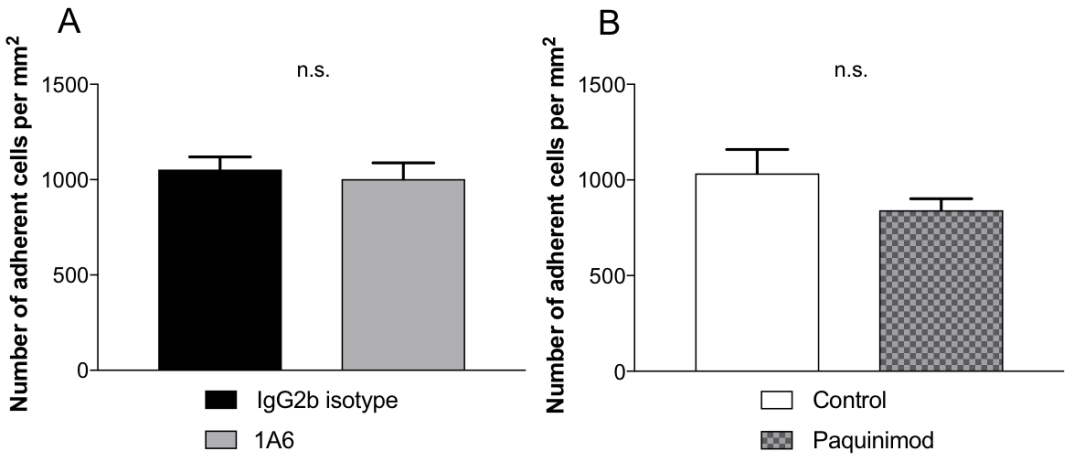

Fig. 15: Pre-treatment with rat anti-mouse TLR4 antibody (1A6) (A) or Paquinimod (B) alone does not impair leukocyte adhesion *in vivo.*

Number of adherent cells per mm^2 were assessed in TNF-α stimulated cremaster muscle venules of mice, pre-treated with either anti TLR4 antibody or IgG2b isotype (A) or with Paquinimod or control substance (B). Unpaired t-test was performed. All values are presented as mean ± SEM.

Interestingly, using pertussis toxin in order to block chemokine dependent activation, the number of adherent cells declined significantly in comparison to controls, if either TLR4 binding site of MRP8/14 was blocked by Paquinimod or anti TLR4 blocking antibody was applied.

In mice pre-treated with anti TLR4 antibody (1A6) and pertussis toxin, only half of the total number of adherent cells was found in comparison to control group with IgG2b isotype and pertussis toxin (499.81 ± 32.21 in n= 28 venules *vs.* 1,088.27 ± 57.37 in n= 30 venules) (Fig. 16 A).

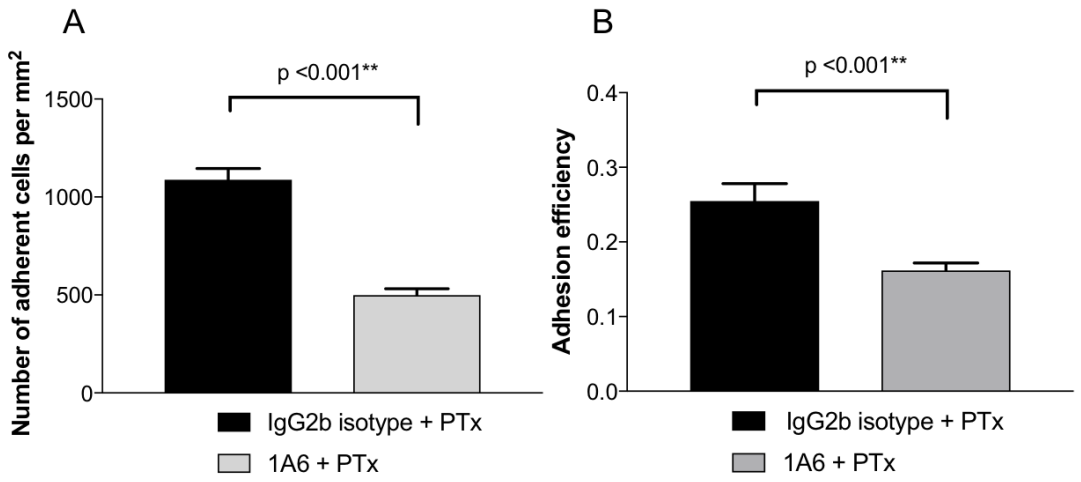

Fig. 16: Leukocyte adhesion is reduced upon blocking of TLR4 and additional pertussis toxin *in vivo.*
Number of adherent cells per mm^2 (A) and adhesion efficiency (B) were assessed in TNF-α stimulated cremaster muscle venules of mice, pre-treated with pertussis toxin and either anti TLR4 antibody or IgG2b isotype. Unpaired t-test was performed. All values are presented as mean ± SEM.

The significant reduction in leukocyte adhesion was also detectable if Paquinimod plus additional pertussis toxin was injected. Here, the total number of adherent cells was 426.52 ± 36.36 for Paquinimod + PTx group (n= 21 venules) in comparison to 1,016.17 ± 84.14 for control + PTx group (n= 16 venules) (Fig. 17 A). These reductions were also found, if leukocyte adhesion efficiencies were calculated (Fig. 16 B + 17 B).

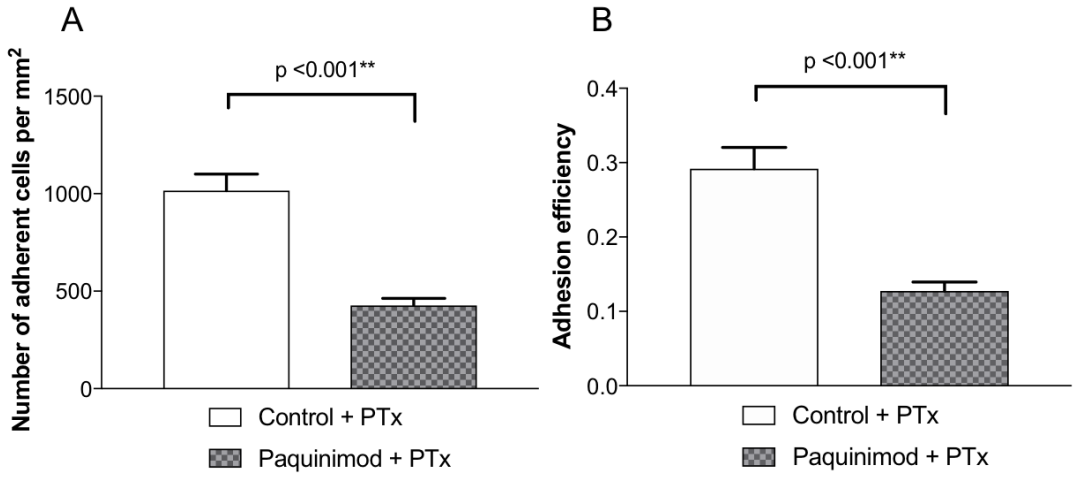

Fig. 17: Leukocyte adhesion is reduced upon blocking of the TLR4 binding site on MRP8/14 with Paquinimod and additional pertussis toxin *in vivo.*
Number of adherent cells per mm^2 (A) and adhesion efficiency (B) were assessed in TNF-α stimulated cremaster muscle venules of mice, pre-treated with pertussis toxin and either Paquinimod or control substance. Unpaired t-test was performed. All values are presented as mean ± SEM.

These results indicate that MRP8/14 is a key player in E-selectin dependent leukocyte adhesion *in vivo* in cremaster muscle venules that were stimulated with TNF-α.

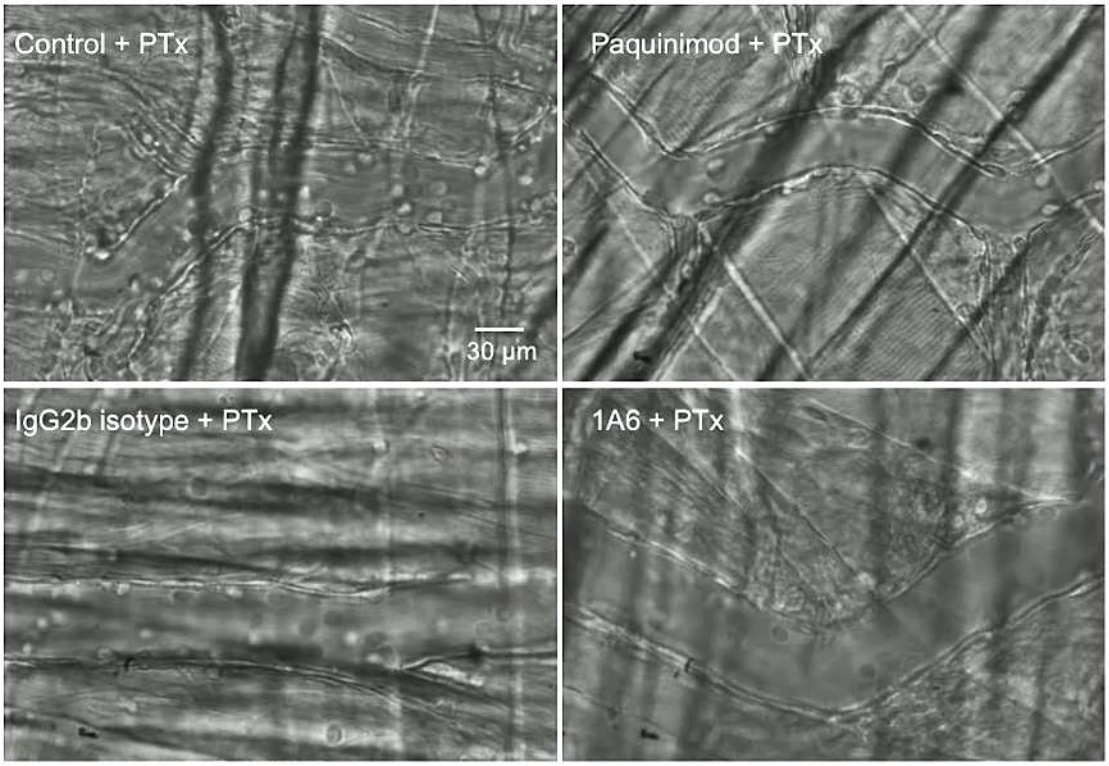

Fig. 18: Representative selection of micrographs showing adherent cells in cremaster muscle venules.
Microscopic pictures of cremaster muscle vessels with visible differences in the number of adherent cells in TNF-α stimulated cremaster muscle venules of C57BL/6 wildtype mice pre-treated with pertussis toxin (PTx) and either control substances (control and IgG2b isotype) or Paquinimod or rat anti-mouse TLR4 antibody 1A6. Scale bar 30µm.

Further, we added pre-treatment with anti E-selectin antibody (9A9) (Group C) to exclude any additional signalling pathways independent of E-selectin.

As expected, additional rat anti-mouse E-selectin antibody (9A9) had no additional effect on our results, demonstrating that the contribution of E-selectin to firm leukocyte adhesion depends on MRP8/14 and TLR4 (Fig. 19).

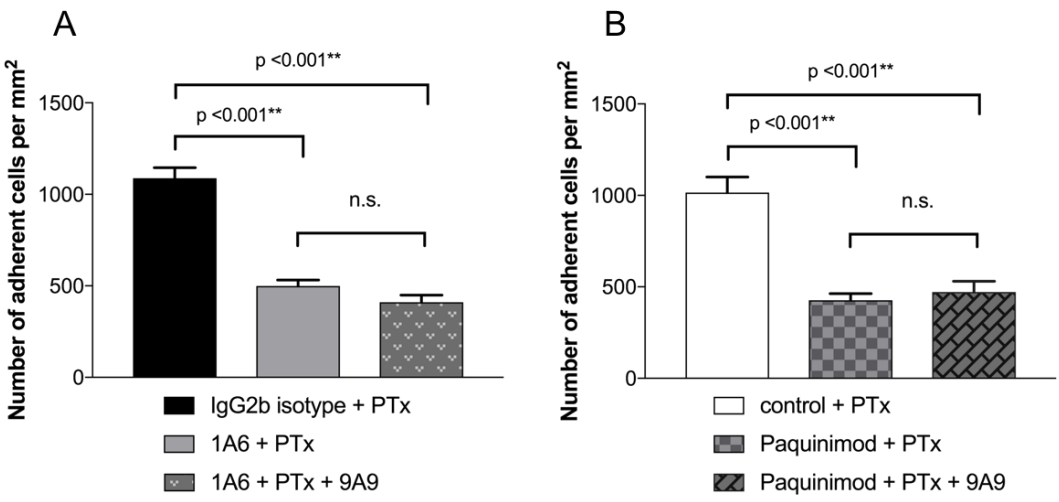

Fig. 19: Contribution of E-selectin to firm leukocyte adhesion depends on MRP8/14 and TLR4 *in vivo*. Numbers of adherent cells were assessed in TNF-α stimulated cremaster muscle venules of mice pre-treated with pertussis toxin and either anti TLR4 antibody alone or with anti E-selectin antibody (9A9) (A) or Paquinimod alone or with anti E-selectin antibody (9A9) (B). For multiple comparisons, one-way analysis of variance (ANOVA) was used with Tukey's *post-hoc* test. All mean values are presented ± SEM.

4.3 Leukocyte transmigration

Finally, we assessed the effect of MRP8/14 on leukocyte transmigration *in vivo.* To do this, mounted cremaster muscle tissue was fixed with PFA 4% and stained with Giemsa. Afterwards, quantification was performed by counting transmigrated cells in a defined field of view of at least 10 vessels with a diameter around 30µm and a length around 230µm.

In line with the findings on leukocyte adhesion, the number of transmigrated cells was significantly reduced in C57BL/6 wildtype mice pre-treated with pertussis toxin and either anti TLR4 antibody (1A6) (429 ± 33 cells in n= 59 venules) (Fig. 20 A) or Paquinimod (575 ± 37 cells in n= 50 venules) (Fig. 20 B) compared to mice that were pre-treated with pertussis toxin and control substances (1029 ± 84 cells in n= 43 venules in isotype IgG2b + PTx (Group A) and 1059 ± 74 cells in n= 41 venules in control + PTx (Group B).

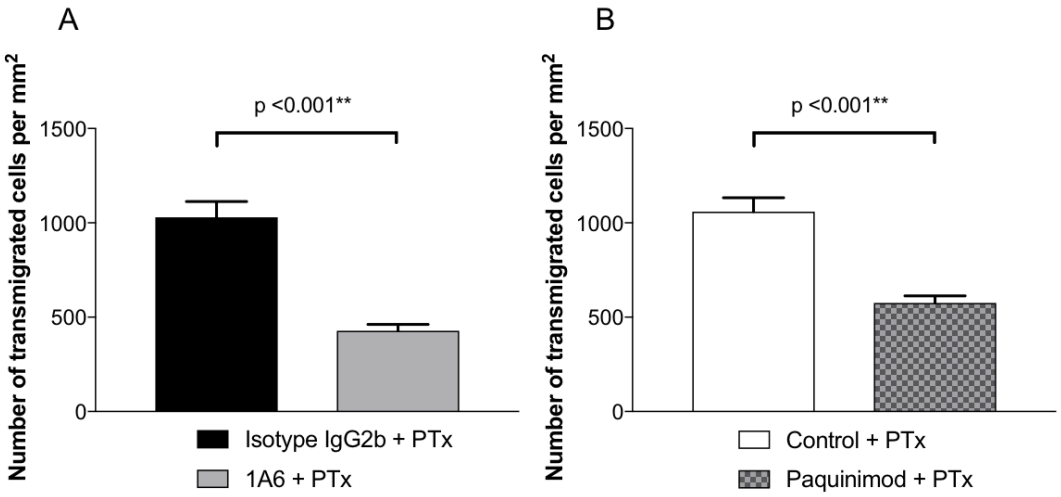

Fig. 20: Leukocyte transmigration is reduced if pertussis toxin was added to anti TLR4 antibody (1A6) or Paquinimod *in vivo*.

Numbers of transmigrated cells were assessed in TNF-α stimulated cremaster muscle tissue of mice that were pre-treated with pertussis toxin and either anti TLR4 antibody (A) or Paquinimod (B) and their control substances. Unpaired t-test was performed. All mean values are presented ± SEM.

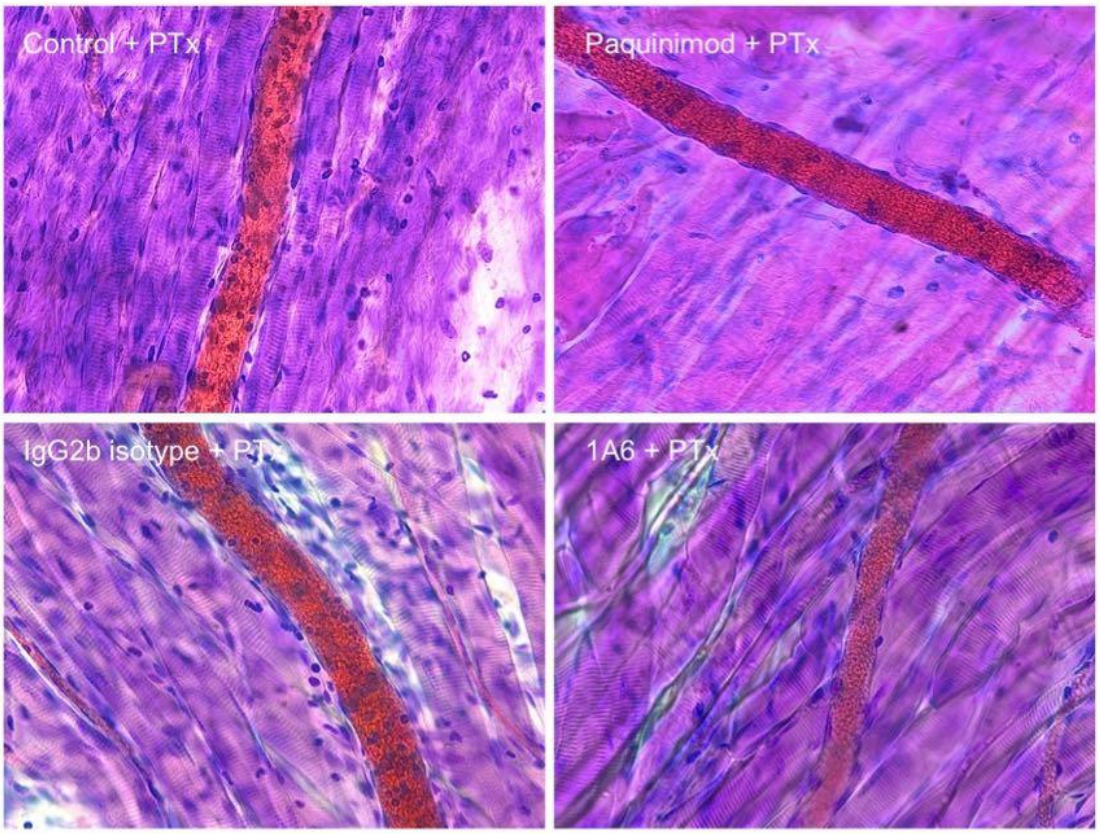

Fig. 21: Micrographs of transmigrated leukocytes after pre-treatment with pertussis toxin and either Paquinimod or rat anti-mouse TLR4 antibody (1A6) around TNF-α stimulated cremaster muscle venules.

Microscopic images illustrating Giemsa-stained cremaster muscle tissues. Transmigrated cells per mm^2 are indicated in a defined field of view using a x100 immersion objective. Pre-treatment with a mix of Paquinimod + PTx or rat anti-mouse TLR4 antibody 1A6 + PTx significantly reduced transmigration.

Also, by assessing different leukocyte populations by morphological criteria, we could show that the reduction in transmigration due to blockade of TLR4 receptor in the presence of pertussis toxin mostly affected polymorphonuclear neutrophils (PMN) (Fig. 22).

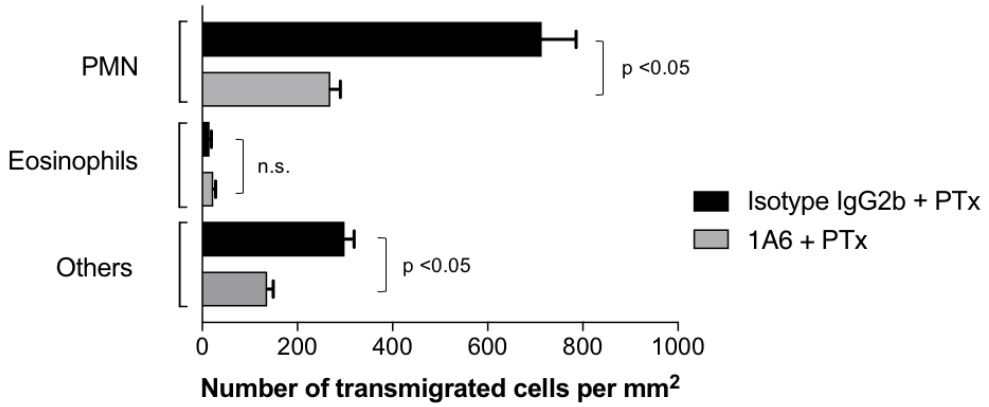

Fig. 22: Reduced transmigration of leukocytes in the presence of anti TLR4 antibody (1A6) with additional pertussis toxin mostly affects polymorphonuclear neutrophils *in vivo*.
Numbers of transmigrated cells were count in cremaster muscle tissue that was stimulated with TNF-α of mice that were pre-treated with pertussis toxin and IgG2b isotype or anti TLR4 antibody. Morphological subdivision showed main reduction of transmigration of polymorphonuclear cells (PMN) and others. Unpaired t-test was performed. All mean values are presented ± SEM.

This effect could also be detected in TNF-α stimulated cremaster muscle tissue of mice that were treated with pertussis toxin and Paquinimod, suggesting that MRP8/14 plays a major role in transmigration of the cells that release the protein upon stimulation with E-selectin (Fig. 23).

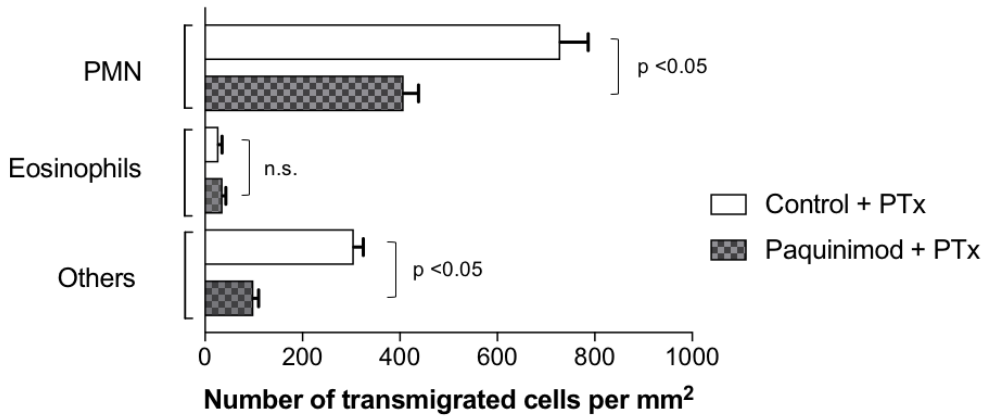

Fig. 23: Reduced transmigration of leukocytes in the presence of Paquinimod with additional pertussis toxin mostly affects polymorphonuclear neutrophils and others *in vivo*.
Numbers of transmigrated cells were count in TNF-α stimulated cremaster muscle tissue of mice that were pre-treated with pertussis toxin and PBS/10%DMSO or Pauquinimod. Morphological subdivision showed main reduction of transmigration of polymorphonuclear cells (PMN) and others. Unpaired t-test was performedAll mean values are presented ± SEM.

5 Discussion

5.1 Discussion of methods

5.1.1 Is intravital microscopy on murine cremaster muscle sufficient to address the aim of our study?

The present study had the aim to identify the functional importance of extracellular MRP8/14 in leukocyte recruitment. To demonstrate the *in vivo* relevance of previous *in vitro* findings, intravital microscopy on murine cremaster muscle tissue was performed.

Principally, the murine cremaster muscle preparation serves as a well-established model to answer different questions relaying on cell-cell interactions under physiological conditions (Baez, 1973; Sperandio *et al.*, 2006). It is an easily accessible, thin tissue that has a higher vessel density than mesenteric tissue in the mouse. In comparison to the murine air pouch or back chamber model, the cremaster muscle is not suitable for chronic experiments, since chronic exteriorization leads to tissue destruction.

Due to heterogeneity of blood flow and vascular architecture in the microcirculation, it was of great importance to assess hemodynamic and geometric parameters (Sperandio *et al.*, 2006). This enabled us to compare different treatments under similar flow conditions in equally large vessels.

Although these standards can also be pre-set in flow chambers, transfer of data from *in vitro* to *in vivo* is limited since flow chambers are coated with only a small selection of adhesion molecules (Sperandio *et al.*, 2006).

Records of postcapillary venules gained within two hours after inflammatory stimuli with TNF-α were analysed manually to gain information on leukocyte behaviour. Personal influence can thereby produce errors. In order to prevent such, most experiments were blinded and all records were analysed by the same person with interim controls by an experienced supervisor.

Scoring and evaluation of Giemsa-stained whole mount cremaster tissue was systematically performed using one consistent field of view, defined by a 250µm

diameter (Zeiss Axioskop 40 microscope with an oil immersion objective x100, 1.25NA). Sequential shift of the cremaster tissue allowed following the dichotomous branching of venules. This preserved accidental analysis of one vessel at two different times.

5.1.2 Is TNF-α a sufficient inducer of sterile inflammation?

Our aim was to show E-selectin dependent MRP8/14 release from neutrophils, inducing slow rolling and adhesion in vivo. Reasonably, E-selectin had to be primarily present in the area of interest. But, in comparison to P-selectin, which is stored in Weibel-Palade bodies of endothelial cells and can be upregulated within 10 minutes after minimal tissue trauma such as exteriorisation, E-selectin is almost undetectable under steady state conditions (Jung *et al.*, 1997). Its upregulation and endothelial expression requires previous upstream biosynthesis that can be induced by strong inflammatory mediators such as TNF-α. Keelan *et al.* elicited the maximum of E-selectin expression on the endothelial cells at approximately two hours after induction (Keelan *et al.*, 1994). So, we injected TNF-α two hours prior to investigation and left the freshly exteriorized cremaster muscle under physiological conditions with superfusion buffer for at least 10 minutes before observation and recording (Smith *et al.*, 2004b). The TNF-α induced inflammation model is a neutrophil-driven model. In comparison to monocytes, whose recruitment takes approximately 8 hours after inflammation, the recruitment of neutrophils is rapidly induced within 2 hours after TNF-α injection (Sperandio *et al.*, 2006).

5.2 Discussion of results

MRP8/14 plays an important role in the clinical context of acute and chronic inflammation. Elevated serum levels serve as clinical laboratory marker in order to observe disease activity as well as the patient's response to medical treatment in juvenile idiopathic arthritis in a very sensitive way (Anink *et al.*, 2015; Foell *et al.*, 2004). Also, concentration of MRP8/14 in feces strongly correlates with ongoing inflammation (Menees *et al.*, 2015) and recently, MRP8/14 became focus of interest on acute coronary syndromes (Averill *et al.*, 2012; Sakuma *et al.*, 2017). Nevertheless, all these clinical implications relay on the stability of MRP8/14 that is locally secreted by polymorphonuclear cells at sites of acute inflammation. Although this local accumulation of MRP8/14 is almost unique in comparison to CRP for example, the exact local function of extracellular MRP8/14 remained unknown.

With the present study, we demonstrate that MRP8/14 serves as a rapid activator of β_2-integrins by binding in an autocrine manner to Toll-like receptor 4 after being secreted by polymorphonuclear cells upon E-selectin dependent activation. Blocking TLR-4 binding or inhibiting a distinctive binding site of MRP8/14 *via* Paquinimod markedly increased leukocyte rolling velocity and significantly reduced leukocyte adhesion as well as transmigration into the inflamed tissue.

5.2.1 The role of MRP8/14 in neutrophil slow rolling and adhesion *in vivo*

In our neutrophil driven, TNF-α induced inflammation model, slow rolling is known to result from inside-out signalling, initiated rather by E- than by P-selectin expressed by endothelial cells (Zarbock *et al.*, 2012). In comparison to trauma-induced inflammation where P-selectin is the main receptor inducing neutrophil rolling with a velocity of approximately 20µm/s, TNF-α induced inflammation model leads to an upregulation of E-selectin that serves as binding partner for PSGL-1 on polymorphonuclear cells. Thus, inside-out signalling leads to rapid activation and extension of the integrin LFA-1 that in turn regulates and slows down rolling velocity (Kunkel *et al.*, 2000; Kuwano *et al.*, 2010; Zarbock *et al.*, 2011). Our previous *in vitro* studies revealed, that E-selectin-PSGL-1 binding also is the strong-

est inducer of MRP8/14 secretion into the extracellular environment. So, we suggested, that this rapid integrin activation by inside-out signalling includes secretion and binding of MRP8/14 in an autocrine fashion to TLR4, expressed on the PMN, resulting in slow rolling and adhesion (Ehrchen *et al.*, 2009; Vogl *et al.*, 2007). Besides the question of the exact secretion modus of MRP8/14 upon E-selectin, our *in vitro* observations on the interplay of MRP8/14 and TLR4 led to two important questions in the context of neutrophil recruitment, that have only been partially answered by *in vitro* experiments:

The first question to answer would be, whether blocking of either extracellular MRP8/14 by Paquinimod or its counter receptor TLR4 by antibody has any effect on rolling velocity or adhesion of leukocytes *in vivo*.

Therefore, we transferred our previous results from *in vitro* flow chamber experiments to the TNF-α induced sterile inflammation model in murine cremaster muscle venules. By blocking either MRP8/14 or TLR4, we could demonstrate that rolling velocity was significantly increased by approximately 50% in both groups. Thus, our results support our hypothesis that E-selectin dependent MRP8/14 release and binding to TLR4 is mandatory for induction of slow rolling. Since rolling velocity increased to the same level in each group, we could also highlight the important role of MRP8/14 as autocrine binding partner for TLR4.

Further, we could demonstrate that TLR4 plays an important role as rapid inducer of the β_2-integrin LFA-1, which underlines a new functional aspect of Toll-like receptors that were mainly known to transduce signals from outside leading to intracellular regulation of transcriptional factors and thereby orchestrating inflammatory responses such as cytokine production, proliferation or cell death (Kawai *et al.*, 2010). In 2014, Chung *et al.* could show that out of the TLR family, TLR2 and TLR5 mediate rapid activation of β_2-integrins in leukocyte adhesion to immobilized ICAM-1 and fibronectin, suggesting that TLR at least could facilitate adhesion of leukocytes during their recruitment (Chung *et al.*, 2014). Similar to this group, we could detect that TLR4 is involved in leukocyte recruitment, mediating LFA-1 activation. Further, we could show, that stimulation of the extracellular activation loop by MRP8/14 binding to TLR4, downstream signalling involves myD88 and consecutively Rap1-GTP activation that was previously described by Stadtmann *et al.* (Stadtmann *et al.*, 2011).

Of note, the affinity and avidity of β_2-integrins can also be affected by leukocytes sensing chemokines on inflamed endothelium (Zarbock *et al.*, 2012). In contrast to E-selectin mediated β_2-integrin activation via PSGL-1 (Kuwano *et al.*, 2010) and our described autocrine loop of MRP8/14, chemokines bind to G-protein-coupled receptors (chemokine receptors) on leukocytes leading to intracellular signalling inducing firm arrest. The group of Smith and Kuwano postulated an overlapping and partially dependent interplay of E-selectin and chemokine induced β_2-integrin activation that are needed for firm leukocyte adhesion (Kuwano *et al.*, 2010; Smith *et al.*, 2004a). Although little is known about the precise intracellular chemokine receptor dependent signalling pathway, Fan *et al.* assume a final common path and connection of chemokine- and E-selectin-dependent signalling which channels its Rap1-activation (Fan *et al.*, 2015). Bearing in mind this synergistic effect, we could not identify any significant decrease of adhesion if the heterodimer itself or its binding partner TLR4 were blocked. But upon concomitant injection of pertussis toxin, that blocks $G_{\alpha i}$-protein-coupled chemokine receptors due to ADP-ribosylation of the $G_{\alpha i}$ protein, the number of adherent PMNs decreased significantly in comparison to controls. As expected, additional blocking of E-selectin did not reduce leukocyte adhesion any further. Hence, we could not only confirm that chemokine and E-selectin synergistically induce inside-out signalling for firm adhesion (Smith *et al.*, 2004a). We also extended these findings by showing that MRP8/14 is a relevant player in mediating E-selectin triggered LFA-1 activation for adhesion *in vivo.*

The second question finally would be whether extracellular MRP8/14 exerts any effects on the microvascular environment influencing slow rolling and adhesion that could not be detected by our *in vitro* findings. Viemann *et al.* demonstrated binding of MRP8/14 on activated human mammary epithelial cells (HMEC), inducing severe reduction of endothelial monolayer integrity, thrombogenic responses and last but not least increased gene transcription of pro-inflammatory chemokines and adhesion molecules such as VCAM-1 and ICAM-1 that could potentially affect leukocyte rolling and adhesion, as well as transmigration due to overall higher surface representation of integrin ligands (Viemann *et al.*, 2005). Also, RAGE was discussed as potential protein receptor for MRP8/14 on endothelial cells as well as MRP8/14 was shown to bind to sulfated glycosaminoglycan structures on HMEC (Robinson *et al.*, 2002). According to the group of Eue *et al.*,

this binding can be triggered by LPS or TNF-α, that is in turn also inducer of sterile inflammation in our experiments (Eue *et al.*, 2002). Our *in vitro* results speak indirectly against the role of RAGE as relevant MRP8/14 ligand since MRP8/14 surface binding only increased in the presence of TLR4. But due to the fact, that we could not fully exclude such additional effect *in vivo,* further investigations will be needed to clarify this issue.

Importantly, the β_2-integrin activation that is dependent on MRP8/14 is restricted to myeloid cells since MRP8/14 is only found in neutrophils and to a smaller extend in monocytes (5%). As it was shown previously by Frosch *et al.*, circulating monocytes actively release MRP8/14 under inflammatory conditions, such as by TNF-α stimulated HUVECs (Frosch *et al.*, 2000). But one has to keep in mind, that stimulation with TNF-α was performed up to 16 hours in comparison to 2 hours in our model, that represents a neutrophil driven acute local inflammation at 2 hours after TNF-α injection. Also, in our *in vitro* studies, we found small levels of MRP8/14 in serum of mice that were PMN depleted, indicating that circulating monocytes potentially confound the release of MRP8/14.

In synopsis with our results it still remains unclear, whether secreted MRP8/14 is able to serve as direct paracrine activator of other PMNs that did not have contact with E-selectin in order to induce their own MRP8/14 release. However, local biological activity of MRP8/14 heterodimer is restricted by tetramer formation in presence of calcium, so paracrine function seems unlikely in this setting (Vogl *et al.*, 2018).

5.2.2 The role of MRP8/14 in neutrophil transmigration *in vivo*

Transmigration is the result of a multi-step process that relays on outside-in signalling events during neutrophil adhesion and crawling. In comparison to rapid β_2-integrin activation that is mandatory for neutrophil adhesion and mainly driven by LFA-1, crawling depends on Mac-1, another β_2-integrin (Phillipson *et al.*, 2006). Mac-1 binds to ICAM-1 and other ligands and directs intraluminal crawling perpendicular to the blood flow. Human MRP14 was reported to act as an indirect affinity regulator of Mac-1 due to increased receptor recognition on neutrophils. Newton *et al.* hereby postulated an increase of Mac-1 affinity that is MRP14 and G-protein dependent (Newton *et al.*, 1998). Static *in vitro* experiments clearly demonstrate limitations to clarify this and further investigations are needed to elucidate a potential effect of MRP8/14 on other integrins than LFA-1 in the pathophysiological circumstance of an acute inflammation.

Integrin-integrin ligand binding upon leukocyte adhesion does not only affect cytoskeletal reorganization and polarization within the PMN by formation of protrusive lamellopodia at the leading edge and contractile uropods at the posterior pole in order to enable crawling (Nourshargh *et al.*, 2014). Crawling also exerts effects on vascular permeability by inducing clustering of endothelial ICAM-1 and an increase in intracellular Ca^{2+} in endothelial cells, that leads to actin cytoskeletal rearrangements. In addition, functional proteins such as VE-Cadherin are instrumental in destabilization of junctions which facilitates transendothelial migration (Vestweber, 2012). In this context, concomitant effects of MRP8/14 on endothelial cells might be relevant in the discussion of the role of MRP8/14 in leukocyte transmigration. Recently, Wang *et al.* described the heterodimeric protein binding towards TLR4 and RAGE as putative receptors on human umbilical endothelial cells (HUVEC), promoting disorganization of endothelial F-actin and zona occludens protein-1 (ZO-1) (Wang *et al.*, 2014a). Also, Viemann *et al.* revealed further importance of MRP8/14 in caspase-dependent endothelial cell death of HMEC-1 (Viemann *et al.*, 2007). As a consequence, endothelial stability is reduced and enables transendothelial migration that could partially explain our results. Hence, we postulate that the reduction of transmigrated PMNs can be due to endothelium but also neutrophil dependent mechanisms exerted by MRP8/14.

On monocytes, intracellular MRP8/14 might also play an important role in outside-in signalling. A study of Vogl *et al.* demonstrated, that intracellular MRP8/14 has a direct impact on microtubule reorganization during transendothelial migration of monocytes. Thereby, the MRP8 subunit functions as direct interacting surface with tubulin filaments. But interestingly, this interaction is inhibited once MRP14 is phosphorylated via p38 MAPK, leading to the conclusion, that MRP14 might be the regulatory subunit. Further, phosphorylation of MRP14 competes with binding to MRP8 in a calcium-dependent manner (Vogl *et al.*, 2004). Conformational changes due to calcium were discussed intensively, direct effects of calcium onto tubulin were excluded. The same group also observed a diminished number of transmigrated granulocytes into skin lesions of MRP14 knock out mice and attributed this observation to the lower amount of tubulin expression in the same population (Vogl *et al.*, 2004). Further, a descriptive study on imaging of leukocyte recruitment showed a significant delay of MRP14 $^{-/-}$ monocytes in migration (Gran *et al.*, 2018). If these results on monocyte transmigration could only be attributed to the intracellular role of MPR8/14 or also include intracellular functions of MRP8/14 during the early recruitment steps, remains subject to further research. We could show that blocking of extracellular MRP8/14 or TLR4 did have a significant impact also on transmigration. But it remains unknown if our observations on reduced number of transmigrated PMNs relay on the effect of extracellular MRP8/14 only and if direct effects of MRP8/14 on endothelial cells as well as its intracellular interaction with tubulin polymerization might contribute to successful transmigration. Additional studies are warranted to elucidate the impact of MRP8/14 in transmigration of PMNs during inflammatory responses.

5.3 Discussion in the context of existing literature

Over the past decade, various functions of MRP8/14 have been revealed. However, the characterization of the heterodimer and its subunits remains complex and incompletely understood, especially concerning its role in inflammation (Pruenster *et al.*, 2016; Wang *et al.*, 2018). The new findings of our study support the well shared concept that MRP8/14 acts as pro-inflammatory molecule due to its key role as promoter of leukocyte recruitment. Based on our findings further investigations were made to elucidate MRP8/14 as potential therapeutic target for immunomodulatory therapies.

Nevertheless, some relevant aspects of other studies on MRP8/14 need to be discussed in synopsis with our results. Assuming that the heterodimer MRP8/14 is the most abundant cytoplasmic protein present in neutrophils, the diverse functional roles of its subunits should not be neglected. Previous studies focussed on characterisation of either MRP8 or MRP14, their conclusions were difficult to interpret in a physiological context. First of all, one has to keep in mind that homology of DNA of human MRP8 resembles only at about 69% to the murine MRP8, which might lead to misinterpretation in translational research (Passey *et al.*, 1999b). Furthermore, chemotactic properties of MRP8 have been discussed extensively. In 1996, Cornish *et al.* observed an elevated recruitment of mononuclear cells as a consequence of chemotaxis after intradermal injection of MRP8 (Cornish *et al.*, 1996). The equivalent effect on neutrophils was described after injection of MRP8, MRP14 or the heterodimer into a murine air pouch (Ryckman *et al.*, 2003a). These findings provided finally a basic principle that MRP8/14 might be related to PMN migration. Complementary, Vogl *et al.* revealed that intracellular Ca^{2+}-dependent phosphorylation of MRP14 influences binding capacities of the MRP8 subunit to microfilaments and thereby possibly inhibiting cells migration (Vogl *et al.*, 2004). Using a functional double knock out model ($MRP14^{-/-}$) helped to evaluate and distinguish the function of MRP8/14 on signal transduction as well as on cellular interaction. New insights by the same group showed, that under chronic exposure to TNF-α keratinocytes and immunocytes of $MRP14^{-/-}$ reveal higher MRP8 gene expression and secretion leading to uncontrollable inflammation (Vogl *et al.*, 2018). Here, the authors conclude that if MRP14 is genetically absent, the homodimer MRP8 will have no counterpart that regulates

and possibly autoinhibits its pro-inflammatory function. On the other hand, it was shown that MRP8, as active component of the heterodimer, binds to TLR4 and thereby induces higher TNF-α expression in macrophages, thereby maintaining a pro-inflammatory vicious circle (Vogl *et al.*, 2007). MRP8 was also considered as a sort of inflammation pre-conditioning protein, inducing in a dose-dependent manner a certain tolerance against endotoxins such as LPS in macrophages (Coveney *et al.*, 2015). In line with these findings, Austermann *et al.* presented results on MRP8/14 priming phagocytes *via* TLR4 and inducing hyporesponsivness even in sterile inflammation (Austermann *et al.*, 2014). Previously, it was demonstrated by Schenten *et al.* that within the heterodimeric protein, that will be secreted from neutrophil-like HL-60 cells and purified neutrophils upon PMA and fMLF stimulation, phosphorylated MRP14 is more important for TLR4 signalling leading to cytokine secretion than unphosphorylated MRP14 (Schenten *et al.*, 2018).

Now taking all these previous studies together, one could see that MRP8/14 plays a critical role in the balance of an inflammatory response of the innate immune system. Subsequently it is not surprising that in excessive and uncontrollable inflammations such as rheumatoid arthritis or psoriasis, higher serum levels of MRP8/14 can be found. Our results define one molecular mechanism that could potentially lead to these serum levels, since autocrine binding of MRP8/14 to TLR4 does not only activate the same neutrophil to roll slower and adhere but might also potentiate the recruitment of other immune cells present in the same vessel. In 2018, Vogl *et al.* could show that the pro-inflammatory function of MRP8/14 at local sites will be abolished by Ca^{2+}- induced tetramer formation of two MRP8/14 heterodimers. In consequence, TLR4/MD2 binding site of the MRP8 subunit will be covered due to conformational change, leading to a loss of biological activity. This tetramerisation is triggered by low pH which is typically present at sites of inflammation. The autoinhibition is only present for heterodimers and has very little relevance for homodimers due to their inability to form tetramers (Vogl *et al.*, 2018). With respect to this study, one could assume that in inflammatory disorders there exists an imbalance of MRP8/14 heterodimer – tetramer formation, possibly due to higher transcription of both subunits that exert themselves several other effects.

6 Concluding remarks

In summary, this study highlights the role of the endogenous protein MRP8/14 as amplifier of innate immune response and its role as pro-inflammatory damage associated molecular pattern.

Our results identify an extracellular autocrine activation pathway that is critical for leukocyte slow rolling and adhesion *in vivo.* Upon E-selectin-PSGL1 interaction neutrophils secrete MRP8/14 that binds to TLR4. In turn, a rapid intracellular signalling cascade is initiated, followed by β_2-integrin activation. Blocking either MRP8/14's binding site to TLR4 with Paquinimod or the receptor itself led to a significant increase in leukocyte rolling velocity *in vivo*. With addition of Pertussis toxin to the experiments, we could show that this pathway is of critical importance for firm arrest of PMN and finally promotes transendothelial migration.

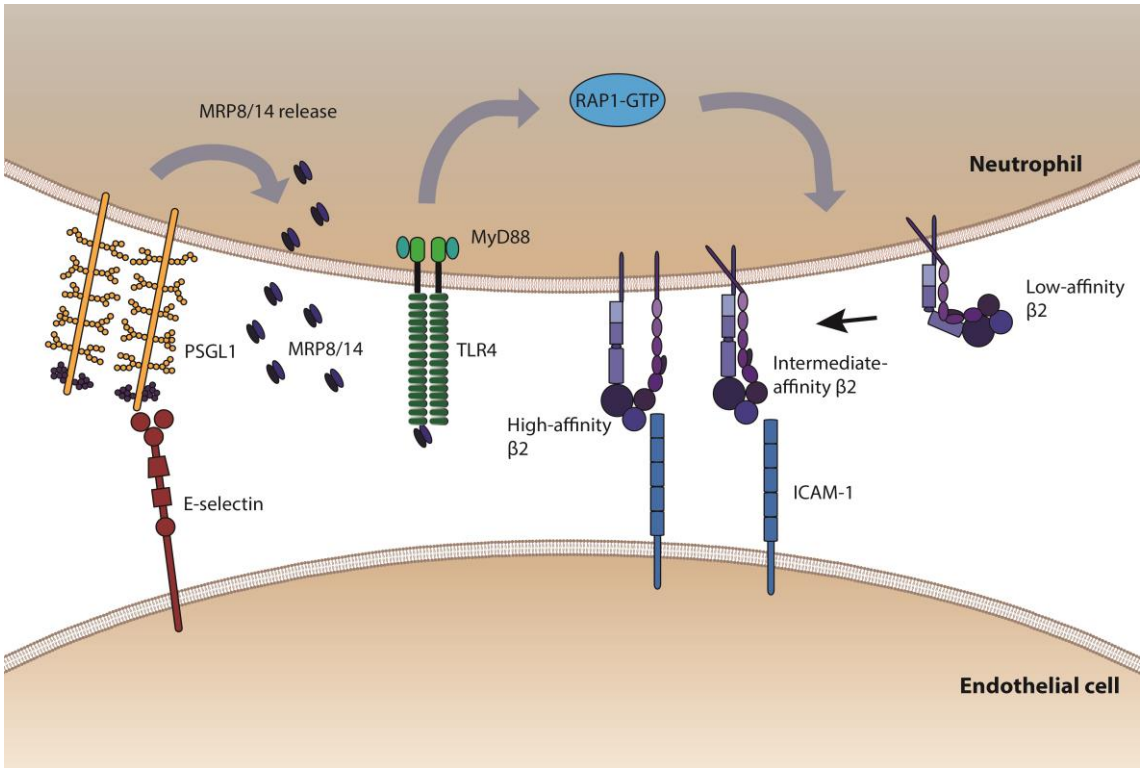

Fig. 24: Schematic illustration of the role of MRP8/14 in leukocyte recruitment *in vivo* (Pruenster *et al.*, 2015).
Rolling on inflamed endothelium, polymorphonuclear cells trigger E-selectin-PSGL-1 dependent release of the heterodimeric protein MRP8/14 that binds extracellularly to TLR in an autocrine manner. This activation loop signals *via* MyD88 and Rap1-GTP a conformational change of β_2-integrin LFA-1, finally leading to slow rolling and firm leukocyte adhesion.

Nevertheless, the protein's effects on its microenvironment such as endothelial cells and other circulating leukocytes as well as its intracellular role still remain unclear, but might be important to clarify the exact role of MRP8/14 during the inflammatory response. Further investigations will be necessary to target MRP8/14 therapeutically as treatment options in acute and chronic inflammatory disorders with unwanted recruitment of innate immune cells.

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