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CXCR1/2 Inhibition in Neutrophil Recruitment

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1 Introduction

Neutrophil granulocytes, the major group of leukocytes in healthy human blood, are terminally differentiated cells of myeloid lineage. Histologically, they can be identified based on their "neutral" - as opposed to eosinophilic or basophilic - cytoplasm, their segmented nucleus, as well as cytoplasmatic granules. They play a key role in innate immunity, and perform modulatory functions in adaptive immunity. Due to this central role in immunity, neutrophils express a wide array of receptors such as G-protein coupled receptors (GPCR), Toll-like receptors (TLR), Leukotriene B4 receptor, immunoglobulin Fc receptors and potent enzymes such as neutrophil elastase (NE), myeloperoxidase (MPO) and matrix metalloprotease 9 (MMP9). Important functions include shaping immune responses, mediating tissue injury and repair, and killing microbes. Several main mechanisms for killing pathogens have been established, including phagocytosis, degranulation and neutrophil extracellular traps (NETs)[1,2]. In some (auto-)immune diseases and conditions however, these powerful tools are involved in the destruction of healthy tissues[3,4]. Additionally, their complex role in cancer pathophysiology is beginning to be elucidated[5]. However, targeting neutrophils in disease has to be balanced with their important physiological roles[6].

In a classic acute inflammation setting, tissue injury directly and indirectly releases a plethora of cytokines, including tumor necrosis factor alpha (TNF-α), chemokines and interleukins through tissue-resident macrophages and other resident cells. Circulating neutrophils and endothelial cells are then activated by elevated concentrations of these chemokines and neutrophils are recruited into tissue where they exert their effector functions, e.g. phagocytosis of the bacteria, attraction of other immune cells or induction of tissue repair. The extravasation or recruitment is a hallmark feature of neutrophils and due to its tight regulation represents a potential target for therapeutic inhibition[7].

1.1 Recruitment Cascade

The recruitment of neutrophils from the blood consists of tethering, rolling, adhesion and transmigration and is visualized in Figure 1. Circulating neutrophils are tethered to the endothelium and begin to roll. This is mediated through selectins on the endothelium (such as E-selectin and P-selectin) and selectin ligands on the neutrophil (such as P-selectin glycoprotein ligand 1/PSGL1, E-selectin ligand 1/ESL1 or cluster of differentiation 44/CD44). The next step is adhesion, where the neutrophil sticks to the endothelium. This process is mediated through the interaction

of integrin ligands on the endothelium (intercellular adhesion molecule 1/ICAM-1 and vascular cell adhesion molecule 1/VCAM-1) and β_2 and β_1 integrins on the neutrophil, respectively. The adhesion is strengthened by conformational change of integrins on the neutrophil and the neutrophil begins crawling in order to search for a suitable spot to transmigrate through the endothelium, either between two endothelial cells, i.e. paracellular route, or through one endothelial cell, i.e. transcellular route. Important molecules required here are neutrophil elastase (NE), very late activation leukocyte antigen 3/VLA-3 and very late activation leukocyte antigen 6/VLA-6[8].

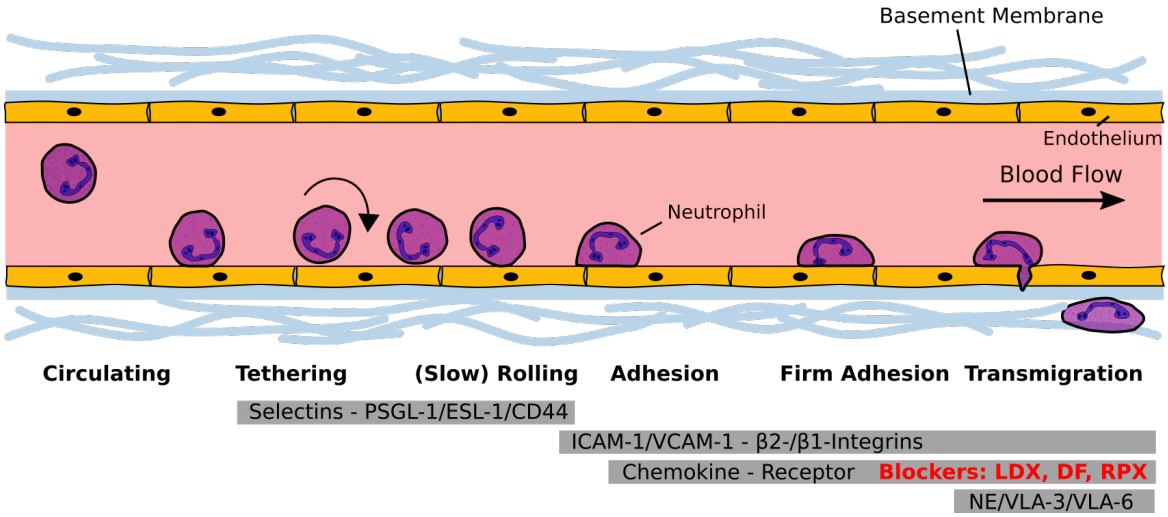

Figure 1: Neutrophil recruitment cascade and involved molecules. Here, the phases of neutrophil recruitment from vessel to tissue including the involved receptors and ligands are visualized. The chemokine receptor inhibitors Ladarixin (LDX), DF2755A (DF) and Reparixin (RPX) are marked in red in the picture. For a detailed explanation, please consult text above.

1.2 CXCR1/2 and Their Chemokines in Neutrophil Recruitment

C-X-C chemokine receptors 1 and 2 (CXCR1 and CXCR2) are G-protein coupled receptors. They are expressed on various immune and non-immune cells including neutrophils, macrophages and endothelial cells. The ligands of CXCR1/2 are C-X-C motif chemokines with an additional glutamic acid-leucine-arginine (ELR) motif. High affinity ligands in humans for CXCR1 are CXCL6 and CXCL8, and for CXCR2 CXCL1-3 and 5-8[9]. In mice and rats, the presence of CXCL8 has not been described so far, so its functional equivalent CXCL1 (also called keratinocyte-derived chemokine/KC) is used instead[10]. In the following section, we discuss the role of

these receptors in neutrophils, and particularily their effects on neutrophil recruitment.

As is typical of GPCRs, upon ligand binding, the α subunit is activated while the $\beta\gamma$ subunits dissociate from the complex. This activation of the $\beta\gamma$ subunit alone is sufficient for triggering chemotaxis amongst other critical neutrophil functions[11].

One well-studied prerequisite for the induction of adhesion through CXCR1/2 in neutrophils is the activation of integrins[12]. Integrins are heterodimeric receptors expressed in many immune cells including neutrophils. One class of integrins expressed in neutrophils are β_2 integrins, which includes for example leukocyte functionary antigen 1 (LFA-1; Integrin $\alpha_L\beta_2$) and macrophage-1 antigen (Mac-1; Integrin $\alpha_M\beta_2$). It has been established that binding of LFA-1 to intercellular adhesion molecule 1 (ICAM-1) is required for neutrophil arrest and adhesion[13].

β_2 integrins exist in three different conformations: low, intermediate and high affinity. Upon activation, the integrin switches from low to intermediate affinity state and the bond between integrin and ligand is strengthened. This change happens as the neutrophil rolls, so that it rolls slower until it finally arrests[13]. β_2 integrins can be activated through the activation of chemokine receptors or through the interaction of E-selectin and PSGL-1 during the rolling stage of neutrophil recruitment in a process called inside-out signaling[14,15]. Inside-out signaling for integrin activation requires at least Src and Syk tyrosine kinases[16,17]. β_2 integrins also serve as signaling receptors (so-called "outside-in" signaling) which primes the cell for subsequent adhesional strengthening and intraluminal crawling. This phase follows arrest and is characterized by neutrophils crawling along the endothelium in order to find a suitable spot to cross it. Here, key molecular players include the β_2 integrin Mac-1 and the integrin ligand ICAM-1[18].

In intraluminal crawling, neutrophils are guided especially by chemokine dependent mechanisms, before they finally cross the endothelium in a process called transmigration. In transmigration, CXCL2 is presented by endothelial cells to neutrophils in order to guide them through the vascular wall and into the tissue[19]. Once in the tissue, the cells continue their migration towards the source of injury.

In fact, one of the first described roles of CXCR1/2 in neutrophils was chemotaxis[20,21]. This means neutrophils can sense a gradient of chemokines and actively migrate towards a higher concentration. Chemokine receptor inhibitors are usually selected for their ability to inhibit this chemotaxis behavior[22,23]. For CXCL8, this chemotaxis is mostly mediated through CXCR1[24]. Required proteins and factors for chemotaxis include Ca^{2+}, PI3K, Janus kinase 3 (JAK3), and tyrosine kinases Cbl and Akt[25–28].

Another effect mediated through CXCR1 is the production of reactive oxygen species (ROS), which serves as an attractant to other immune cells, as well as a defense against pathogens[2,29].

The activation of CXCR1/2 also leads to changes in Ca^{2+} levels. Upon G protein activation and $\beta\gamma$ dissociation, phospholipase C is activated which cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositoltriphosphate (IP_3). IP_3 in turn activates IP_3-sensitive receptors on the endoplasmatic reticulum (ER) which release stored Ca^{2+} into the cytoplasm. Through increases in cytoplasmic Ca^{2+} levels calcium channels on the plasma membrane open and allow extracellular Ca^{2+} to flow in. This is also called store-operated calcium entry (SOCE)[30]. Calcium signaling in neutrophils has been demonstrated to be essential for β_2 integrin activation, migration, as well as phagocytosis, ROS production and degranulation. Important intermediate players include Orai1, Stromal interaction molecule 1 and 2 (STIM1, STIM2), and other non-specific cation channels such as transient receptor potential (TRP) and P2X channels[31].

After ligand binding, the receptors CXCR1 and 2 are endocytosed (internalized), cleared of their ligand and can be "recycled" back to the membrane. The importance of this process, which is dependent on G protein coupled receptor kinase (GRK), β-arrestin and AP-2 adapter protein[32,33], for neutrophil recruitment as a whole is not yet well understood. However it seems to be independent of G protein signaling, occur only at higher chemokine concentrations - 10-fold higher than required for inducing a Ca^{2+} response -, and thus not required for physiological neutrophil chemotaxis[34]. For CXCR2 but not for CXCR1, different ligands have different profiles of cAMP generation, β-arrestin recruitment, and receptor internalization[35].

The chemokines CXCL8 and CXCL1

The chemokine CXCL8, more commonly known as interleukin 8 (IL-8), is a variable-length protein that can be secreted by many cells, including monocytes, macrophages, fibroblasts, hepatocytes, epithelial and endothelial cells[20]. It is synthesized as a 99 amino acid long precursor and then cleaved or truncated depending on cell type and stimulus[36]. Many of the aforementioned cells secrete CXCL8 in response to stimulation with IL-1β or TNF-α. CXCL8 is the prototypical chemokine of CXCR1 and 2, in fact the previous names of these receptors (IL-8 receptor 1 and 2) reflect exactly this relationship[29].

CXCL8's main physiological role is as a chemoattractant for neutrophils in order to get to the site of inflammation through activation of CXCR1/2 and Duffy antigen receptor[24,37]. Intradermal application of CXCL8 leads to local inflammation, edema

and neutrophil accumulation at the site of injection. Intravascular injection of CXCL8 leads to severe granulocytopenia, followed by granulocytosis[38]. CXCL8 and other chemokines are mostly presented to neutrophils on endothelial cells bound to heparan sulfates[2].

A CXCL8 homologue has not yet been discovered in mice. Therefore, for modeling of the CXCR1/2-CXCL8 pathway in mice other ligands of CXCR2 such as CXCL1/KC or CXCL2 (also called macrophage inflammatory peptide 2 or MIP-2) are used[39,40]. However, in rats and mice it has been demonstrated that intravascular application of recombinant human CXCL8 also leads to arrest of rolling neutrophils[41,42].

In vitro and *in vivo*, local application of CXCL1 and to a lesser extent CXCL8 leads to an increase in adhesion of neutrophils to the vessel walls and accumulation of neutrophils in tissue[42]. $G_{\alpha i2}$ is required for this arrest, which is why this effect can be suppressed using Pertussis toxin[43]. As mentioned, a molecular correlate of this observation is the activation of β_2 integrins.

In summary, CXC chemokine receptors 1 and 2 are G-protein coupled receptors which bind various CXC chemokines, where the promiment examples are CXCL8 in humans and CXCL1 (KC) in mice. Important functions of these receptors on a cellular level include chemotaxis, adhesion, transmigration and on a molecular/signaling level Ca^{2+} influx, ROS production and β_2 integrins activation. In response to high chemokine concentrations, the receptors are endocytosed, cleared of the ligand and recycled back on to the plasma membrane.

1.3 Pathophysiology of the CXCL8-CXCR1/2 axis

Lung diseases

In classic acute bacterial or fungal infections, e.g. with *Candida albicans* or *Escherichia coli*, production of CXCL8 and subsequent activation of CXCR1/2 leads to leukocyte recruitment and activation, which is a prerequisite for killing microbes at the infection site[44]. This means inhibition of these receptors would not be beneficial, but hinder healing. One exception is *Klebsiella pneumoniae* pneumonia, where the opposite is true: disease is improved upon CXCR1/2 inhibition as evidenced by lower airway neutrophil count and lower lung myeloperoxidase levels, among others[45].

In **acute lung injury** (ALI) and its late stage called acute respiratory distress syndrome (ARDS), loss of endothelial and epithelial barriers leads to lung edema and impaired gas exchange, while influx of immune cells especially neutrophils and

release of cytokines further damage lung tissue. Even with treatment, ALI may progress to ARDS and/or irreversible lung fibrosis. With a mortality of 29-42% for ARDS, it is evident that there are is an unmet need in the treatment of this condition. Currently ALI/ARDS patients receive supportive treatment, mainly in the form of mechanical ventilation[46]. The implication of neutrophils and CXCL8 in the pathophysiology of this disease complex have been clearly described[47]. Some research using inhibitors has been done in experimental animal models of this disease. In mice, CXCR2 blocking antibodies or CXCR2 genetic loss attenuated sterile ALI severity[48,49]. Similarily, inflammatory activity was diminished in murine cigarette smoke and organic barn dust induced ALI after small molecule and peptide CXCR1/2 inhibitor treatment[50,51].

Another lung disease with neutrophil and CXCR1/2 involvement is **chronic obstructive pulmonary disease** (COPD). It is a progressive lung disease characterized by an overshooting immune reaction leading to mucus hypersecretion, obstructive bronchiolitis and emphysema. Current treatment (sympathomimetics, anticholinergics, inhaled corticosteroids) is only symptomatic and unable to stop progression of the disease[52]. CXCL8, its mRNA, and neutrophilic enzymes such as NE are present in the sputum of COPD patients, where higher levels correlate with higher disease severity[53,54]. *In vitro* inhibition of CXCR2 on neutrophils from COPD patients' sputum reduced their chemotaxis to sputum supernatant[55]. Multiple CXCR2 inhibitors were tested in clinical trials for COPD, including small molecule inhibitors and monoclonal anti-CXCL8 antibodies. All tested compounds led to improvements in markers such as dyspnea, sputum or blood neutrophils, but not in overall disease severity[56,57]. Danirixin slightly improved the occurrence of exacerbations and COPD scores, and is currently undergoing a dose ranging study[58–61]. Despite promising preclinical evidence however, inhibiting cytokines and specifically CXCL8 in COPD has so far only shown very limited clinical therapeutic effect[62].

Asthma is also a chronic progressive airway disease, characterized by hypersecretion, bronchospasm, edema and long-term airway remodeling. Attacks can be caused by a wide range of factors, the most common being allergen exposure and viral/bacterial infections. Airway obstruction in COPD and asthma can be quantified using the forced exhaled air volume in 1 second (FEV1). Treatment is similar to COPD and intervenes at two pathophysiological levels: bronchodilators (especially β sympathomimetics) for acute attacks and anti-inflammatory drugs (especially inhaled corticosteroids) for middle to long term prevention of attacks. Despite the fact some researchers argue for a role of neutrophils in the early phase of an attack and

neutrophil recruitment is seen in the late phase, their exact role in asthma remains elusive[63,64]. Similar to COPD, an increase of CXCL8 can be seen in the sputum before an asthma attack[44]. Clear evidence of the role of the CXCR1/2-CXCL8 axis in asthma so far is limited to hypersecretion and angiogenesis[64,65]. Clinical trials with small molecule inhibitors have been undertaken[66,67]. One inhibitor reduced neutrophil recruitment to the lung/sputum and lowered systemic absolute neutrophil count (ANC), however both compounds showed only little clinical benefit in terms of ameliorating FEV1 or preventing attacks/exacerbations[66,67].

Autoimmune diseases

The role of neutrophils and the CXCL8-CXCR1/2 axis including its inhibition are beginning to be unraveled for a variety of autoimmune diseases[6].

Type 1 diabetes (T1D) is an autoimmune disease, where the insulin producing beta islets of the pancreas are progressively destroyed, leading to an unchecked build-up of blood glucose and all its consequences. Pathophysiologically, immune cells including neutrophils and T cells attack and destroy these cells. Mounting evidence shows the CXCL8-CXCR1/2 axis plays an important role in the recruitment of neutrophils to the pancreatic tissue[68]. Traditionally, T1D is treated with lifelong parenteral insulin substitution. However, new immunomodulatory drugs, including CXCR1/2 inhibitors, are starting to emerge: treatment of mice with a CXCR2 inhibitor in an experimental T1D model attenuated neutrophil recruitment to the pancreas almost completely[69]. Reparixin was tested in mice and humans regarding the outcome of islet cell transplantions and was found to consistently improve it as evidenced by elevated C-peptide levels and lower insulin requirement[70].

Psoriasis is a chronic autoimmune disorder which manifests itself primarily on the skin, but may affect other organs such as joints. The skin inflammation is characterized by erythematosquamous plaques at typical localizations. Pathophysiologically, it is classified as a complex genetic deregulation of the immune system mediated by T cells. Well researched players include Th_{17} cells, and the interleukins IL-17 and IL-23. Currently, psoriasis is treated with topicals, phototherapy, and systemic immunomodulants like retinoids, methotrexate, cyclosporine A, and antibodies[71,72]. Even though CXCL8 and CXCR1/2 are not main areas of research[72], there is evidence they are present in psoriatic skin lesions and contribute to its pathogenesis[73,74]. CXCR2 seems to be over-expressed in these lesions, while CXCR1 does not seem to be detectable[75]. CXCL8 staining revealed some parts of skin lesions, as well as accumulating neutrophils to be positive[76]. However, the exact role of neutrophils and the CXCL8-CXCR1/2 axis, and its ther-

apeutic potential in psoriasis remains to be elucidated.

Rheumatoid arthritis (RA) is a chronic autoimmune disease which leads to painful deformation and destruction of joints. Key pathophysiological features include the so-called rheumatoid factor (autoantibodies) and an overproduction of TNFα, leading to persistent sterile inflammation of the synovia. Current treatment recommendations include non-steroidal anti-inflammatory drugs (NSAIDs) like ibuprofen, disease-modifying anti-rheumatic drugs (DMARDs) like methotrexate, and antibodies like anti-TNFα. Similar to psoriasis, its prevalence of 0.5-1% precludes a big interest in novel therapies[77]. The involvement of neutrophils and CXCL8 is well documented in literature: CXCL8 can be detected at high levels in serum, as well as synovial fluid of RA patients[78]. Mononuclear phagocytes from RA patients spontaneously produce CXCL8[79]. The synovial membrane and other structures in RA can be positively stained for CXCL8[80]. Neutrophils can be detected in synovial fluid very early, and it is thought they are a key player in orchestrating the immune response in RA through the production of various cytokines and ROS[81]. In arthritis models in mice[82,83] and rats[84,85] it was demonstrated that small molecule inhibition of CXCR1/2 leads to reduced disease activity markers, including PMN recruitment and hyperalgesia.

Ulcerative colitis (UC) is a chronic autoimmune disease of the intestinal mucosa, mainly the rectum and colon. The inflammation of the intestine generates symptoms such as bloody diarrhea, malabsorption and pain, usually in a biphasic manner (flare-up and remission). Discovered pathophysiological features include the relevance of innate lymphoid cells, T helper cells, IL-13 and IL-4 as drivers of the disease. Also, dysbiosis and TLR2 and 4 upregulation is seen, though it is unclear whether these are causes or consequences. Current treatment options include 5-aminosalicylates, corticosteroids, 5-mercaptopurine, anti-TNF antibodies, and – as ultima ratio – proctocolectomy[86]. Neutrophils, CXCL8 and its receptors, also seem to be involved in the pathogenesis of UC: depletion of PMN and inhibition of leukocyte adhesion in a rat model attenuated experimental UC, and in humans, CXCL8 and CXCR1/2 expression is increased in UC and can be correlated with different disease phases and severity[87]. Neutrophil infiltration is also one of the major criteria in two histological grading systems for UC[88]. In a CXCR2 knockout study in mice, Buanne and colleagues found that Cxcr2$^{-/-}$ mice were protected to a certain degree from experimental UC, as evidenced by reduced PMN infiltration, lower disease scores, improved histopathology, and lower MPO activity[89].

Malignancies

The role of the CXCL8-CXCR1/2 axis in malignancies has been well established. Tumors interact in many ways with the immune system, and induce angiogenesis to sustain growth[90]. Neutrophils and other immune cells have ambivalent roles in tumors[5].

While the overall role of neutrophils in malignancies is complex, tumor-associated neutrophils (TAN) can be broadly classified based on their anti-tumor (N1) or pro-tumor (N2) phenotypes. Though this phenotype often changes in the course of the disease, many reports indicate an overall pro-tumor (N2) role for neutrophils[5,6]. Specifically, it is thought that neutrophils are recruited to the tumor tissue and by releasing their enzymes and destroying the extracellular matrix (ECM), they pave the way for other cells to grow in this space[91]. CXCR2 knockout studies in mice provide evidence that at least for prostate[92], pancreatic[93], breast[94], colorectal[95] and lung cancer[96], absence of the receptor interferes with disease progression in terms of reduced tumor volume, and less angiogenesis. Studies using small molecule inhibitors instead of knockout animals are starting to emerge, for example inhibition of malignant melanoma cell growth[97,98], colon cancer cell metastasis formation[99] and prostate cancer cell growth and vascularization[100]. In Ras-driven cancers, inhibition of CXCL8 using an antibody attenuates their growth[101]. Overall, inhibition of the CXCL8-CXCR1/2 axis in malignancies seems to have a beneficial effect in clinical animal disease models.

Other diseases

Alcoholic steatohepatitis (ASH) is a sterile inflammation of the liver resulting from excessive long-term alcohol consumption. ASH usually manifests itself through liver failure, and if left untreated leads to cirrhosis and eventually end-stage liver disease and death. Pathophysiologically, chronic alcohol consumption severely disturbs the liver fatty acid, ROS and enzyme metabolism. Treatment is usually symptomatic, and abstinence does not guarantee remission of the disease. Immunomodulators like corticosteroids and anti-TNFα drugs have been tested with mixed results[102]. Chemokines which seem to be involved in the recruitment of neutrophils to liver tissue include CXCL2 and to a lesser extent CXCL1[103]. To date, evidence of the direct involvement of CXCL8 in ASH is lacking, however patients might still profit from CXCR1/2 inhibition, since these receptors also bind CXCL1 and 2. In fact, in a murine ASH model CXCR1/2 pepducins were able to stop progression as well as reverse disease progression to some extent[104].

Sepsis is a clinical condition characterized by acute organ dysfunction and infection of the blood, with mortality rates around 20-50%. All organs can be affected, though lung and cardiovascular system play a major role early in the course, while at later time points brain and kidney injury are predominant. Septic shock is a possible and severe complication. Pathophysiologically, early pro-inflammatory pathways lead to collateral damage to the organs, while late anti-inflammatory signals preclude an increased susceptibility to secondary infections. Treatment currently consists of antibiotics, and supportive intensive care ("hit early, hit hard")[105]. Neutrophils, being key cells of the innate immune system, are involved in the pathogenesis of sepsis and related clinical models of vascular inflammation[106]. It is well-described that septic neutrophils exhibit significant migration defects. While CXCR2 expression on neutrophils is significantly decreased in comparison with healthy control[107], CXCL8 in sepsis has been described as a biomarker of disease severity and predictor of outcome[108,109]. Blocking CXCR1/2 has lead to very mixed results in experimental sepsis models: while CXCR1/2 inhibition[110] and CXCR2 knockout[111] lead to increased survival in septic peritonitis, CXCR1 activation is a protective factor in *Candida albicans* sepsis[112]. To this end, the exact role of CXCR1/2 in sepsis therefore remains unclear and might depend on the dose and timing of CXCR1/2 activation/inhibition.

Ischemia reperfusion (I/R) injury is a condition which results from reapplying blood flow to tissues which before were ischemic, where it was observed that tissue damage is much greater than can be explained by ischemia alone. This type of condition can affect all organs and tissues, but is predominantly seen in patients with myocardial infarction, stroke and after hepatic and renal transplants. Dependent on the size and ischemia time of the tissue, remote neutrophil accumulation can be seen in the lung, which manifests itself clinically as pulmonary edema. On a molecular level, ROS production by neutrophils leads to direct and indirect tissue damage. Treatment consists of supportive measures such as hypothermia, the goal being to limit the organism's oxygen need[113]. A plethora of novel therapeutics targeting the immune system are currently being investigated[114]. Involvement of neutrophils appears to be negligible for at least very severe cerebral ischemia in rats[115], while direct involvement of the CXCL8-CXCR1/2 axis so far has been demonstrated at least in myocardial infarction[116]. *In vivo* inhibition studies show that CXCR1/2 inhibition limits neutrophil influx to I/R areals[22,117–122]. A suppression of tissue damage was observed in several studies in rat liver[118,119], rat cerebral[22,120] and rat gut[121,122] I/R models, while in another experiment using a mouse cerebral I/R model no effects on tissue damage were noted[117]. In summary, there are many

reports demonstrating that CXCR1/2 inhibition in I/R clinical disease models can limit neutrophil influx, and possibly associated tissue damage.

Alzheimer's disease (AD) is a chronic degenerative disease of the brain characterized by accelerating dementia. Amyloid β (Aβ) peptide, a variable length peptide cleaved from amyloid precursor protein (APP), accumulates in the extracellular spaces of the brain. In the intracellular space, the cytoskeleton physiology is disturbed leading to accumulation of tau proteins. Alterations of enzymes, which are responsible for cleaving APP, can shift the balance to overproduction. The accumulation of these proteins finally leads to microglial activation, loss of synapses and cell death. The atrophy progresses in such a manner that it generates the dementia symptoms. Treatment is purely symptomatic, consisting of cholinesterase inhibitors for cognitive symptoms, and neuroleptics for psychiatric symptoms[123]. The role of CXCR2 in AD pathophysiology is starting to emerge. Stimulation with CXCL8 increases Aβ production *in vitro*. Furthermore, small molecule inhibition or CXCR2 silencing inhibited the production of two amyloid β peptides *in vitro*[124]. These findings were later confirmed by the same group in a murine AD model *in vivo* using Cxcr2$^{-/-}$ mice[125]. Small molecule CXCR1/2 inhibition also inhibited Aβ production, among other markers for AD, in rats[126].

In summary, CXCR1/2 and its main chemokine in humans CXCL8 are involved in many diseases and conditions. While for some diseases like ischemia/reperfusion injury, cancer AD and RA important therapeutic effects of CXCR1/2 inhibition are beginning to be unraveled, in others such as asthma, COPD, there seems to be little clinical benefit of CXCR1/2 inhibition. For acute infections including sepsis there seem to be conflicting roles of the CXCL8-CXCR1/2 axis. Finally, for diseases like ASH, PKD, UC, psoriasis, and T1D, there is still little known about the efficacy of CXCR1/2 inhibition despite promising first hints.

1.4 Inhibitors of CXCR1/2

A variety of inhibitors of CXCR1 and/or 2 from different classes have been described to date (see **Table 1**).

One of the first to be used was the toxin of *Bordetella pertussis* (Pertussis toxin; PTx), which was first discovered to inhibit neutrophil activation[127], and later CXCL1-mediated adhesion in mice *in vivo*[42]. On a molecular level, it catalyzes the ADP-ribosylation of the Gα subunits $G_{\alpha i1-3}$ and $G_{\alpha o1-2}$ which prevents downstream G protein signaling. It is noteworthy that in clinical models e.g. peritonitis, PTx only partially blocks neutrophil recruitment[42]. Because of its inherent wide range of

(side-)effects on the human body, it is not used in the therapy of diseases. However, it remains a valuable tool in the research of chemokine-mediated neutrophil activation, and also was used in this project.

The diaryl urea class of inhibitors includes the compounds SB225002[128], SB-332235[84], SB656933[129] and GSK1325756 (Danirixin)[130,131]. *In vitro* and *in vivo* experiments were conducted with these compounds proving their efficacy. Clinical models included arthritis and ALI models in rats and peritonitis models in mice. Danirixin was also investigated in humans in phase 1 and phase 2 clinical trials for COPD, where first results show slight improvement of diseases activity with no relevant adverse events[58–61].

Another class of inhibitors are boronic acid containing molecules which includes the compounds SX-517[143], SX-576[142,148] and SX-862[144,145]. SX-517 and SX-576 showed lowering of neutrophil influx and activation in human *in vitro* and mouse/rat *in vivo* models. SX-862 showed inhibition of myeloid-derived suppressor cell trafficking *in vivo* in mouse advanced malignant tumor models.

Compounds from Dompè (L'Aquila, Italy) include Ladarixin[119], Reparixin (Repertaxin)[118], DF2162[85,138] and DF2755A[137]. These compounds were tested for their efficacy in *in vitro* chemotaxis assays, as well as rat liver I/R, arthritis, and mouse T1D and nociception models. Further clinical trials have been conducted with some of the compounds (Table 2).

Some inhibitors such as Antileukinate, NAc-PGP, CXCL8 K11R G31P or pepducins are peptide-based. Antileukinate was first described in 1995 and tested in neutrophil activation, chemotaxis, and recruitment *in vitro* and *in vivo* models[147,149]. N-Acetyl-Proline-Guanine-Proline (NAc-PGP) is a peptide which was found in the degraded extracellular matrix following airway inflammation and neutrophil influx[150]. Its isomer DD-NAc-PGP was later discovered to be a competitive CXCL8 antagonist in chemotaxis assays[132]. CXCL8(3-73) K11R G31P (short G31P) is a CXCL8 analogue with two mutations (at positions 11 and 31, respectively), and was reported to have a higher affinity on CXCR1 and 2 than native CXCL8, while suppressing neutrophil activation and chemotaxis[151,152]. It was also effective in attenuating pulmonary inflammation in an experimental K. pneumoniae pneumonia guinea pig model[45].

Pepducins are lipid-conjugated proteins which target intracellular loops of G proteins. Lipids, such as palmitate, are appended N-terminally to intracellular loops, e.g. i3 or i1, of G-protein coupled receptors. The lipid allows these molecules to float in the cell membrane and disrupt the activation of G-proteins via these receptor intracellular loops[153]. They are named after the receptor they target, then the

Table 1: Overview of CXCR1/2 inhibitors.

Class	Name	References
Interference with G protein signaling		
Lipid-conjugated peptide	Pepducin x1/2pal-i1	[104]
Peptide	Pertussis toxin	[42]
Competition or binding of CXCL8		
Mutated peptide	CXCL8 K11R G31P	[100]
Small peptide	DD-NAc-PGP isomer	[132]
Protein	TNF-stimulated gene 6 protein (TSG6)	[133]
Allosteric inhibition		
Diarylurea	SKF83589	[134]
	SB225002	[128]
	SB656933	[129]
	SB332235	[84]
	GSK1325756 (Danirixin)	[131]
Cyanoguanidine	SB468477	[135]
Ketoprofen derivate	Reparixin (Repertaxin)	[136]
unknown	Ladarixin (DF2156A)	[22]
	DF2755A	[137]
	DF2162	[138]
	SCH-527123 (Navarixin)	[139]
Bicyclic thiazolopyrimidine	AZD-8309	[140]
Quinoxaline	PD0220245	[141]
Boronic acid	SX-576	[142]
	SX-517	[143]
	SX-862	[144,145]
Unknown		
unknown	SB455821	[146]
Protein	Antileukinate	[147]

The table is grouped by mechanism of action and chemical class of the inhibitor. For details, see section 1.4.

conjugated lipid, and finally the intracellular loop, for example x1/2-pal-i1 is a pepducin targeting CXCR1/2 (x1/2), has palmitate conjugated (pal), and interacts with the first intracellular loop (i1). Pepducin x1/2-pal-i3 and x1/2-LCA-i1 inhibit neutrophils *in vitro* in calcium signaling assays, as well *in vivo* in a murine peritonitis model. The group also reported that the administration of the pepducins protected the mice from death due to sepsis, even if the administration occured delayed[154]. Pepducin x1/2-pal-i1 was synthesized and tested in experimental murine alcoholic steatohepatitis (mASH)[104]. It reduced incidence and mortality of mASH, while signs of reversal of mASH, downregulation of chemokines, and reduction of neutrophil influx in the liver could also be observed.

In summary, different classes of compounds inhibiting CXCR1/2 have been described so far. While for some classes such as diaryl urea and compounds from Dompè, there is already a large body of preclinical data and first clinical trials, other classes like peptide-based and boronic acid containing inhibitors were so far only investigated *in vitro* and a limited amount of preclinical disease models.

Table 2: Clinical trials with CXCR1/2 inhibitors.

Drug	Indication	Sponsor	Ph	Status	Ref
AZD5069	Asthma, Bronchiectasis, COPD	Astra-Zeneca	2 c	c c	NCT01704495 NCT01255592
AZD8309	Airway inflammation	Astra-Zeneca	-	c	NCT00860821
Ladarixin	Insulin-dependent T1D	Dompè	2	c	NCT02814838
Reparixin	Lung transplant I/R	Dompè	2	c	NCT00224406
Reparixin	Post surgical I/R following coronary artery bypass graft	Dompè	1	c	[122]
Reparixin	T1D islet cell transplantation	Dompè	2	c	NCT01220856
SB 656933	Ulcerative colitis	Glaxo-Smith-Kline	2	t	NCT00748410
SCH 527123 (MK-7123)	COPD Asthma	MSD	2	c c	NCT01006616 NCT00688467
anti-CXCL8 antibody	COPD	Abgenix Inc	2	c	[57]
Danirixin	COPD	Glaxo-Smith-Kline	1 2	c c c c	NCT01209052 NCT01209104 NCT02130193 NCT03034967
SX-682	Metastatic melanoma	Syntrix Biosystems	1	r	NCT03161431

Ph = Phase. Status: c = completed, t = terminated, r = recruiting. References are either published results or ID from ClinicalTrials.gov of National Library of Medicine at the US National Institutes of Health (NCT; https://clinicaltrials.gov).

2 Aim of the Study

In many diseases, a role of neutrophils and CXCR1/2 have been described to be of pathophysiological relevance. Studies using inhibitory compounds have seen a reduction of neutrophil influx in many cases and in some cases even improvement of disease characteristics including clinical scores. Basic research shows that CXCR1/2 are mainly involved in neutrophil adhesion, transmigration and chemotaxis. While for chemotaxis there is solid data on the effect of CXCR1/2 inhibition, its effects on the recruitment cascade have not yet been conclusively clarified.

Therefore, the aim of this project is to elucidate the effects of CXCR1/2 inhibition on neutrophil recruitment, especially rolling, adhesion and transmigration, using *in vitro* and *in vivo* methods and the new small molecule inhibitors Ladarixin, Reparixin and DF2755A. This will help in better understanding chemokine signaling and inhibition in neutrophils, and ultimately lead to new therapeutic approaches in the treatment of CXCR1/2 dependent diseases.

3 Materials and Methods

3.1 Laboratory Animals

For this study, C57Bl/6 mice were used as wildtype animals (Charles River Laboratories, Sulzfeld, Germany). For multi-photon fluorescence microscopy, *Lyz2*^{GFP} mice on C57Bl/6 background were used[155]. All animals were held at the Core Facility Animal Models (CAM), Biomedical Centre Munich, Munich, Germany. All animal experiments were approved by Regierung von Oberbayern, Munich, Germany under AZ 55.2-1-54-2531-122/12.

3.2 Compounds

3.2.1 Buffers and Solutions

Superfusion buffer for Cremaster muscle

132 mM	NaCl
4.7 mM	KCl
2.66 mM	$CaCl_2$
2.13 mM	$MgCl_2$
18 mM	$NaHCO_3$

HEPES-buffered HBSS

1 mM	$CaCl_2$
1 mM	$MgCl_2$
0.1%	Glucose
10 mM	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)
0.25%	Bovine serum albumin (BSA)
7.4 $\pm$ 0.02	adjust pH using NaOH or HCl

Mouse anesthesia solution per kilogram of body weight

100 mg/kg	Ketamine
20 mg/kg	Xylazine
ad	0.9% NaCl

3.2.2 Antibodies

The primary and secondary antibodies used in the experiments are listed below (Tables 3 and 4).

Table 3: Primary and labeled antibodies.

Antibody	Reactivity	Isotype	Dye	Clone	Manufacturer
E-selectin (CD62E)	rat anti-mouse	IgG2	-	9A9	BioXCell
PECAM-1 (CD31)	rat anti-mouse	IgG2	Alexa Fluor 546	390	BioLegend
Neutrophil Elastase	rabbit anti-mouse	IgG	-	polyclonal	AbCam
VLA-3 (CD49c)	mouse anti-mouse	IgG1	-	42/CD49c	BD Biosciences
VLA-6 (CD49f)	rabbit anti-mouse	IgG2	Alexa Fluor 647	GoH3	BioLegend

Table 4: Secondary antibodies.

Antibody/Reactivity	Dye	Manufacturer
Donkey anti-rabbit	Alexa Fluor 488	Invitrogen/Thermo Fisher
Goat anti-rat	Alexa Fluor 546	Invitrogen/Thermo Fisher

3.2.3 Chemokine Receptor Inhibitors

The compounds Ladarixin, Reparixin and DF2755A were generously provided to us by Dompè S.p.a (L'Aquila, Italy). For the *in vitro* experiments, we used a target concentration of 5 µM, and for *in vivo* 15 or 30 µg/g body weight, which were the concentrations reported to lie well within the range of inhibition of Ladarixin[119], Reparixin[118] and DF2755A[137].

3.3 Isolation of Human Neutrophils

Blood was drawn from healthy human donors after obtaining their consent. Heparin was applied to the syringe at a concentration of 430 international units (IU) Heparin per 50ml of blood. Around 20-40 ml of blood were drawn. Polymorphprep (Alera

Technologies, Oslo, Norway) was then added, and the sample was prepared according to its application sheet. Briefly, the Polymorphprep solution was added in a 1:1 ratio on top of the blood, then the sample was centrifuged. The top plasma and peripheral blood mononuclear cell (PBMC) layers were carefully removed, before removing the polymorphonuclear (PMN) cell layer containing the neutrophils. They were then washed twice with phosphate-buffered normal saline (PBS).

As a medium for temporarily storing the neutrophils HEPES-buffered Hanks's salt solution (HBSS) was used (recipe see above).

The isolated neutrophils were then stained using Tuercks solution, counted in an improved Neubauer chamber, and the HBSS buffer was added in order to obtain a concentration of 1x10^6 cells/ml. Before applying them to the flow chambers, they were incubated with the compounds or controls.

3.4 Isolation of Murine Neutrophils

C57Bl/6 mice were sacrificed and bone marrow from the hip, femur and tibia bones was flushed out with PBS. After washing the cells, PMNs were isolated using a Percoll (Sigma-Aldrich, St. Louis, MO, USA) solution at 1.08 and 1.11 g/ml density. Cells were counted and resuspended in HBSS at 1x10^6/ml as described above for human neutrophils. Subsequently, they were incubated with compounds/controls for 1h at 37 ℃ as indicated.

3.5 In Vitro Flow Chambers

μ-Slide VI 0.1 (ibidi GmbH, Martinsried, Germany) plastic microflow chambers were coated with the specified recombinant proteins at these concentrations: E-selectin 5 µg/ml, ICAM-1 4 µg/ml, CXCL8 10 µg/ml. The proteins were diluted using PBS with 0.1% BSA. This approach and the concentrations were established before by the group[156]. 10 µl of this coating solution was applied to each chamber. After overnight incubation at 4 ℃, Casein in PBS was applied to block unspecific binding sites. After another 2 hours of incubation at room temperature, the chambers were flushed with normal saline (0.9% NaCl).

In parallel, 1 ml aliquots of the isolated neutrophil suspension (prepared as described before) were incubated for 2 hours at 37 ℃ with 5% CO_2 with the compounds Ladarixin, Reparixin (at the indicated concentrations), and the controls normal saline, Pertussis Toxin (PTx; 200 ng/ml).

Finally, the flow chambers were placed under an inverse microscope (Zeiss; Objective Nikon Fluor 20x 0.75) with a heating pad set to 35 ℃. The samples were

drawn up in a syringe and applied using a precision pump (Harvard Apparatus) at a calculated shear rate of 1 dyne/cm^2 while videos were recorded using an MC-1002 CCD camera (Bildsysteme Horn, Aalen, Germany) and VirtualDub software.

3.5.1 Data Analysis

Five to six minutes after the start of the flow the number of rolling and adherent cells was analyzed. The number of rolling cells was counted as follows: the video was set to the specified time point. Then, a line was drawn perpendicular to the vessel wall. When the video was played back for one minute, the number of cells which passed the line was counted. Similarily, the number of adherent cells was determined: the video was set to the specified time point, whereafter all the cells were marked. When the video was played back for one minute, the cells which moved less than one times their diameter (as determined by the mark) were considered adherent.

3.6 Intravital Microscopy of the Mouse Cremaster Muscle

The cremaster muscle is a thin muscle surrounding the spermatic cord and testis in mammals on each side. Due to its ease of access and thinness, the cremaster muscle can be used to study leukocyte interactions with the endothelium and tissue *in vivo*. Its use as a model has been first described in 1973[157].

The procedure consists of anaesthetizing the mouse using ketamine and xylazine (recipe see above). The anesthesia is repeated at 1/3 of the above mentioned dose every hour. The mouse is placed on a heatbed set to 37℃ and catheters are placed in the trachea to stabilize breathing during the surgery and experiment, and carotid artery to have safe intravascular access in order to apply substances and draw blood. The scrotum is then cut open, the cremaster muscle is taken out and freed of connective tissue. Lastly, it is cut open lengthwise and pinned to the stage. For an example preparation, see Figure 2. During the experiment, it is superfused with 37℃ warm superfusion solution (recipe see above).

In this project different approaches using the cremaster muscle were used.

3.6.1 Chemokine-induced Inflammation of the Cremaster Muscle

Inhibitor or saline or pertussis toxin (control) was injected intraperitoneally into C57Bl/6 mice (Charles River Laboratories, MA, US) at the indicated concentrations, together with anti-E-selectin antibody at a concentration of 100 µg/mouse where indicated. 1 hour later, 600 ng of recombinant murine KC (ImmunoTools GmbH,

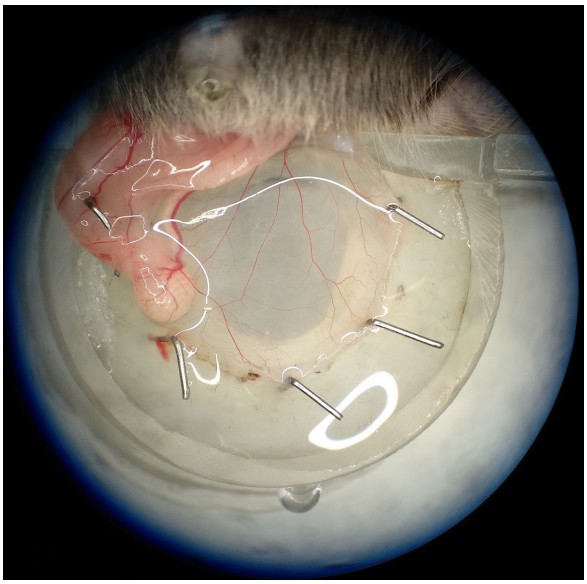

Figure 2: Example of Cremaster Muscle Preparation. The picture shows the cremaster muscle of an anesthesized C57Bl/6 mouse after preparation and ready for microscopy, as seen through the preparation microscope. The blood-filled vasculature is clearly visible. The testicle which was wrapped in the muscle is pinned to the stage on the left side (own picture).

Friesoythe, Germany) or recombinant murine CXCL2 (R&D) in 200 µl saline was injected into the mouse scrotum. After another hour, a carotid artery catheter was placed, the cremaster muscle was exteriorized and 4-6 vessels per mouse were recorded as described below. After that, where indicated, the cremaster muscles were fixed with 4% paraformaldehyde (PFA) on glass slides, colored using Giemsa-Wright stain and embedded using EUKITT medium (O. Kindler GmbH, Bobingen, Germany).

3.6.2 Chemokine-induced Neutrophil Arrest in the Cremaster Muscle

Inhibitor or saline (control) or pertussis toxin (PTx, control) was injected i.p. into C57Bl/6 mice (Charles River Laboratories, MA, US) at a final concentration of 30 µg/g body weight (for PTx 4 µg/mouse). Two hours later, a carotid artery catheter was placed, the cremaster muscle was exteriorized and the mouse placed on the stage under the microscope. A suitable vessel was recorded for 1 min, before 600 ng CXCL1 or CXCL8 per mouse was applied through the carotid artery[158]. The recording was continued for >5 min thereafter. At the end of the experiments, systemic blood was taken from the carotid artery and analyzed for leukocyte count in an IDEXX ProCyte DX hemocytometer (IDEXX Laboratories, ME, US).

3.6.3 Data Analysis of Adhesion and Rolling

The mouse was then placed on a stage under a BX51WI microscope with water immersion objective 40x 0.8 NA (both Olympus, Tokyo, Japan) and videos of post-capillary venules 20-40 µm in diameter were recorded using a CCD camera (CF8/1, Kappa). Videos were captured using VirtualDub recording software. Vessel diameters, lengths and rolling velocities were analyzed using Fiji software and the MTrackJ plugin, respectively. The number of rolling and adherent cells was counted as previously described in section 3.5.1. For the chemokine-induced arrest, cells were counted before and after applying the chemokine, respectively.

3.6.4 Data Analysis of Transmigration

The Giemsa-Wright stained cremaster muscles were placed under a Leica DM2500 microscope equipped with a DMC2900 CMOS camera. A HCX PL FLUOTAR 100x/1.40 Oil in bright field was used for counting the number of perivascular neutrophils, eosinophils, and other WBCs around vessels with a diameter 20-40 µm. For each mouse, 3-5 field of views were counted.

3.7 Multiphoton Imaging of Neutrophil Transmigration in the Cremaster Muscle

A dose of 600 ng CXCL2 and fluorescent anti-PECAM-1 Alexa Fluor 546 antibody was injected intrascrotally into *Lyz2*^{GFP} (LyE-GFP) mice. Two hours later, the mice were anesthesized and the cremaster muscle was exteriorized as previously described. Images were recorded at the BMC Bioimaging Core Facility with a Leica SP8 MP upright laser scanning microscope equipped with a 25x1.0 water immersion objective. A pulsed InSight DS+ laser with a set wavelength of 840 nm was used as a light source. External, non-descanned hybrid photo detectors (HyDs) were used to acquire images at a resolution of 1024x1024 pixels. Two channels, corresponding to GFP and Alexa Fluor 546 signals, were captured in a Z stack with a layer height of 4 µm.

An injury was set in the cremaster using the same laser: the multi-photon laser was used to scan a 50x50 µm area between vessels 50 times at a high power setting. Power settings were varied until an injury in the signals could be observed. Immediately thereafter, video was captured for 30 min.

Videos were analyzed using Fiji software, and the Image Stabilizer plugin. The Z stacks were combined in a maximum intensity projection, and the GFP intensity

was measured in a 50x50 µm box around the laser injury. Since the intensities varied with each experiment, a relative intensity based on the first intensity was calculated.

3.8 Immunofluorescence Staining of Murine Neutrophils

Murine bone marrow neutrophils were isolated as described above. After treatment with controls/compounds, they were seeded for 30 min at 37℃, 5% CO_2 onto μ-Slide 8-well glass slides (ibidi GmbH, Martinsried, Germany) coated with PECAM-1/ICAM-1/chemokine at 2/8/10 µg/ml or 2% BSA (control) respectively. After fixation with 4% PFA (15min; RT) and permeabilization with 0.1% Triton X-100 and 2% BSA in PBS (1h; RT), primary antibodies were incubated overnight at 4 ℃. Thereafter, the secondary antibodies (Alexa Fluor 488 and Alexa Fluor 546) were incubated for 1h at RT. Lastly, labeled anti CD49f antibody was incubated in a single step overnight at 4 ℃. The cells were embedded in PermaFluor.

Confocal microscopy was performed with an inverted Leica SP8X WLL microscope at the BMC Bioimaging Core Facility, equipped with a WLL2 laser and acusto-optical beam splitter. Images were acquired with a 40x1.2 objective. The following fluorescence settings were used: Alexa Fluor 488 (excitation 498 nm; emission 508-535 nm), Alexa Fluor 546 (excitation 555 nm; emission 565-620 nm), Alexa Fluor 647 (excitation 652 nm; emission 662-720 nm). Channels were recorderd sequentially to minimize bleed-through. All channels were recorded with hybrid photo detectors (HyDs). As many cells as possible per FOV were captured using Z stacks with the "system optimized" setting for Z stack height.

The number of cells showing ring formation in each channel was counted using Fiji software.

3.9 Statistical Analysis

R and GraphPad software was used for statistical analyses. Comparison of two experimental groups was done using Student's t-test `t.test(...)`, independent or paired - depending on the experimental setting. Comparison of more than two groups was performed using ANOVA `aov(...)` and Dunnett's post-hoc test `glht(..., linfct=mcp(treatment="Dunnett")))`. p values > 0.05 were considered statistically significant.

4 Results

In the table below the results of the experiments on the effects of Ladarixin, Reparixin and DF2755A on the different phases of neutrophil recruitment are summarized. They are elaborated on in the following subsections.

Table 5: Summary of results

	CXCL1	**CXCL2**	**CXCL8**	**human**	**mouse**
in vitro					
Rolling	-	-	X	•	
Adhesion	-	-	X	•	
in vivo					
Rolling	-	-	-		•
Acute Adhesion	X	-	X		•
Adhesion	X	X	X		•
Transmigration	✓	✓	✓		•
Interstitial migration	-	✓	-		•

This table summarizes the results of experiments done on the different phases of neutrophil recruitment in this study. Legend: - not investigated, X no significant effect of inhibitor vs. saline control, ✓inhibition of this phase of neutrophil recruitment vs. saline control, • experiments performed in this species. Please see following section for details.

4.1 *In Vitro* Rolling and Adhesion

To evaluate the first two stages of neutrophil recruitment - rolling and adhesion - a microflow chamber assay using isolated human neutrophils was utilized as previously described in sections 3.3 and 3.5. For the first set of experiments, the chemokine CXCL8 was immobilized in flow chambers as part of the coating solution, together with E-selectin and ICAM-1. Here, treatment of cells with Ladarixin showed 18.3 $\pm$ 5.4 rolling cells/FOV, versus 14.18 $\pm$ 4.5 for NaCl treatment, and 18 $\pm$ 2.14 for Reparixin (Figure 3; numbers always mean $\pm$ standard error of mean). For adhesion, the saline control showed 28.9 $\pm$ 4.7 cells/FOV, Ladarixin treated assays 23.3 $\pm$ 5.9 and Reparixin treated assays 35.7 $\pm$ 6.1 cells/FOV.

Overall, treatment with Ladarixin or Reparixin did not significantly alter the number of neither rolling nor adherent cells versus saline control in flow chambers.

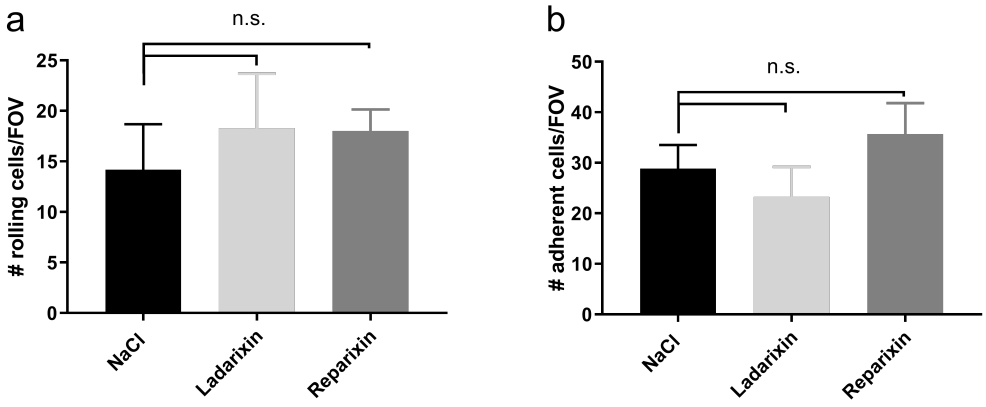

Figure 3: Ladarixin and Reparixin do not influence neutrophil rolling and adhesion *in vitro* in response to CXCL8. a. Number of rolling leukocytes per field of view of microflow chambers coated with E-Selectin/ICAM-1/CXCL8. b. Number of adherent leukocytes per field of view of microflow chambers as described in a. $n \geq 3$, mean $\pm$ SEM, n.s. using 2-way ANOVA

In order to investigate the response to CXCL8, a second assay was performed where the chemokine was not coated onto the chambers, but rather the cells were pre-treated with it immediately before applying them to the chamber. Also, pertussis toxin (PTx) was added as a positive control. As can be seen in Figure 4, treatment of cells with Ladarixin yielded 45.8 $\pm$ 9.4 adherent cells/FOV, and Reparixin 53.8 $\pm$ 12.1 adherent cells/FOV. Saline treated assays showed 37.8 $\pm$ 4.5, and PTx 18.2 $\pm$ 1.7 adherent cells/FOV. The difference between NaCl and PTx treatment groups is statistically significant at p = 0.0042, the differences between NaCl and Ladarixin, and NaCl and Reparixin treatment groups are not.

Overall, the treatment of neutrophils with PTx led to a significantly reduced number of adherent cells compared to saline control as expected. Treatment of PMNs with the compounds Ladarixin or Reparixin did not however significantly influence the number of adherent cells.

Together, this data shows that Ladarixin and Reparixin do not significantly influence human PMN adhesion *in vitro* in response to CXCL8, as evidenced by a significantly reduced number of adherent neutrophils in a flow chamber assay.

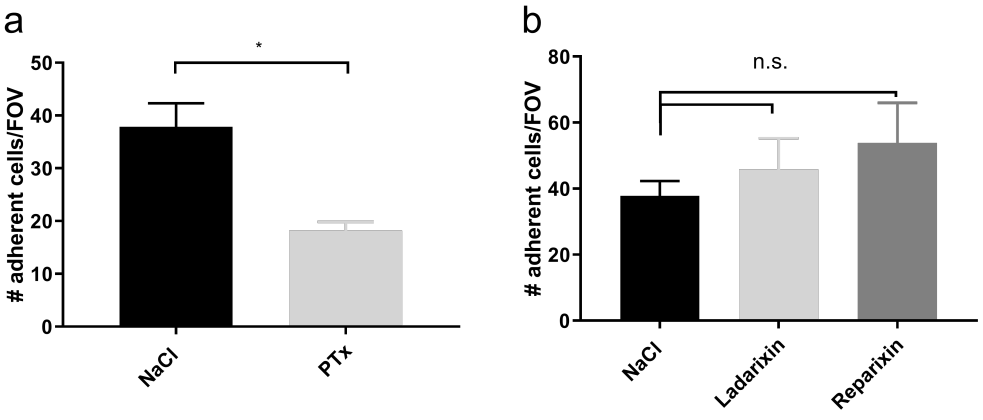

Figure 4: Ladarixin and Reparixin do not influence neutrophil adhesion *in vitro* in response to CXCL8. Number of adherent leukocytes per field of view of microflow chambers coated with E-Selectin/ICAM-1 after treatment with CXCL8 (10 ng/ml). a. Cells were treated with normal saline (NaCl) or Pertussis toxin (PTx, 200 ng/ml). $n \geq 3$, mean $\pm$ SEM, $p < 0.05$ using Students' t-test. b. Cells were treated with normal saline (NaCl), Ladarixin (50 µM) or Reparixin (50 µM). $n \geq 3$, mean $\pm$ SEM, n.s. using 2-way ANOVA.

4.2 *In Vivo* Rolling and Adhesion

4.2.1 Acute Induction of Arrest

Based on the fact that CXCR1/2 chemokines are involved in the induction of neutrophil arrest, and as shown by the application of CXCL1 and CXCL8 in postcapillary venules of the cremaster muscle[41,42], we decided to test inhibitors regarding this feature. Experiments were conducted as previously described in section 3.6.2. Briefly, wildtype mice were treated i.p. with either compounds or controls, and placed under the microscope. While recording the video, they then received chemokine intravascularly through the cartoid artery catheter.

In the first set of experiments (Figure 5), the application of intravascular CXCL1 in Ladarixin and saline (control) treated mice both lead to an immediate and significant increase in the number of adherent cells after the injection as compared to before. For saline control, the number of adherent cells increased from 276.7 $\pm$ 45.1 to 620.4 $\pm$ 115.7 per mm^2 before and after injection of CXCL1, respectively. For Ladarixin, adherent cells per mm^2 of vessel increased from 369 $\pm$ 77.8 pre-injection to 607.7 $\pm$ 79.5 post-injection.

Next we investigated the effects of Ladarixin, Reparixin and DF2755A in response to CXCL8 in the same model, and also added Pertussis toxin as a second control (Figure 6). The number of adherent cells per mm^2 of vessel wall in NaCl treated

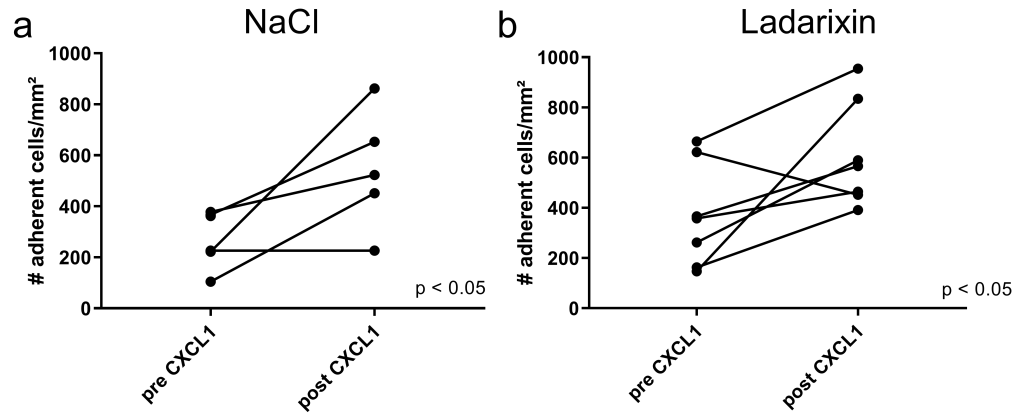

Figure 5: Ladarixin does not influence acute neutrophil arrest *in vivo* in response to CXCL1. Number of adherent leukocytes per mm^2 of vessel in the mouse cremaster muscle, before and after intravascular injection of 600 ng CXCL1. Mice were pre-treated with **a.** NaCl or **b.** Ladarixin (15 µg/g body weight) i.p. 3 hours before the experiment. n $\geq$ 3 mice per group. p < 0.05 where indicated (*) using paired Students' t-test.

mice increased from 268.5 $\pm$ 35.2 pre-injection to 475 $\pm$ 31.5 post-injection, in Ladarixin treated mice from 363.8 $\pm$ 33.3 pre-injection to 625.2 $\pm$ 32.58 post-injection, and in DF2755A treated mice from 338.6 $\pm$ 31.7 pre-injection to 501.5 $\pm$ 45.3 post-injection. In PTx treated mice, the number of adherent cells/mm^2 slightly increased from 291.4 $\pm$ 22.4 to 360.3 $\pm$ 59.69 post-injection, this increase being statistically non-significant.

As expected, the change in neutrophil count per mm^2 in Pertussis toxin treated mice did not significantly increase after chemokine injection. In contrast, mice treated with Ladarixin, Reparixin and DF2755A showed a significant increase after chemokine injection, similar to saline control.

In summary, here we have shown that Ladarixin, Reparixin and DF2755A do not influence acute neutrophil arrest in response to intravascularly applied chemokines. This is evidenced by significant increases in neutrophil adhesion immediately after chemokine application in mice treated with the compounds, similar to saline treated mice.

4.2.2 Induction of Tissue Inflammation

A second well-studied effect of chemokines in the cremaster muscle model is the induction of tissue and vessel inflammation when injected intrascrotally before microscopy. In short, wildtype mice were treated with compounds or controls i.p.,

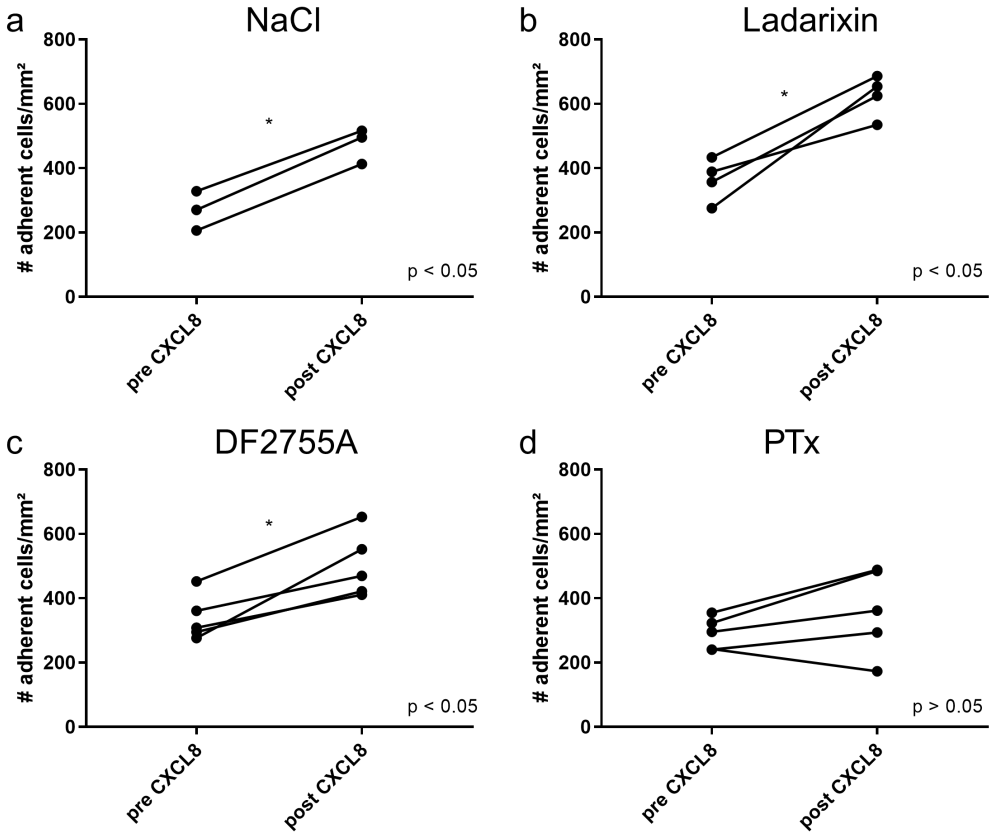

Figure 6: Ladarixin and Reparixin do not influence neutrophil adhesion *in vivo* in response to CXCL8. Number of adherent leukocytes per mm^2 of vessel in mouse cremaster, before and after intravascular injection of 600 ng CXCL8. Mice were pre-treated with NaCl, Ladarixin or Reparixin (both 30 µg/g body weight) or Pertussis toxin (4 µg/mouse) i.p. 3 hours before the experiment. **a.** NaCl pre-treatment, n = 3 mice. **b.** Ladarixin pre-treatment, n = 3 mice. **c.** Reparixin pre-treatment, n = 3 mice. **d.** Pertussis toxin pre-treatment, n = 5 mice, n.s. $p < 0.05$ where indicated (*) using paired Students' t-test.

then chemokine intrascrotally before the number of rolling and adherent cells were counted (see section 3.6.1 for details).

For rolling, the number of rolling cells in saline treated mice was 23.6 $\pm$ 6.8/FOV versus 18.1 $\pm$ 4.7/FOV for Ladarixin treated mice, the difference being statistically not significant. For adherent cells, saline treated mice showed 637.8 $\pm$ 106.2 adherent cells/mm^2 and Ladarixin treated mice 642.4 $\pm$ 64.7, the the difference being statistically not significant (Figure 7). Overall there is no significant difference in neither the number of adherent nor rolling cells in mice treated with Ladarixin i.p. as compared to mice treated with saline control i.p.

Because of recent findings of differential functions of CXCL1 and CXCL2 regard-

ing neutrophil recruitment[19], we hypothesized that our previous results might be explained by differential inhibition of chemokines and decided to conduct further experiments using this model, the two chemokines CXCL1 and CXCL2 and the inhibitor DF2755A. To investigate this, we performed another set of experiments, whose results are summarized in Figure 8a (CXCL1 chemokine) and 8b (CXCL2 chemokine). NaCl/CXCL1 treated control mice showed 566.3 $\pm$ 85.6 adherent cells/mm^2, and DF2755A/CXCL1 treated mice 453.4 $\pm$ 99.8 adherent cells/mm^2. For CXCL2 chemokine, NaCl treated mice showed 502.5 $\pm$ 102 and DF2755A treated mice 458.7 $\pm$ 79.7 adherent cells/mm^2. The differences between the respective groups are statistically non significant. Overall there is again no significant difference in the number of adherent cells upon stimulation with either CXCL1 or CXCL2 and inhibition of CXCR1/2 through DF2755A in comparison with saline control.

These results show that Ladarixin and DF2755A do not influence neutrophil rolling and adhesion, as evidenced by non-significant differences in neutrophil adhesion counts, and additionally neutrophil rolling counts in the case of Ladarixin.

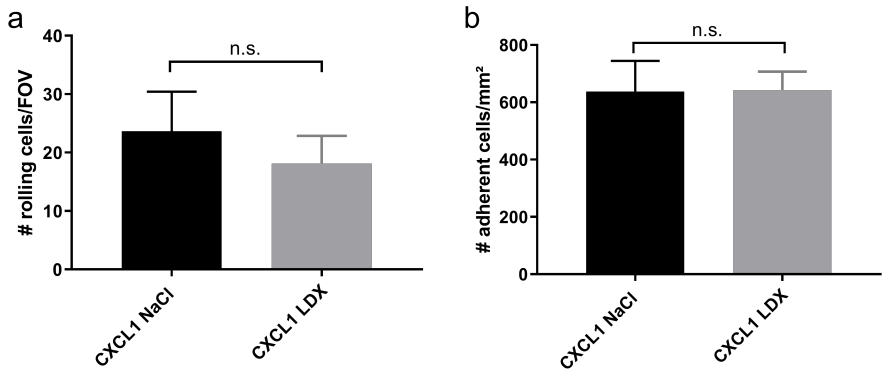

Figure 7: Ladarixin does not influence neutrophil adhesion and rolling *in vivo* in response to CXCL1. Data obtained 3 hours after injection of 600ng CXCL1 intrascrotally, and 4 hours after initial inhibitor/control i.p. injection. Number of **a.** rolling cells per field of view, and **b.** adherent cells per mm^2 of vessel in mouse cremaster. n(NaCl) = 2 mice, n(Ladarixin) = 3 mice, differences are p>0.05 (n.s.) using Students' t-test.

4.3 *In Vivo* Transmigration

As previously discussed, another important role of chemokines in neutrophil recruitment is the induction of transmigration. Therefore, we investigated the effects

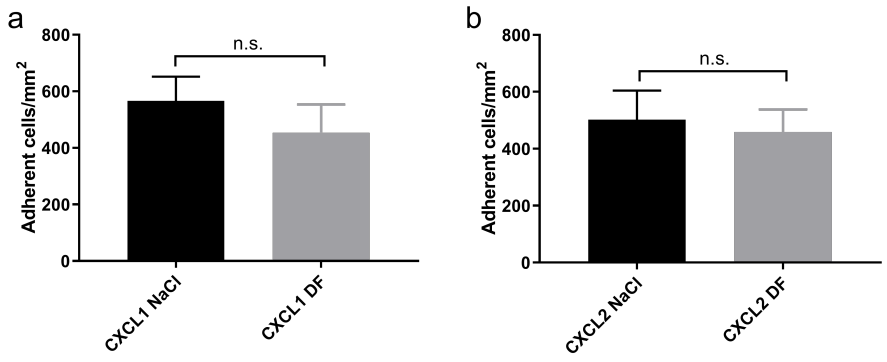

Figure 8: DF2755A does not influence neutrophil adhesion *in vivo* in response to CXCL1 or CXCL2. Data obtained 3 hours after injection of 600ng chemokine intrascrotally, and 4 hours after initial inhibitor/control i.p. injection. Number of adherent cells per mm^2 vessel in mouse cremaster: **a.** in response to CXCL1, **b.** in response to CXCL2. Differences are p>0.05 (n.s.) using unpaired Students' t-test. n = 5 mice for all groups, errors bars show SEM.

of DF2755A on CXCL1 and CXCL2, as well as sterile inflammation induced transmigration.

The first set of experiments used an *in vivo* approach described in detail in section 3.7. Briefly, *Lyz2*^{GFP} neutrophil reporter mice received the compound or saline control i.p., followed by CXCL2 and labelled Anti-PECAM-1 antibody intrascrotally. This antibody stains the microvasculature. Then, neutrophil transmigration behavior was observed using a multiphoton microscope after sterile laser injury using GFP intensity at injury site as a parameter.

As shown in Figure 10a, the mean relative GFP signal intensity increased from 1, the relative start point set for each video directly following the laser injury, to a peak of 4.8 $\pm$ 0.8 (28 minutes) for NaCl treated mice and 1.6 $\pm$ 0.2 for DF2755A treated mice. From time point 16 min onwards, the difference between the two groups are statistically significant. Overall there was a significant difference in the mean GFP intensity at the injury site, which corresponds to the number of neutrophils, between saline and DF2755A treated mice from 16 min after laser injury onwards. Representative images from the two groups can be seen in Figure 10b. Therefore we had first indications that DF2755A inhibits neutrophil migration to the injury site (interstitial migration) as well as transmigration.

To confirm the effect on transmigration, we analyzed the Giemsa-stained cremaster muscles of the mice used in the experiments for Figure 8 as described in detail in section 3.6.4. In short, after the *in vivo* microscopy experiments the cremaster muscles were Giemsa stained and the number of perivascular leukocytes was

counted under 100x magnification. NaCl/CXCL1 treated mice had a mean number of 1822 $\pm$ 200 perivascular cells/mm^2, while DF/CXCL1 treated mice showed 770 $\pm$ 99.4 perivascular cells/mm^2 (Figure 9a). For CXCL2 chemokine, NaCl treated mice showed 1284 $\pm$ 153.6 perivascular cells/mm^2, and DF2755A treated mice 724.1 $\pm$ 109.6 perivascular cells/mm^2 (Figure 9b). The differences between the respective groups were statistically significant. The difference between NaCl/CXCL1 and NaCl/CXCL2 is not statistically significant ($p > 0.6$, not shown in figure). Overall, the number of transmigrated cells per mm^2 was significantly lower in both CXCL1- and CXCL2-treated mice compared to saline control. Since neutrophils make up the majority of leukocytes, we concluded that DF2755A treatment inhibits neutrophil transmigration in response to CXCL1 and CXCL2. To extend and confirm these

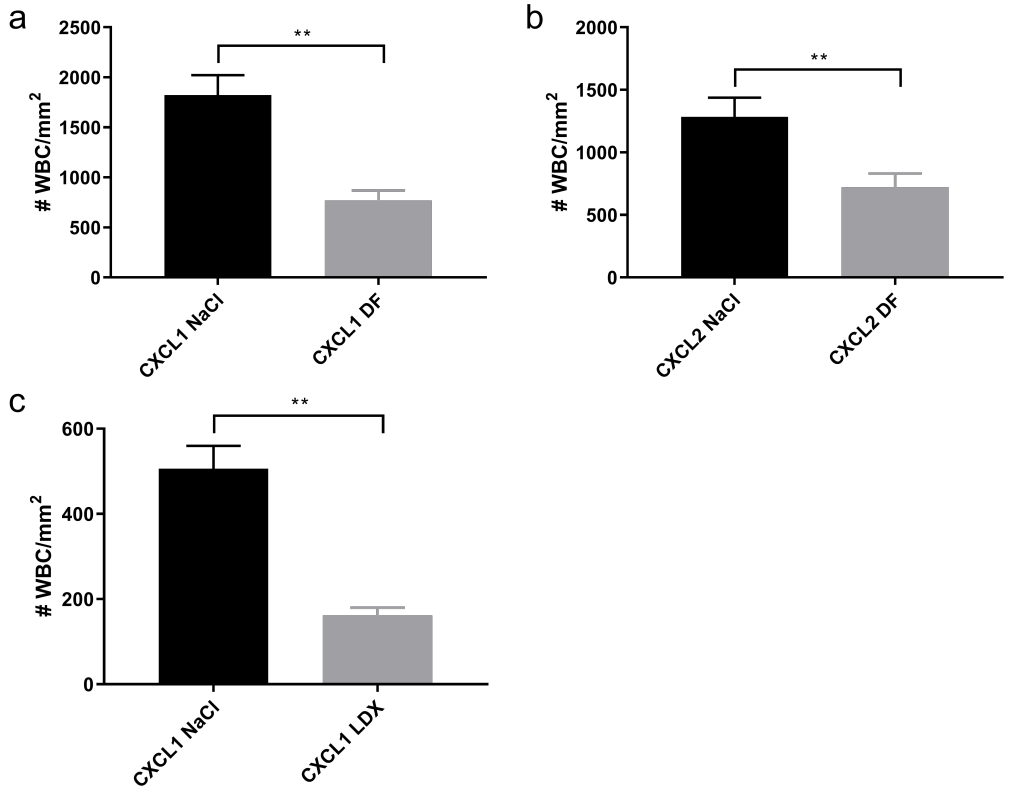

Figure 9: DF2755A and Ladarixin reduce neutrophil transmigration *in vivo* in response to CXCL1 or CXCL2. Data from C57Bl6 mice 3 hours after 600 ng CXCL1/CXCL2 intrascrotally, and 4 hours after initial DF2755A or Ladarixin (both 30µg/g body weight) or NaCl control i.p. injection. Number of perivascular leukocytes per mm^2 tissue in Giemsa-stained mouse cremaster: **a.** DF2755A versus saline pre-treatment, CXCL1 chemokine. **b.** DF2755A versus saline treatment, CXCL2 treatment. **c.** Ladarixin versus saline treatment, CXCL1 treatment. n = 5 mice for all groups, errors bars show SEM, $p < 0.01$ (*) using unpaired Students' t-test.

results, we analyzed cremaster muscles after Ladarixin treatment versus saline control in the same way. Saline treated control mice showed 506.5 $\pm$ 53.5, and Ladarixin treated mice 161.9 $\pm$ 17.8 perivascular cells/mm^2. The difference is statistically significant. Therefore, the number of transmigrated leukocytes is significantly smaller in Ladarixin treated mice in comparison to saline control.

Therefore, overall we can conclude that chemokine receptor inhibition using DF-2755A or Ladarixin inhibits neutrophil transmigration in response to CXCL1, as well as interstitial migration and transmigration in response to CXCL2 for DF2755A.

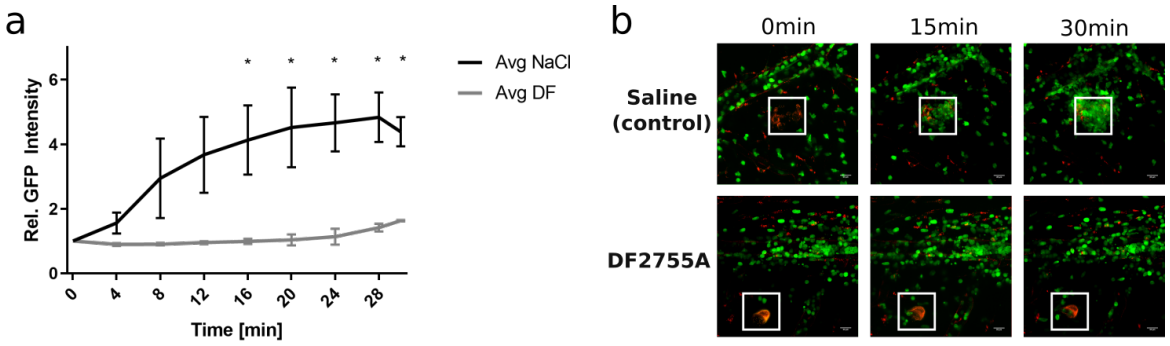

Figure 10: DF2755A inhibits Neutrophil Migration towards Sterile Inflammation Site. a. Neutrophils (green) and microvasculature (red, anti–PECAM-1) of CXCL2–stimulated mouse cremaster muscle 0 min, 15 min and 30 min after laser injury marked by white box. b. Quantification of neutrophil transmigration in DF2755A-treated vs. saline-treated mice using relative GFP intensity at injury site, where time point 0 has the relative intensity 1. $p < 0.05$ where indicated (*) using Students t-test.

4.4 *In Vitro* Vesicle Mobilization to the Plasma Membrane

In search for a possible explanation of this inhibition, we turned to a method which had been established by our group before[159]. Since neutrophil transmigration depends on translocation of neutrophil elastase (NE), integrins $\alpha 3\beta 1$ (VLA-3) and $\alpha 6\beta 1$ (VLA-6) to the neutrophil surface (see section 1.1), we hypothesized that blocking CXCR1/2 blocks this translocation and subsequent transmigration. The successful translocation can be observed using confocal fluorescence microscopy as a ring-like structure, corresponding to the protein being enriched at the plasma membrane. In this assay, isolated murine neutrophils were incubated with compounds/controls and then seeded onto glass slides coated with P-Selectin, ICAM-1 and CXCL1. The cells were then stained against VLA-3, VLA-6 and NE, and evaluated for ring formation.

As can be seen in figure 11b, for the NE channel we found 9.2% $\pm$ 3% ring formation for NaCl treated cells on BSA coating (control) versus 25.4% $\pm$ 4.3% for NaCl treated cells on CXCL1 coating, which is a significant increase. For NE channel and DF treated cells, 11.4% $\pm$ 5.3% ring formation was observed for BSA coating and 8.4% $\pm$ 2.8% for CXCL1 coating, which is not a statistically significant difference. In the VLA-3 channel (figure 11d), 8.4% $\pm$ 2.3% of NaCl treated cells on BSA versus 32.6% $\pm$ 6.9% of NaCl treated cells on CXCL1 showed ring formation, which is a statistically significant difference. For DF treated cells, 14.5% $\pm$ 3% on BSA versus 16.3% $\pm$ 6.3% on CXCL1 coating, which is not a statistically significant difference. In the VLA-6 channel (figure 11f), 11.8% $\pm$ 5% of NaCl treated cells on BSA versus 23.4% $\pm$ 5.9% of NaCl treated cells on CXCL1 showed ring formation, which is not a statistically significant difference. For DF treated cells, 8.2% $\pm$ 3.5% on BSA versus 11% $\pm$ 3.6% on CXCL1 coating, which is not a statistically significant difference. In figure 11a, c and e, representative images for each channel/treatment/chemokine combination is shown. In summary, DF2755A treatment of cells did not lead to a statistically significant increase in ring formation in the NE and VLA-3 channels compared to NaCl treated cells. For VLA-6, either treatment did not lead to a statistically significant difference.

Therefore our data suggests that DF2755A inhibits the translocation of VLA-3 and NE to the plasma membrane upon CXCL1 stimulation.

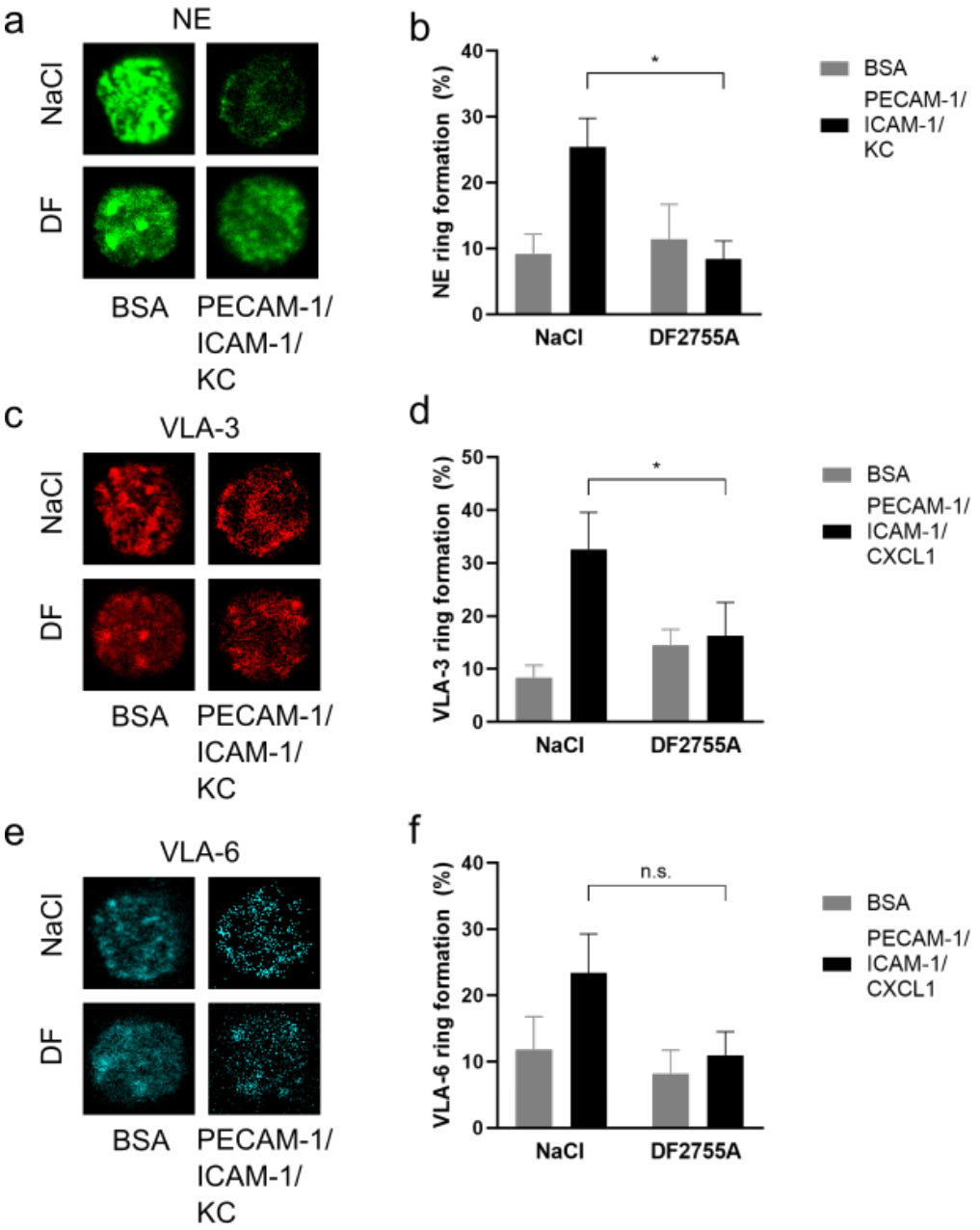

Figure 11: DF2755A inhibits the translocation of NE and VLA-3 to the plasma membrane in response to CXCL1. Data from neutrophils isolated from C57Bl6 mice, then treated with 5 µM DF2755A in NaCl or NaCl, respectively. Representative cells and percentage of cells with ring formation for **a, b, c.** NE channel, or **d, e, f.** VLA-3 channel, or **g, h, i.** VLA-6 channel, and slides with BSA or full coating (PECAM-1/ICAM-1/CXCL1 or short CXCL1) as indicated. $p < 0.05$ where indicated (*) using Students t-test.

5 Discussion

In this project, we addressed the effects of inhibiting CXCR1/2 through small molecule inhibitors on neutrophil recruitment. Together, the results demonstrate that the inhibition of these chemokine receptors by the specific CXCR1/2 inhibitors Ladarixin, Reparixin and DF2755A does not have an effect on neutrophil rolling and adhesion, but on transendothelial and interstitial migration (Section 4). The data also indicates that one possible mechanism for this inhibition of transmigration is the inhibition of mobilization of NE and VLA-3 to the cell membrane (Fig. 10).

The main *in vitro* methods for this study were coated microflow chambers and immunofluorescence staining of murine neutrophils. *In vivo* methods included experiments using intravital microscopy of the mouse cremaster muscle in brightfield and using two-photon microscope and the analysis of said cremaster muscles using Giemsa stains.

Microflow chambers are a widely-used method to study leukocyte interactions with selected proteins[156,159]. However, like many *in vitro* systems, they are somewhat unreliable necessitating a high *n* number and translating to high statistic spread. For example in one of our flow chamber experiments, a mean number of 14 cells could be observed rolling with a standard deviation 5 and a range 3 - 30 in raw data (Figure 3, NaCl). It is plausible that this is due to different activation states of the isolated neutrophils, e.g. due to premature activation *in vitro*. The method could thus be improved by e.g. temperature controlling the isolated neutrophils (at body temperature).

Intravital microscopy provides the unique opportunity to observe cells at work in their physiological milieu, which contributes to the validity of research using these methods. That being said, one limiting factor of this and most other studies on the topic are concerning the dynamics of chemokines. In some adhesion experiments, a quick burst of chemokines triggers adhesion. Many diseases discussed above are of chronic nature and their pathophysiology most probably depends on continous lower dose chemokine concentrations, which weakens the value of these experiments. Additionally, our experiments have been performed in line with chemokine concentrations previously established in literature, not validated concentrations seen in diseases. For example, a dose of 600 ng CXCL8 intravascularly was used in the experiments. Using a molecular weight of 14.7 kDa[160] and a typical blood volume of 1.46 ml per mouse, we get a concentration of around 28 nM vs. a mean measured concentration of 2.5 nM CXCL8 in sputum of COPD patients[53]. To this end, the bridge between our preclinical experiments and clinical diseases and

models has not been completed yet.

Pertussis toxin as a positive inhibitory control has historical roots, since it has been used since the 1980s for leukocyte adhesion experiments[42,127]. Its broad mechanism as a G_{ai2} protein inhibitor may lead to other, to date not investigated, effects on signaling. Therefore, the relevance and specificity of experiments using this compound could be improved using other, more specific inhibitors such as blocking anti-CXCR1/2 antibodies or Cxcr2$^{-/-}$ mice, both of which have already been used in research[161].

Therefore, experiments with more physiological chemokine dynamics profiles and more specific controls, would need to be established in order to further validate these results and bring this research closer "from bench to bedside."

While extensive *in vivo* studies on neutrophil recruitment were performed and presented, the experiments on vesicle mobilization experiments still lack more data. Confirming these results *in vivo*, e.g. using anti-NE/VLA-3 staining in the mouse cremaster, would be required before drawing final conclusions.

Also, some combinations of chemokines and inhibitors such as Reparixin or Ladarixin and CXCL2 *in vitro* and *in vivo* could be added to further strengthen the data and resulting conclusions (see Table 5). Conversely, the multi-photon imaging experiments could be repeated with CXCL1 and/or CXCL8 as chemokines. However it is unlikely this will produce different results and change the conclusion: transmigration counts in Giemsa stained cremaster were performed with both CXCL2 and CXCL1, and comparing both groups showed non-significance (section 4.3). Therefore it can be presumed repeating the multi-photon experiments with CXCL1 will not produce different results than CXCL2.

Surprisingly, there was a statistically significant difference in perivascular cells/mm^2 between saline controls in DF/CXCL1 and LDX/CXCL1 experiments. NaCl/CXCL1 treated mice in DF experiments had a mean number of perivascular cells of 1822 $\pm$ 200 (Figure 9a). In LDX experiments, NaCl/CXCL1 treated control mice showed 506.5 $\pm$ 53.5 perivascular cells per mm^2. The p value using unpaired t test is < 0.01. The experiments have been performed by different researchers with different levels of experience in animal experiments in general and cremaster experiment specifically. Even if the experimental conditions were the same globally, it is plausible that this difference in animal handling and experiment experience, e.g. handling of mice during injections, preparation of the cremaster and handling of the cremaster once exteriorized, explains some of the difference in extravasated cells. A possible mechanism is through induction of stress hormones such as cortisol, which suppress the immune system including neutrophils. On the other hand, part of the difference

could be explained through inter-observer variability, which is a known bias in many diagnostic tests depending on observer interpretation of images[162,163].

In one other study investigating this issue using similar methods, contradicting results on the number of adherent cells *in vivo* were found using Reparixin as an inhibitor[136]. However, the experiments performed there were limited with only n=4 mice per group and only Reparixin/CXCL1 as test compounds. Additionally, not the whole recruitment cascade was analysed. This difference in results highlights the difficulty of *in vivo* cremaster experiments, and the subsequent need for a sufficiently high number of experiment sets and animals within them.

As has been described in the literature before, CXCL1, CXCL8 and CXCR1/2 are involved in the induction of arrest/adhesion and transmigration in the neutrophil recruitment cascade[2]. On one hand, our results confirm that chemokines play an important role in neutrophil transmigration. On the other hand, the results concerning rolling and adhesion were unexpected. While indeed, as described before, with PTx we observed a reduction in neutrophil arrest and adhesion, with the new small molecule inhibitors this reduction could not be observed. Histopathological studies on leukocyte transmigration, similar to those performed here, revealed an inhibitory effect of PTx on this stage as well[164].

In the present study, with all used inhibitors, the chemokine can still bind to the receptor. In the case of PTx this is due to it binding only to the G protein subunits, not the receptor itself. For DF2755A, Ladarixin and Reparixin this was demonstrated in radioactive ligand binding assays[118,119,137]. Therefore, the explanation for the differential inhibitory behavior of PTx and the new small molecule inhibitors may lie between binding of the ligand and triggering GPCR signaling. Alternatively or additionally, the small molecule inhibitors and/or PTx might inhibit other to-date unknown signaling pathways.

While biased **a**gonism was already discussed for these receptors (section 1.2), this biased **anta**gonism was only described with CXCR4 so far[165]. In that study, the authors showed that using a CXCR4 peptide antagonist, they could inhibit signaling and chemotaxis through this receptor, but not cause receptor recycling or tolerance. Similarily, in our study the small molecule inhibitors and/or PTx might only inhibit part of the signaling through CXCR1/2, respectively.

Concerning other pathways, recently it was reported that CXCR2 associates with the receptor CCRL2, probably also a G-protein coupled receptor, which is a prerequisite for CXCR2-mediated neutrophil recruitment[166]. More importantly, the study specifically showed CCRL2 is required for CXCR2-mediated neutrophil arrest and β_2 integrin activation. Now in our case it might be possible that PTx blocks this

receptor in addition to CXCR1/2, which could explain the difference in the adhesion/arrest experiments. A key question here would be if CCRL2 is also required for transmigration.

Regarding chemokines, properties which could explain this difference include dimerization and elongation/truncation. For CXCL8, a report described different affinites and intracellular signaling responses to CXCL8 monomer versus dimer[167]. Furthermore, it was shown recently that CXCL12 heterodimerizes with a galectin to modulate leukocyte chemotaxis and recruitment[168]. Taken together, PTx and/or the small molecule inhibitors might differentially interfere with chemokine dimerization which modulates intracellular signaling and ultimately the recruitment phenotype.

Chemokines appear in different elongated and truncated forms which display different biological characteristics[36]. In that study, there was no significant difference in neutrophil recruitment of elongated, truncated and intact CXCL8; however a difference of effect on *in vitro* chemotaxis was observed. No data has been published so far on inhibiting the different forms of chemokines, so even though this might not explain our results, the question still remains what effect different inhibitors have on the different forms of CXCL8.

From a basic research standpoint, the results of this study maybe open up more questions than they answer. Especially the missing link between chemokine inhibition and normal rolling/adhesion is an important open question for future research. Possible explanations could include biased antagonism at receptor level or other new pathways like CCRL2.

From a clinical standpoint, small molecule chemokine receptor inhibitors could provide a very specific inhibition of neutrophils with limited side effects to other parts of the immune system. Therefore, they have immense potential as immunomodulators. Although there are promising first hints for some diseases like I/R, AD and RA, it remains to be elucidated in which clinical diseases/conditions and under which circumstances they benefit the patient. At the same time, testing other neutrophil chemokine receptor inhibitors for the whole spectrum of neutrophil recruitment like in this study could ultimately provide useful additional information for their clinical use. In combining different (neutrophil) inhibitors it may possible to more finely tune the inhibition of neutrophil recruitment and ultimately tissue damage and disease activity.

6 Summary

Neutrophils are key cells of the innate immune system and are the first cells arriving in acutely inflamed tissue. For this purpose, neutrophils have to extravasate out of the intravascular compartment into tissue, a prerequisite for their effector function. While selectins are responsible for tethering and rolling, the chemokine receptors CXCR1 and 2 in conjunction with leukocyte integrins provide the signaling and activation events for firm adhesion on the endothelium and subsequent transmigration into tissue. Neutrophils and CXCR1 and 2 chemokine receptors have been found to play a role in the pathophysiology of many (auto-)immune diseases and conditions including ischemia/reperfusion injury, rheumatoid arthritis and Alzheimer's disease. Ladarixin, Reparixin and DF2755A are new small molecule inhibitors of CXCR1 and 2. It has been extensively shown that they reduce neutrophil recruitment and subsequent tissue injury in multiple clinical models including experimental acute lung injury, ischemia/reperfusion injury and type 1 diabetes mellitus. However, the exact mechanisms of this therapeutic inhibition have not yet been fully revealed. Therefore, in this project, we addressed the effects of these drugs on neutrophil recruitment using *in vitro* microflow chambers and *in vivo* mouse cremaster muscle models with brightfield and two-photon laser scanning microscopy. As receptor ligands we used CXCL1, CXCL2 and CXCL8. In summary, we found that *in vitro*, CXCR1 and 2 inhibition by Ladarixin, Reparixin and DF2755A does not significantly influence human neutrophil rolling and adhesion versus saline control. *In vivo*, we found that the inhibitors also do not significantly influence neutrophil rolling and adhesion, but neutrophil transmigration versus saline controls. This first in-depth study of small molecule inhibitors and their effect of the recruitment cascade therefore helps to explain the molecular mechanism of these compounds and provides a base for optimizing the treatment of (auto-)immune conditions and diseases using small molecule chemokine inhibitors. Furthermore, the differential role of chemokine inhibition in neutrophils seen here suggests the existence of new, not yet discovered players in neutrophil recruitment.

7 Zusammenfassung

Neutrophile Granulozyten spielen eine entscheidende Rolle im angeborenen Immunsystem und sind die ersten Zellen, die bei akuter Entzündung ins Gewebe rekrutiert werden. Dafür migrieren sie aus den Gefäßen ins Gewebe. Selectine sind zuständig für das Tethering und Rollen, die Chemokinrezeptoren CXCR1 und 2 aktivieren zusammen mit Leukozytenintegrinen die benötigten Signalwege für feste Adhesion auf den Endothelzellen und die darauffolgende Transmigration ins Gewebe. Frühere Untersuchungen zeigen, dass Neutrophile und CXCR1 und 2 Chemokinrezeptoren eine Rolle in der Pathophysiologie verschiedener (Auto-)Immunerkrankungen spielen, unter anderem Ischämie/Reperfusionsschäden, rheumatoide Arthritis und Alzheimer-Demenz. Ladarixin, Reparixin und DF2755A sind neue kleinmolekulare Inhibitoren von CXCR1 und 2. Es wurde bereits einschlägig gezeigt, dass diese Substanzen Neutrophilenrekrutierung und damit einhergehende Gewebszerstörung in mehreren klinischen Modellen, z.B. experimentelles akutes Lungenversagen, Ischämie/Reperfusionsschäden und Diabetes mellitus Typ 1, verhindern. Aber die genauen Mechanismen dieser therapeutischen Hemmung sind noch nicht vollständig aufgeklärt. Daher haben wir in dem vorliegenden Projekt die Effekte dieser Substanzen auf Neutrophilenrekrutierung mithilfe *in vitro* Flusskammern und *in vivo* Maus-Cremastermodellen unter Zuhilfenahme von Licht- und Zweiphotonenmikroskopie untersucht. Als Rezeptorliganden haben wir CXCL1, CXCL2 und CXCL8 verwendet. Zusammengefasst haben wir gefunden, dass CXCR1 und 2 Blockade mittels der oben gennanten Substanzen - Ladarixin, Reparixin und DF2755A - das Rollen und die Adhäsion von humanen Neutrophilen *in vitro* nicht beeinflusst. Die *in vivo* Mausexperimente zeigten ebenfalls, dass die Inhibitoren keinen signifikanten Einfluss auf Rollen und Adhäsion von Neutrophilen haben, wohl aber auf die Transmigration von Neutrophilen. Diese erste ausführliche Untersuchung von Chemokinrezeptorinhibitoren und deren Wirkung auf die Neutrophilenrekrutierungskaskade hilft daher die molekularen Mechanismen dieser neuen Substanzen besser zu verstehen und bereitet den Weg für die Optimierung der Therapie verschiedener (Auto-)Immunerkrankungen mit ihnen. Darüberhinaus deutet die hier gesehene differenzelle Chemokinrezeptorblockade bei Neutrophilen auf neue, bisher noch nicht bekannte Akteure in der Neutrophilenrekrutierung hin.

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9 Abbrevation list

AD Alzheimer's disease

ALI Acute lung injury

ANC Absolute neutrophil count

ANOVA Analysis of variance

ARDS Acute respiratory distress syndrome

ASH Alcoholic steatohepatitis

BSA Bovine serum albumin

CCL*n* C-C chemokine ligand *n* (CCL2 = MCP-1 = monocyte chemoattractant protein 1, CCL5 = RANTES = regulated on activation, normal T cell expressed and secreted)

CCR*n* C-C chemokine receptor *n*

CCRL*n* C-C chemokine receptor-like *n*

COPD Chronic obstructive pulmonary disease

CXCL*n* C-X-C chemokine receptor ligand *n* (CXCL8 = IL-8 = interleukin 8; CXCL1 = KC = keratinocyte-derived chemokine)

CXCR*n* C-X-C chemokine receptor *n*

DF DF2755A

ECM Extracellular matrix

GPCR G-protein coupled receptor

HPF High power field (100x magnification)

ICAM Intercellular adhesion molecule

I/R Ischemia-reperfusion (injury)

LDX Ladarixin

LFA-1 Lymphocyte function-associated antigen 1 (CD18/CD11a; $\alpha L\beta 2$)

mRNA Messenger ribonucleic acid

NE Neutrophil elastase

NET Neutrophil extracellular traps

RPX Reparixin

PBMC Peripheral blood mononuclear cell

PBS Phosphate-buffered saline

PKD Polycystic kidney disease

PMN Polymorphonuclear leukocyte

PSGL-1 P-selectin glycoprotein ligand 1

PTx Pertussis toxin

RA Rheumatoid arthritis

STIM Stromal interaction molecule

T1D Type 1 diabetes mellitus

TNF-α Tumor necrosis factor α

TRP Transient receptor potential

UC Ulcerative colitis

VLA-3 Very late antigen 3 (α3β1, CD49c/CD29)

VLA-6 Very late antigen 6 (α6β1, CD49f/CD29)

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11 Appendix

11.1 List of Figures

11.2 List of Tables

12 Affidavit

I,

Sebastian Sitaru

hereby declare, that the submitted thesis entitled

CXCR1/2 Inhibition in Neutrophil Recruitment

is my own work. I have only used the sources indicated and have not made unauthorised use of services of a third party. Where the work of others has been quoted or reproduced, the source is always given.
I further declare that the submitted thesis or parts thereof have not been presented as part of an examination degree to any other university.

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