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# *The Effects of Ladarixin in Leukocyte Recruitment*

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# 1 INTRODUCTION

Neutrophilic granulocytes make up the greatest part of our leukocytes in peripheral blood. In exact numbers this means 40–70% of 4000-10 000 white blood cells per µl whole blood. In the mouse, those numbers are lower but still relevant with 10-30% of blood leukocytes[2–4]. Neutrophils are one of the first immune cells arriving at sites of tissue injury or infection. After differentiation in the bone marrow from myeloid precursor over myeloblast, promyelocyte, myelocyte, metamyelocyte, band cell to, finally, the segmented/polymorphonuclear neutrophil, also called PMN[5], the free flowing cells released into the vasculature can read inflammatory signals on the vascular endothelium. Those are mostly passed on by tissue resident immune cells and induce the neutrophil recruitment cascade consisting of: tethering, rolling, activation and firm adhesion to the inflamed vascular wall followed by intraluminal crawling to find a suitable transmigration spot. During this final step of emigration not only the endothelium, but also the underlying vascular basement membrane (BM) and pericyte layer must be overcome[6]. This process has been elucidated in great detail over the last decades and will be further explained in an own chapter. Reaching the site of injury or microbial infection, neutrophils make use of their phagocytic capabilities and the content of their many intracellular granules, to which they owe their name “granulocyte”, to defend us against harmful pathogens[5].

This has long thought to be their only task. But in the past years the scientific community had to realize that in their short-lived life, comprised of a half-life in circulation of around 8 hours in mice and humans[7], neutrophils might do a lot more than previously expected. New findings suggest neutrophils not only on the fighting front against bacteria and fungi, but also against viruses and intracellular pathogens. Next to their important and diverse involvement in the innate immune response, they also appear to be steering and interacting with B- and T-cells from the adaptive immune system. A contribution to the resolution of inflammation and remodeling is also being discussed. Besides positive new revelations about neutrophil functionality, their role in many different diseases became quite apparent. Obesity and diabetes, atherosclerosis and thrombus formation, chronic and acute lung diseases, allergic and anaphylactic reactions, autoimmune diseases or cancer as well as sepsis and neurodegenerative diseases are some of the examples where neutrophil engagement has been reported[8,9]. While the relevance and exact mechanisms of neutrophil involvement are still unclear in many cases, this nevertheless gives an opportunity to better understand some of those diseases and is stirring up hope for new, specifically targeted therapies. As most

neutrophil-derived or associated pathologies arise through neutropenia or neutrophils with lacking or excessive activity, new treatment ideas are directed towards enhancing or inhibiting neutrophil function and increasing or decreasing neutrophil numbers. Also a certain "derailment" of neutrophil capabilities can cause or fuel disease, in which case bringing the neutrophil back on track would be a helpful strategy[9].

This thesis will focus on one of those approaches, as the herein tested small molecule inhibitor Ladarixin, targeting neutrophil chemokine receptors CXCR1 and 2, has shown to successfully reduce neutrophil extravasation and related tissue damage. But the question of "How?" remained and will now be partly unraveled in this dissertation.

## *1.1 The Neutrophil Recruitment Cascade*

How immune cells reach their place of use has been a topic of great interest for many years. Fortunately for neutrophils, most of the required recruitment steps have been described in detail, so today there is quite a lot of knowledge and literature regarding the neutrophil recruitment cascade.

When tissue gets injured or infected, so-called danger- or pathogen-associated molecular patterns (DAMPS/PAMPS) are released which lead resident innate immune cells, like macrophages, dendritic cells or mast cells, to spread pro-inflammatory mediators. Either those, or direct pathogen contact via the pathogen recognition receptors (PRRs), signals the endothelium of the nearby microvasculature to upregulate adhesion and rolling relevant molecules like E- and P-selectin. In the initial phase pre-stored P-selectin (from Weibel-Palade-Bodies) will most likely be the main player, as E-selectin needs to be upregulated via transcription. Both murine and human E-/P-selectins bind neutrophil expressed P-selectin glycoprotein ligand-1 (PSGL-1)[5,10,11]. This results in the first step of the recruitment cascade: the tethering or capturing of neutrophils from blood flow. The subsequent rolling along the inflamed vessel wall is realized by rapid building and breaking of such selectin-PSGL-1 bonds, also involving membrane "tethers" and "slings" to slow down the process[12]. While rolling, neutrophils encounter chemokines, also presented on the activated endothelium. Here, the ELR-CXC chemokines are important, but not the only contributors. They are distinguished by the combination of glutamate-leucin-arginine before their amino-terminal CXC motif and

include CXCL8 (IL-8, “IL” for interleukin) in humans and the mouse counterpart: CXCL1 (also keratinocyte chemoattractant/KC). Also, the chemokines CXCL2, CXCL3, CXCL5 and CXCL7 are involved. Neutrophils are equipped with the matching ELR-CXC chemokine receptors, called CXCR1 and CXCR2. Binding results in activation of neutrophils, paving the way for the following firm adhesion and intracellular signaling that supports succeeding steps like crawling and chemotaxis, transmigration, degranulation and the initiation of the nicotinamide-adenine-dinucleotide-phosphate (NADPH) oxidase pathway[13–15]. As most neutrophils might already be pre-primed by earlier exposure to cytokines or PAMPs, full activation can be seen as a two-step process, necessary for reaching the neutrophils maximum defense capabilities[16]. Tethering and activation are the prerequisites for the so called “inside-out-signaling”, where the extracellular inflammatory signal gets passed on by the G-protein-coupled chemokine receptors or other receptor systems, resulting in a conformational change of the constitutively and highly expressed surface β2-integrins: Lymphocyte function-associated antigen 1 (LFA1, αLβ2 or CD11a/CD18, with “CD” for cluster of differentiation) and macrophage-1-antigen (MAC1, αMβ2 or CD11b/CD18). They switch from their compact and bent resting form to the extended one, with an initially closed then open conformation. This transition translates into higher affinity states towards their endothelial ligands: Intercellular adhesion molecule 1 and 2 (ICAM1/2). While the extended but closed conformation represents intermediate affinity and is needed for slowing the neutrophil further down, the fully extended open form shows the highest affinity and mediates firm adhesion to the vessel wall by binding of LFA1 or MAC1 to ICAM1/2[17]. To impede later detachment, integrin clustering can be observed as a method of “adhesion strengthening”. This ligation between ICAM1/2 and LFA1 or MAC1 initiates the “outside-in-signaling”. The external bond activates intracellular pathways by conformational changes and transducers like protein tyrosine kinases (PTKs) leading to neutrophil spreading and crawling. As a result, neutrophils can travel quite some distance while probing the surroundings to find a suitable spot to leave the vessel. This is implemented mainly by MAC1 and ICAM1. The exact role of ICAM2 in this setting is still unclear[6,10,18]. The diapedesis through the monolayer of the endothelium is observed mostly paracellular at endothelial cell junctions and takes about 2-5 minutes. But also the more time-consuming transcellular route occurs (20-30 minutes)[6,19]. Regarding this transendothelial migration (TEM), many endothelial cell adhesion molecules and receptors have been identified that seem to operate in the following sequential manner from the apical endothelium down to the basement membrane: ICAM2, junctional adhesion molecules (JAMs), endothelial cell-selective adhesion molecule (ESAM), platelet endothelial cell adhesion molecule-1 (PECAM-1), Poliovirus receptor (PVR), CD99 and CD99L. Although this list is far from complete and their interplay, importance and exact

role is not entirely clear yet, more light has been shed on this part of transmigration than on the following steps of overcoming the vascular basement membrane and pericyte layer[10].

### *1.1.1 Overcoming the Basement Membrane – Squeezing or Degradation?*

The migration through the vascular basement membrane with its embedded pericytes still remains incompletely defined, compared to the many preceding processes. This lamina, underlying the endothelial cells, gives structural support and has been discovered to also mediate functional cues. The basement membrane components of this 100-300nm thick layer are produced by surrounding endothelial and pericyte cells and include mainly two different network-forming laminin isotypes and collagen type IV, interconnected (non-covalent) by other components such as nidogens and perlecan. Both collagen IV and laminins have the ability to self-assemble into polymeric structures and crosslink via covalent bonds. Laminins are composed of three subunits: an α, β and γ chain, with the α-chain as cell-binding part. In our context, meaning the endothelial BM of postcapillary venules, the two isoforms laminin 411 (also laminin 8 or α4β1γ1) and laminin 511 (laminin 10/α5β1γ1) occur. While laminin 411 is found evenly everywhere, laminin 511 has regions of high, low or no expression. In studies, areas of low laminin 511 content combined with less collagen IV and nidogen-2 expression (not perlecan) as well as associated pericyte gaps proved to be the preferred exit sites for neutrophils[20–22].

Regarding the question of how neutrophils overcome the BM barrier, two competing hypotheses exist. As the average BM pore size measures around 50nm[22], an easy wandering through, even by deformed neutrophils, seems rather unlikely. The first theory states that neutrophils might squeeze through non-proteolytically enlarged gaps at the before mentioned “low expression regions” (LER). This is supported by the assumed looser cross-linking between laminin 411 and collagen IV, compared to the “anti-migratory” laminin 511[23]. Also structural changes conveyed by mechanical/”pulling” forces from the endothelial layer during TEM are being discussed[24,25]. Another argument is the fact that neutrophils constantly move through the BM network without leaving any trace[26].
The second theory goes in the direction of degrading or disassembling the venular BM in order to breach through. Irrespective of which theory, this part of the venular wall penetration seems to be more complex and laborious than the previous steps of transendothelial migration, as it takes more than three times longer, up to 15 minutes[5]. Also, BM penetration was shown via *in-vitro* experiments to only take place with the overlying endothelium, supporting the idea that

TEM not only prepares the neutrophil for the following diapedesis, but possibly also the BM by creating mechanical forces and BM structural changes [22,27,28]. This thesis is focused on the second possible hypothesis. The neutrophil is equipped with a large selection of enzymes with antimicrobial activity. Some of them were found to successfully degrade components of the BM. Different studies have shown temporary enlargement of LERs during neutrophil transmigration and found laminin on extravasated neutrophils, which was not produced by themselves but associated with a cell surface integrin called very late antigen 6 (VLA-6, also integrin α6β1 or CD49f)[20]. Mainly VLA-6, but to a smaller extent also VLA-3 (α3β1/CD49c), are upregulated upon neutrophil activation[29,30] and are widely known as the "laminin-binding" integrins, which leaves them with the task of initially providing contact to the obstructing BM[31]. Additionally, the neutrophil-derived serine protease neutrophil elastase (NE) was shown to be able to cut the β1 chain from the laminin heterotrimer and thereby degrade the tight BM network while also releasing potential chemotactic fragments[32]. Besides laminin, NE and the other serine proteases proteinase-3 and cathepsin G can successfully degrade a variety of extracellular matrix (ECM) components such as collagen IV, elastin, fibronectin and vitronectin. Also the three matrix metalloproteinases (MMPs) MMP-8 (or neutrophil collagenase), MMP-9 (gelatinase) and MMP-25 (also called MT6-MMP or leukolysin) showed proteolytic activity against ECM parts like collagens, laminin, fibronectin, proteoglycans and gelatin and were affiliated with BM disassembly as well as making way for interstitial migration[33,34]. This specific degradation machinery waits prestored in intracellular vesicles to be translocated to the cell surface and then released. The neutrophil elastase, as well as proteinase-3 and cathepsin-G can be found in primary azurophilic granules, MMP-8 resides in the specific granules, MMP-9 mainly in the gelatinase granules, while MMP-25 is dispersed over different granules and the cell surface[33]. The BM-contact-providing integrins VLA-3 and VLA-6 are allocated to the secretory vesicles[35]. This "degradation-theory" for BM transmigration is supported by studies showing reduced neutrophil influx, when serine proteases lack activation in a mouse dipeptidyl peptidase 1 (DPP1) knockout model[36] . Similar circumstances were observed in wound healing experiments with MMP-8 deficient mice[37]. Regarding NE, VLA-3 and -6, multiple papers describe their importance during this process: Lerman et al. (2014) showed a reduced amount of extravasated neutrophils in a murine sepsis model after peptide blockade or knockout of VLA-3[38]. Antibody-inhibition of VLA-6 presented neutrophils stuck between the endothelium and the BM unable to pass over into the tissue[30,39]. This was complemented by *in-vitro* and *in-vivo* studies, where NE blockade presented with reduced perivascular basement membrane penetration[40,41]. This sums up to neutrophils being unable to traverse the vascular BM or being "stuck" before this final layer upon knockout or specific inhibition of the three players VLA-3/6 and NE. Further assessing these findings, some

of the studies noticed a missing mobilization of those intracellularly stored compounds from their granule storage to the cell surface, their site of action, after respective inhibition as the underlying cause[30,31,35,39–41]. Although, as of today, multiple arguments and experimental results for both hypotheses exist, the involvement of the neutrophil-derived serine protease NE is very probable in view of current data, also from our lab, and is therefore the focus of this thesis.

## *1.2 Which Granule has what?*

The neutrophil's intracellular space is packed with multiple granular structures that can be distinguished into different subtypes by their time of appearance, content, surface proteins or also staining reactivity. Granules develop sequentially due to alternately timed protein expression during maturation in the bone marrow until a mature neutrophil finally holds three granule sets and additional secretory vesicles, which stem from endocytic origin (*Figure 1*)[1].

The first granules to develop during the promyelocyte stage, as the name already implies, are the **primary or azurophilic granules** (peroxidase-positive). The production of the affiliated granule content has already begun in the preceding myeloblast and represents a broad antimicrobial weaponry, including oxidant-producing myeloperoxidase (MPO), lysozyme, bacterial-permeability increasing protein, defensins and proteases, like azurocidin (only in humans), cathepsin G, proteinase 3 and our enzyme of interest, the neutrophil elastase (NE). Azurophilic granules can be subdivided into defensin-poor and defensin-rich, although the purpose or relevance of this finding is unclear and this circumstance only applies to human neutrophils, as murine neutrophils do not harbor defensins[1,5]. Another categorization splits the primary granules according to their target. While one subset is aligned for fusion with the phagosome in order to deliver the antimicrobial content at a high concentration, another group of granules is equipped with a signaling machinery that enables the granule translocation to the cell surface[42].

In the following stages of neutrophil development, the **secondary or specific granules** appear, succeeded by the **tertiary or also gelatinase called granules**. In contrast to the azurophilic granules, those granules are mostly meant for cell surface exocytosis. Their production time and protein content overlaps as they also share their functionality. Next to aiding the extravasation and migration process they create a hostile tissue environment for

immigrated microorganisms. Therefore, they carry integrins like MAC-1, chemotactic and cytokine receptors as the formyl peptide receptor 1 (fPR1), the ECM-degrading proteases MMP-8 (collagenase), MMP-9 (gelatinase) and MMP-25, and antimicrobial effectors such as lactoferrin, lysozyme, secretory phospholipase, or NADPH components. Although they share similar functions and protein assets, they differ in lactoferrin and gelatinase expression. While secondary/specific granules only hold lactoferrin (lactoferrin+, gelatinase-; also, neutrophil gelatinase-associated lipocalin +/NGAL+), tertiary/gelatinase granules owe their name to the gelatinase+/lactoferrin- protein content. To complicate this even more, also "hybrid" granules were detected, containing both the lactoferrin and gelatinase marker (lactoferrin+, gelatinase+)[1,43].

The last "granule" type, the **secretory vesicles (SVs),** are unique in many aspects. While all other granules are formed during maturation and therefore have prestored content upon release from the bone marrow, the secretory vesicles continue to develop in the mature segmented neutrophil. As mentioned, they are formed by a still unidentified endocytosis process and retain cell surface as well as serum proteins for later release. This includes transmembrane proteins, providing contact to the endothelium in the extravasation process like the crawling-associated integrin MAC1, also cytokine and chemokine receptors like the studied CXCR2 receptor, and others as the fPR1[1,44]. SVs also seem to be meant for cell surface deployment, but how exactly they reach this aim and whether endocytosis is their sole source for content, is still under debate[45].

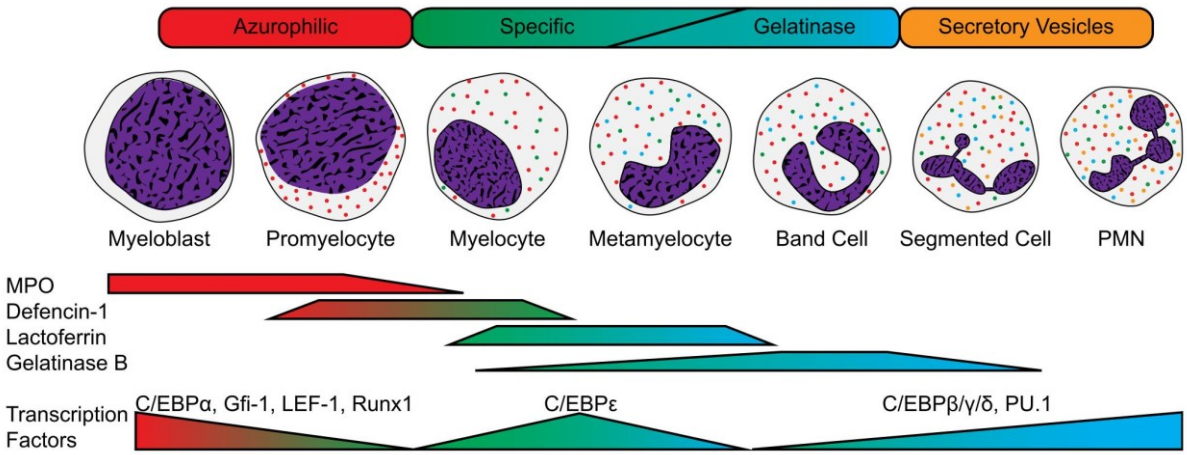


**Figure 1: Granule development during neutrophil maturation[1]**

This figure from Yin et al. (2018) shows the neutrophil's maturation process which is accompanied by the sequential development of their granula (upper row). Time-dependent protein expression (middle row) is regulated by changing transcription factors (lower row).

Since neutrophils never cease to surprise, another, possibly new granule type has been discovered in 2009 by Rørvig et al.: They found a population enriched with the microbe-binding ficolin-1, which is part of the lectin complement pathway that aids the recognition and marking of invading microorganisms[46].

All the mentioned granule subsets seem to follow a strategically and functionally rational order of degranulation. More recent results show that this sequential "deployment" is realized by different degrees of inflammatory stimuli, resulting in a slight to major change of intracellular calcium levels. With the SV being the most sensible to inflammatory stimuli, needing only a minor increase in calcium levels, they are the first ones to translocate their content to the cell surface in order to provide the machinery for initial neutrophil adhesion to the vasculature. With more intense inflammatory activation and calcium signals, degranulation continues with the gelatinase, then "hybrid" and specific granules, followed by the azurophilic granules with the highest threshold. The ficolin-1 rich granules seem to be timed in between the gelatinase granules and SVs. This cascade also coincides with the tasks and functions of the different granule components during neutrophil extravasation from the initial adhesion and activation to final pathogen elimination[1,43,44,47].

### *1.2.1 The Intracellular Signaling Cascade – How Granules reach the Surface*

The greatest part of granule content is meant for degranulation or connection with the cell surface to support the neutrophil in its many functions throughout the transmigration and defense process. This accurately timed mobilization from intracellular storage to the cell membrane is realized by intracellular signaling, which, at least up until today, has been elucidated to some extent for the azurophilic, specific and gelatinase granules, while the trafficking of SVs is still in the dark[1]. In this thesis the focus is on the translocation of the protease neutrophil elastase from its storage in the azurophilic granule to the cell surface as important requirement for the BM-degrading activities in the process of extravasation. Here, a small GTPase ("GTP" for guanosine triphosphate) called Rab27a with its effectors synaptotagmin-like protein 1 (JFC1) and Munc 13-4 have been identified as important players. Different studies, also from our lab, have shown that NE, as well as the two laminin-binding and therefore BM-contact-providing integrins VLA-3 and VLA-6, are stored in Rab27a-positive intracellular granules, which need to be successfully mobilized to the cell membrane in order to penetrate the vascular BM. Otherwise, emigrating neutrophils become stuck in between the endothelial and basement membrane layer, unable to continue their extravasation[39,41,48]. While the major effector JFC1 is only associated with azurophilic granule release, Munc 13-4

coordinates a larger spectrum of different organelles next to azurophilic degranulation[48,49]. They also differentiate in their purpose: JFC1 regulates the granule transport together with the effectors gem interacting protein (GIMP) and RhoA and provides contact between Rab27a on the granule and phosphoinositides on the inner cell membrane. Munc 13-4 on the other hand immobilizes the Rab27a-positive vesicle in that position and helps to induce membrane fusion[50,51]. In an *in-vitro* setting this VLA-3/VLA-6/NE-"ring formation" (because of the microscopically observed accumulation of the stained proteins on the cell surface, thus forming a ring) is induced by exposure to PECAM1/ICAM1 and a stimulating chemokine like CXCL1[31,35,41,52]. Using this method with additional *in-vivo* corroboration, Kurz et al. (2016) also discovered the involvement of the mammalian sterile 20-like kinase 1 (MST1) in this intracellular signaling cascade[31]. It seems to cooperate with the Rab27a effector protein JFC1, but not Munc 13-4, and colocalizes with Rab27a in low density granules and at the plasma membrane, since Rab27a as well as the two effectors were also found to form a ring after PECAM1/ICAM1/CXCL1 stimulation[35]. MST1 deficiency results in impaired VLA-3/VLA-6 and NE translocation, causing a failure in vascular BM penetration. The consequences of this can be observed in a known immunodeficiency syndrome caused by a loss-of-function mutation in the MST1 encoding gene STK4 (serine threonine kinase 4), which also includes recurring acute bacterial infections[31].

But how do Rab27a and the effectors know when it is time for degranulation? The upstream signaling events are quite complex and not yet fully understood. The azurophilic granules hold the highest threshold for degranulation (see subsection *1.2*), meaning they need the greatest amount of intracellular free calcium[1,47]. Such calcium levels are induced by strong activation of the adherent neutrophil via chemokine signaling, for example through the G-protein coupled receptors (GPCRs) CXCR1/2 and simultaneous stimulation of the pathogen related Fc-Receptor[1,53]. GPCRs and their ligands (CXCL-chemokines for CXCR1/2 or N-formyl-methionyl-leucyl-phenylalanine (fMLP) for fPR1) lead, upon activation, to the replacement of guanosine diphosphate (GDP) with GTP by guanin-exchange-factors (GEFs) at the α-subunit of the G-Protein and the dissociation of the β and γ unit of the original heterotrimer. Further downstream signaling via phospolipase Cβ (PLCβ), inositol-3-phophate (IP3) and diacylglycerol (DAG) then leads to calcium release from the endoplasmatic reticulum (ER; via IP3) or the extracellular space (via DAG, proteinkinase C and store-operated-$Ca^{2+}$-channels)[1,54,55]. A similar end-stage pathway can be found for calcium influx signaled through the FcγRIIA and even complement receptors on the neutrophils surface, although a very strong GPCR activation alone by a high amount of ligands, e.g., released CXCL-chemokines, might be sufficient to release azurophilic granules[1,56]. Next to mobilizing enough calcium for this degranulation, another GTPase of the Rho-family called Rac2 is specifically important in the

downstream signaling for primary granule release. Rac2 seems to be an initiator of actin cytoskeleton reorganization, without which granule transport to the plasma membrane is massively impaired[1,57]. Recently, our lab also focused on the role of SRC family kinases (SFKs) as central players in the outside-in-signaling process[35]. In the neutrophil recruitment cascade (see chapter *1.1* for details) this step is conveyed by firm adhesion between the integrins LFA1/MAC1 and their endothelial ligands ICAM1/2. This "outside" signal results in internal signaling leading to adhesion strengthening, spreading and polarization by cytoskeletal rearrangements and subsequent crawling. Because the intracellular integrin end is short and not enzymatic, further signaling has to be undertaken by recruited proteins with this capability, one of them being the SRC family kinases. The results for mice deficient in neutrophil SRC kinases Hck/Fgr/Lyn showed not only weaknesses in before mentioned post-arrest modifications but surprisingly also in vascular BM penetration. Following this lead, impaired Rab27a-dependent vesicle transport was observed with missing mobilization of the main actors NE, VLA-3, VLA-6, but also the signaling participants Rab27a, JFC1 and Munc 13-4, to the cell surface upon PECAM1/ICAM1/CXCL1 stimulation. After finding SFK translocation to the cell membrane in wildtype neutrophils with the same stimulus, it is probable that SRC family kinases regulate BM relevant vesicle trafficking upstream of Rab27a[35]. Next to that, the kinase Hck was found to colocalize with azurophilic granules after GPCR signaling, which in general showed to trigger SFKs[58]. A central role for SFKs in granule translocation is also supported by the findings that SFKs are involved in Rac2 activation, the FcγRIIA signaling for calcium release and a rather unexplored degranulation pathway via p38 mitogen-activated protein kinase (p38MAPK)[1,59–61].

The final fusion with the cell surface is then achieved with the help of a SNARE complex (soluble N-ethylmaleimide-sensitive-factor attachment receptor), whose establishment was previously inhibited by calmodulin and complexin. Here the calcium comes back into play as it both liberates the SNARE complex after an intracellular calcium boost while the calcium-sensing synaptotagmin induces membrane merger[1,62–64]. A schematic overview of the described processes can be found in *Figure 2*, without claim to completeness.

In summary, there are many findings for different signaling and degranulation pathways, which are still incompletely understood. But various initial extracellular stimuli seem to work together and reunite in two main degranulation initiators: calcium and Rac2. The initial essentials for azurophilic, and therefore NE degranulation, are signaling cascades induced by firm adhesion, strong chemokine activation or also pathogen contact. Those result, via SFK involvement, in elevated intracellular calcium levels and activation of Rac2 as major degranulation mediators,

followed by Rab27a, JFC1 and Munc 13-4 aiding the vesicle transport to the fusing SNARE complex.

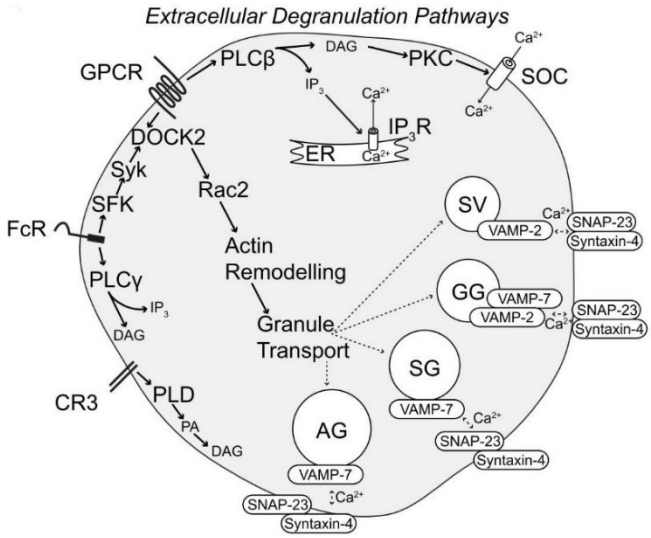


**Figure 2: The complex signaling cascades for neutrophil degranulation[1]**
This schematic overview form Yin et al. (2018) shows the complexity of signaling for granule release. Main initiators are GPCRs signaling via PLCβ, next to Fc- or complement-receptors. Joint downstream pathways lead to actin remodeling via Rac2 and intracellular calcium peaks. Recently, also the involvement of SFKs has been further elucidated. The final membrane fusion is aided by the SNARE complex. (For detailed elaboration see chapter *1.2.1*)

## *1.2.2 The Neutrophil Elastase*

The neutrophil elastase (NE) is a serine protease preproduced and stored in neutrophil primary or azurophilic granules next to the more broadly known myeloperoxidase (MPO) and two other serine proteases, proteinase 3 and cathepsin G. All three serine proteases are part of the chymotrypsin family and though similar in many aspects, meant for their own specific tasks. NE is encoded by the ELANE gene (also known as ELA2) and occurs not only in neutrophils, but also in monocytes (low concentrations), eosinophils and mast cells[65,66]. Several ELANE gene mutations can cause two main forms of congenital neutropenia: cyclic neutropenia and severe congenital neutropenia (SCN)[65]. The initial inactive pro-NE needs to be cleaved by the cysteine protease cathepsin C (also known as dipeptidyl peptidase 1/DPP1) to reach an active state, a typical mechanism for activating enzymes[36,66]. In this form, NE is stored in azurophilic granules, awaiting degranulation upon inflammatory stimuli either into intracellular phagolysosomes or the extracellular compartment with parts staying attached to the neutrophil cell surface[67]. With the ability to degrade elastin, fibronectin, laminin, collagens and proteoglycans next to a variety of plasma proteins, uncontrolled enzymatic activity needs to be prevented by the means of endogenous inhibitors. This task is carried out by protease inhibitors of the serpin (α1-Proteinase inhibitor/α1-Antitrypsin (α1-PI), Monocyte/neutrophil

elastase inhibitor (MNEI), Proteinase inhibitor 6/9 (PI6/9)) and chelonianin (Secretory leukocyte proteinase inhibitor (SLPI), elafin) families, as well as by α2-macrogobulin[66,68]. While NE was at first only recognized for its degrading capabilities in the ECM or intracellular phagosome compartment during inflammation, today this view has broadened. NE shows bactericidal capacities through cleaving virulence factors like flagellin and membrane parts of gram-negative bacteria, as well as abilities useful in fungal defense[66,69,70]. Recently, NE has also been found to be involved in the formation of neutrophil extracellular traps (NETs). After an MPO-dependent activation, NE with its cleavage capacities is necessary for chromatin decondensation and the actual NET release via degradation of histones and actin. The antimicrobial abilities of NETs also stem from primary granule content. As the interest in NETs has grown over the past years, also because of their suspected involvement in many chronic and acute inflammatory diseases, the curiosity towards NE participation has also increased[71]. Next to holding more host defense mechanisms than previously expected, NE is also a strong regulator at the local inflammatory site. Cleaving inflammatory mediators, processing or inducing chemokines and cytokines, cleavage or stimulation of receptors, activation of proteases (like MMP-9)[40] and even lymphocytes and platelets, as well as inactivating inhibitors are some of the recently discovered pro- or anti-inflammatory capacities of neutrophil elastase[66]. One specific example in this context is the newly uncovered involvement of NE in the intracellular cleavage and trafficking of Gasdermin-D (GSDMD), a driving protein in inflammasome activation and pyroptosis[72]. Another topic is the very unclear und highly controversial matter of BM penetration, where NE has been suspected and shown to be an important component. Through translocation to the cell surface during degranulation, NE can unfold its degrading capabilities, especially towards Laminin, and help overcome this final barrier in transmigration[31,32,35,40,41,48,73].

Of note, when the activity of NE is not kept properly in check in inflammatory settings, massive tissue destruction caused by the broad substrate specificity can be the consequence[40]. Examples are different lung diseases, like chronic obstructive pulmonary disease (COPD), acute respiratory distress syndrome (ARDS), cystic fibrosis (CF) or ischemic-reperfusion injury (I/R)[66]. After PMN recruitment via acute or chronic inflammatory stimuli, evidence suggests on one hand edema- and emphysema-causing endothelial toxicity and ECM degradation, on the other hand secretagogue and airway obstructing abilities of extracellular NE, which would be typical with CF and COPD[66,68,74]. Other cases of suspected NE involvement are bullous pemphigoid, arthritis, glomerulonephritis, delayed wound healing and acute promyelocytic leukemia (APL)[66,68,75].

How and why a protease like NE stays active in certain immunological settings regardless of endogenous inhibitors and thereby opens a path towards major organ damage, is still a debated topic with different hypotheses[66]. Answering those questions however is of great importance for future pharmacological advancements, as it will be a challenge to inhibit NE's harmful side while still upholding capacities relevant for host defense.

## *1.3 The CXCR1/2-Receptors: Functions and Pharmacological Targeting*

### *1.3.1 The CXCR1/2 Receptors, their Ligands and Functions*

The neutrophil's surface is equipped with a lot of different receptors. Many of those are able to react to specific protagonists of an inflammatory process. These include G-protein-coupled-receptors (GPCRs), Fc-receptors and adhesion receptors (selectins/selectin ligands/integrins), cytokine receptors and Toll-like-receptors. Since this thesis is about new findings regarding an inhibitor interacting with the GPCRs CXCR1/2, the focus is on those receptors and their ligands, physiological function, and role in different diseases. Next to the mentioned CXCR1/2, also receptors for leukotriene B4, platelet activating factor, complement fragment C5a as well as the fMLP receptors (fPRs) and the CCR1/2/5 receptors for CC-chemokines like CCL5 belong to this group. They all have in common that upon ligand binding they show a strong chemotactic reaction[8,14,76].

In humans and mice, the CXCR1 and 2 receptors are the crucial chemokine receptors on neutrophils with CXCR2 being predominant[9,15]. Both are not only present on neutrophils, but also on T-lymphocytes, natural killer cells, inflammatory macrophages, monocytes, mast cells, basophils, endothelial and epithelial cells, fibroblasts, keratinocytes, melanocytes, smooth muscle cells, hepatocytes and even neurons[77–79]. The chemokine receptors are composed of seven helical transmembrane regions (TMs) and are intracellularly coupled to a pertussis toxin sensitive heterotrimeric G-protein of the $G_{i/o}$ -family (for details regarding the intracellular signaling cascade see the associated chapter *1.2.1*)[14]. In the context of neutrophil recruitment their ligands consist of chemokines from the ELR-CXC family, which are defined by a glutamate-leucine-arginine part located before their CXC motif[13]. In humans the ELR-CXC

chemokines CXCL6 and 8 bind to both CXCR1 and 2, while CXCL5, 7 and CXCL1-3 specifically target CXCR2. Though this is similar in mice, the chemokine CXCL8 has not been identified in mice so far. But murine CXCL1 (mCXCL1/mKC) has shown to be equivalent for CXCL8 (IL-8) and has therefore prevailed as substitute in the scientific community for murine approaches[15].

The CXCR1/2 receptors and their respective chemokine ligands are attributed with important physiological tasks during neutrophil recruitment to an inflammatory site. After initial tethering to the vasculature, neutrophils get into close contact with the inflamed endothelium. This enables the neutrophil receptor CXCR1/2 to interact with ELR-CXC chemokines, which are immobilized by glycosaminoglycans (GAGs) like heparan sulfate on the endothelium[80]. This interaction activates the neutrophil, further leading to the “inside-out-signaling” and induction of firm adhesion. In addition, it is an important requirement for subsequent steps, initiating neutrophil spreading and crawling (“outside-in-signaling”)[5]. Here, neutrophil CXCR1/2 again come into play while they guide the moving neutrophil alongside a chemokine gradient. Also the succeeding interstitial navigation can be supported by CXCR1/2-chemokine interaction, but is mostly substituted by so called “end-stage” chemoattractants such as fMLP, which signal through own receptors[5]. CXCR1/2 activation also conveys complex signaling pathways leading to degranulation and initiation of the NADPH oxidase complex, resulting in the production of reactive oxygen species (ROS) for immune defense[14,79]. This last task has been discovered to be a specific property of the CXCR1 receptor with signaling through phospholipase D (PLD)[81,82], while degranulation is conveyed by both[81]. This is not only fundamental for pathogen elimination, but also for overcoming the venular BM. With degranulation, NE is translocated to the cell surface and in part released from the azurophilic granules (further information in chapter *1.1.1/1.2.1*) in combination with Laminin/BM-contact-providing integrins (VLA-3, VLA-6). Different studies have shown this mechanism to be essential for reaching the interstitial tissue beyond the vascular BM[30,31,35,38–41,48]. The interaction of CXCR1/2 and their chemokines is an important component of neutrophil recruitment. Another task involves the neutrophil’s emigration from the bone marrow, where CXCR2 is upregulated upon granulocyte-colony stimulating factor (G-CSF) signals and critical for their release[9,83]. Next to that, CXCR1/2 seem to modulate angiogenic activities and drive endothelial cell functionalities like proliferation and migration as, for example, in the context of vascular wound healing, where the CXCL1-CXCR2 interaction ameliorated the regeneration of vascular endothelial cells *in-vitro* and *in-vivo* [78,84,85]. Since the many different and important functionalities of the CXCR1/2 receptors were unraveled, also their contribution to disease development or worsening became apparent[86]. Some examples are lung diseases like asthma and chronic obstructive pulmonary disease (COPD)[87], diabetes[88], autoimmune diseases like

rheumatoid arthritis (RA)[89,90], inflammatory bowel diseases[91], cancer[79], Alzheimer's disease[92] as well as sepsis[93].
But next to their many involvements, the active time period of a CXCR1/2 receptor might also be limited. Upon ligand binding, the receptors can be desensitized by a G-protein coupled receptor kinase (GEK)-dependent phosphorylation with subsequent β-arrestin recruitment. This can initiate receptor endocytosis mediated by clathrin, AP-2, Rab5 and dynamin. After internalization, the chemokine ligand is cleaved, and the receptor recycled back to the cell surface for a probable next signaling round. At lower ligand concentrations, a CXCR1/2 oscillation between sensitized and desensitized states has been observed, allowing restimulation of neutrophils[94]. A special trait of CXCR1 in this context is the much faster re-expression on the cell surface after internalization, while for CXCR2 this recycling process takes longer[93].

Although CXCR1/2 and the corresponding ligands have an important role in host defense, they are not the only ones with such capabilities. Other GPCRs, as mentioned before, are fPRs for fMLP and CCR1/2/5 for CCL5, which, upon ligand binding, are as well capable of regulating chemotaxis, priming, adhesion and activation, next to ROS induction and degranulation and are therefore studied in this thesis[14,76].

### *1.3.2 Pharmacological Targeting of CXCR1/2*

As mentioned in the chapter above, the CXCR1/2-ELR-CXC-chemokine axis was found to be a key driver in the pathogenesis and progression of several diseases. Although in some, the relevance of their involvement must be further determined, targeting CXCR1/2 or their respective chemokines offers a promising treatment option in many cases[86]. This chapter will give an overview on CXCR1/2 inhibitor Ladarixin and other existing CXCR1/2 inhibitors as well as potential clinical applications of those substances.

#### 1.3.2.1 Ladarixin

Ladarixin, or DF2156A, is a non-competitive and allosteric small molecule CXCR1/2 inhibitor developed by Dompé Farmaceutici (L'Aquila, Italy). The chemical structure is displayed in *Figure 3*. It partly prevents receptor activation by respective chemokines from a conserved site

and impedes neutrophil recruitment and thereby the inflammatory response with a half-maximal inhibitory concentration ($IC_{50}$) of 1-2nM. Ladarixin binds via multiple polar interactions and a direct ionic bond to Lysin[99] (TM2 of CXCR1) and Aspartat[293] (TM7 of CXCR2) without changing the mechanisms or affinity of endogenous ligand binding. This means, only the chemokine's signal transduction is most likely inhibited by Ladarixin. Although acting as dual CXCR1/2 inhibitor, the affinity towards CXCR2 was measured to be around 100 folds higher[78,95]. The contrary is the case with the primarily CXCR1 inhibiting predecessor Reparixin (Repertaxin)[96], which was tested in phase 2 trials for metastatic triple-negative breast cancer[97], graft dysfunction after single or bilateral lung transplantation, as well as kidney transplantation[77,98]. A phase 3 trial for impeding graft loss and functionality after pancreatic islet transplantation was also conducted with Reparixin[99]. Here, the newest development is an exciting phase 3 trial on hospitalized patients with severe COVID-19 pneumonia, which has just completed the recruiting stage (REP0220/ClinicalTrials.gov Identifier: NCT04878055), after a promising clinical phase 2 trial[100,101].

Ladarixin shows enhanced pharmacokinetic features when compared to Reparixin. With a longer *in-vivo* half-life of 19 hours after intravenous (i.v.) application, it is now suitable for continuous administration in a chronic disease setting, as it is also orally available. Besides that, Ladarixin has proven to be well tolerated and safe in all examined doses in human trials and has been successfully used and tested in a phase 2 trial for adult patients with new-onset type 1 diabetes (T1D)[78,87,95,96]. The final results were just published in September 2022 and showed that Ladarixin treatment had metabolic benefits in a subgroup of patients with lower fasting C-peptide and high insulin demand, although the primary endpoint was not met[102,103]. As those initial results need further studies, a phase 3 trial called GLADIATOR (ClinicalTrials.gov Identifier: NCT04628481) was announced by Dompé in January 2021, which is currently in the recruiting stage and will focus on new-onset T1D in adults with additional low residual β-cell function[104]. Fitting to this topic, *in-vivo* experiments with non-obese diabetic (NOD) mice revealed the inhibition of pancreatic insulitis and immune cell mediated damage after destructive streptozotocin injections through Ladarixin treatment, which obviated and even reversed a diabetic condition[105]. The human trial results are also complemented by a recent study from 2021 with *in-vitro* diabetes and obesity models: Experiments with 3T3-L1 adipocyte cells and primary human cells from adipose patients for translational corroboration showed Ladarixin to dampen the diabetes-typical high-glucose and/or low-level-inflammation-dependent amplification of key inflammatory mediators, especially inhibiting CXCR2 and CXCL1 overexpression. With respect to glucose/insulin pathways in a hyperglycemic/inflammatory environment, Ladarixin also seemed to restore adipocyte functionality by normalizing insulin-mediated signaling via protein kinase B (AKT),

phosphoinositide 3-kinase (PI3K) and the insulin receptor substrate (IRS) as well as the expression of glucose transporters (GLUT1/4) and adiponectin levels. This indicates a pathological role of the CXCR1/2-axis and beneficial effects of Ladarixin in obesity related type 2 diabetes (T2D), which has resulted in an ongoing phase 2 trial (EudraCT Number**:** 2020-003296-18) with pre-diabetical adults and elderly patients[88].

In initial *in-vitro* tests with human PMNs, Ladarixin decreased CXCL8, CXCL6, CXCL1 and CXCL5-mediated migration in a concentration-dependent manner[78,96]. CCL2, C5a or fMLP driven chemotaxis was unhindered. In an *in-vivo* rat model, Ladarixin was then also investigated regarding effects in an ischemia/reperfusion (I/R) experiment of the brain, since CXC chemokines are involved in leukocyte influx and additional associated tissue damage after reperfusion called “reperfusion-injury” [96,106]. Here, a reduced neutrophil invasion of the infarct area, a smaller final infarct size accompanied by a better neurological outcome was observed[96]. Another rat I/R *in-vivo* experiment of the liver, representative of the mechanisms in human organ transplant, showed Ladarixin to be very potent as one single administration inhibited PMN and additionally monocyte influx after reperfusion and abrogated associated tissue damage[78]. Ladarixin was also tested in different murine lung disease models, where CXCR2 is not only found on immune cells, but also lung structural cells[107]: It reduced inflammation, lung tissue damage and thereby preserved lung function in a “classical” T-helper 2 cell/eosinophilic asthma model, a T-helper 17 cell glucocorticoid-resistant asthma model as well as in a bleomycin induced fibrosis experiment and a cigarette-smoke generated Influenza exacerbation[87]. As CXCR1/2 and associated chemokines were found to be important mediators in melanoma progression, Ladarixin was examined in different *in-vitro* and *in-vivo* mouse melanoma models. The results show decreased melanoma cell motility, increased apoptosis by downregulation of survival supporting signals (AKT, nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-kB)), which was also promoted by induction of M1 tumoricidal macrophages. Also, an inhibition of angiogenesis and aldehyde dehydrogenase ($ALDH^+$)-dependent tumor “rejuvenation” was observed. All independent of the actual tumor genetics[95]. Those anti-angiogenic, anti-migratory and anti-proliferative capacities were also noticed in independent *in-vitro* HUVEC (human umbilical vein endothelial cells) experiments with CXCL8 as stimulus and an *in-vivo* sponge approach, where additionally the reduction of the angiogenesis-supporting cytokine tumor necrosis factor-α (TNFα) was detected. This led to the assumption that Ladarixin is potent in modulating tumor- as well as inflammation-dependent angiogenesis[78].

Regarding specificity, Ladarixin showed no interference with other chemokine receptors of the CC/CXC family and did also not disturb chemotaxis towards a fMLP or C5a gradient or affect

spontaneous cell movements without chemokines present[78,96]. Finally, a reduction in neutrophil counts by different Ladarixin doses could not be observed.[95]

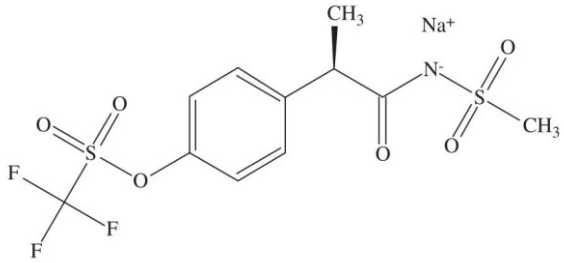


**Figure 3: Chemical structure of Ladarixin/DF2156A[95]**
A dual, allosteric, non-competitive small molecule inhibitor of CXCR1/2. (Figure adapted from Garau et al. (2006))

### 1.3.2.2 Other small molecule CXCR1/2 inhibitors

Next to Dompé, there are also other companies, which have developed promising candidates for CXCR1/2 inhibition using small molecules. One of them is GlaxoSmithKline (GSK). Their predominantly CXCR2 inhibiting diarylurea-class includes SKF83589, SB225002, SB265610, SB332235, and SB656933 (Elubrixin), which where successively developed with the aim of optimized pharmacokinetics and tested *in-vitro* and *in-vivo*. While the initial SKF83589 and the successor SB225002 were not further pursued, the optimized SB656933 (Elubrixin), the analogue of SB332235, went into clinical studies for COPD, CF and ulcerative colitis (UC)[77,108]. Here, the phase 2 trial for CF showed no improvement in lung function or respiratory symptoms. Although the inflammatory biomarkers in the sputum of patients were reduced, still, the systemic inflammation markers in the blood were elevated[109]. Trials for UC were terminated, followed by a general termination of Elubrixin for "undisclosed reasons"[110]. Another candidate, developed from SB332235, is Danirixin (GSK1325756): an oral, competitive, allosteric, selective CXCR2 inhibitor, whose effects are completely reversible. The promising substance is in clinical development for COPD and acute viral respiratory infections with multiple completed clinical trials[111]: The phase 2 results regarding influenza showed Danirixin to be well tolerated with no relevant adverse events and indicated a faster clinical response and decreased serum levels of NE-degraded elastin[112,113]. In COPD phase 2 trials, Danirixin influenced respiratory symptoms and the patients' overall health including the duration of exacerbations, not the amount occurring, in a positive way[114]. Nevertheless, GSK had to terminate Danirixin development for COPD because of insufficient efficacy.[110,115]. But other applications, like in cancer immunotherapy, and also a range of newly developed and modified substances are still on the horizon[110].

SCH527123 or Navarixin (also MK-7132) by Schering Plough is another potent allosteric inhibitor, this time of CXCR1 and 2. Navarixin also has reversible kinetics, but works in a non-competitive, insurmountable manner[111,116]. Promising preclinical tests range from pulmonary disease models for COPD and Influenza to tumor models with colorectal cancer and melanoma cell lines [95,117,118,119,95,120]. Those findings were transferred into clinical trials, where different phase 2 trials were carried out for COPD. Here, Navarixin showed significant improvement of $FEV_1$ (forced expiratory volume in 1 second) as marker for lung functionality and reduced sputum neutrophils, especially in current smokers, but also induced dose-dependent neutropenia[121]. Other phase 2 studies were concentrated on asthma, where no respiratory improvement could be reached[87]. But, as with Elubrixin and Danirixin, clinical trials were ended after phase 2 because of a lack of efficacy. The newest development, however, is a trial investigating Navarixin in advanced or metastatic solid tumors (NCT03473925) with results still pending[110].

AstraZeneca first developed a substance called AZD8309, a fused pyrimidine series-based, reversible, and selective CXCR2-antagonist[107], which was used in phase 1 COPD and RA clinical trials[77]. The oral administration of AZD8309 was tested in an airway neutrophilia trial with inhaled lipopolysaccharide (LPS) in healthy volunteers as model for neutrophil-steered airway diseases like CF, asthma or COPD and significantly showed a decrease in sputum neutrophil counts, NE activity and CXCL1 amount[122]. Although development reportedly stopped in 2007[107], a recent study applied the substance in a murine pancreatitis model. Here, AZD8309 was potent in diminishing neutrophil emigration and activation of intrapancreatic proteases, which led to fewer pancreatic tissue damage and disease severity[123]. The newer allosteric, CXCR2 inhibiting, and slowly reversible AZD5069[124] was assessed on safety and pharmacokinetics in multiple phase 1 trials[125]. A phase 1 trial on the combination with Enzalutamide in metastatic castration-resistant prostate cancer (NCT03177187) has recently been started[110]. Phase 2 trials include uncontrolled persistent and severe asthma with no successful reduction in the amount of exacerbations[126] and a trial with bronchiectasis, where sputum neutrophil counts could be decreased, but sputum and blood levels of some assessed inflammatory mediators remained increased and the subsequent clinical outcome was not changed[127]. In another phase 2 study with COPD patients (NCT01233232), AZD5069 was generally well tolerated but reversible droppings of blood neutrophil counts could be observed in some patients. This circumstance had already been discovered in healthy volunteers[128,129]. The final results for an application of the compound in solid tumors or relapsed metastatic squamous cell carcinomas of the head and neck are still pending (NCT02499328)[130]. One last

compound that should be mentioned in this context is called AZ10397767. It is thiazolopyrimidine-based and supposed to inhibit both CXCR2 and CCR2 with good oral bioavailability[107]. It has only been tested in *in-vitro* and *in-vivo* models with lung adenocarcinoma tumor spheroids and xenografts, where the substance diminished neutrophil infiltration and tumor growth while lacking any effect on vessel density or angiogenesis[131]. Since the neutrophil elastase makes up an important part of this Ladarixin thesis, it is interesting to know that AstraZeneca also developed a novel, selective, reversible and orally applicable NE inhibitor named AZD9668[132].

The next group are boronic-acid containing, non-competitive and selective CXCR1/2 antagonists. The first one discovered in this novel group was SX-517, which was not further pursued because of instability[133,134]. This was followed by SX-576, which still lacked satisfying aqueous solubility, and SX-682[135,134]. SX-682 now proved to be efficient in hindering problematic immunosuppressive myeloid-derived suppressor cell (MDSC) influx into head and neck cancer/oral and lung syngeneic carcinoma models, thereby enhancing the therapeutic effect of natural killer cell-, as well as T-cell-immunotherapy[136,137]. In another murine model for castration-resistant prostate cancer the combination of SX-682 with immune checkpoint blocking agents revealed remarkable potency, again mediated via MDSC attenuation[138]. Hoping for similar results in humans and advances in cases of resistance to cancer therapy, a phase 1 trial for melanoma treatment has been initiated with SX-682, where it will be applied alone or together with Pembrolizumab (NCT03161431)[139].

## *1.4 Preceding experimental Results by the Sperandio Lab*

In the past few years our Lab has conducted a series of experiments in order to investigate the detailed mechanisms with whom Ladarixin influences the neutrophil recruitment cascade. In initial *in-vitro* microflow-chamber tests with isolated human neutrophils, Ladarixin pretreatment showed no capacity to interfere with the first steps of rolling and adhesion. Those experiments were conducted with flow-chambers coated with CXCL8, E-selectin and ICAM-1. The results were analyzed for rolling and adhesion behavior of human neutrophils and compared to a control group treated with normal saline. Additional flow-chamber assays were performed with pertussis toxin as control. Pertussis toxin expectedly reduced the amount of adherent human neutrophils, while Ladarixin again showed no effect on adhesion under those

conditions. In a following *in-vivo* cremaster muscle model, Ladarixin or NaCl (isotonic NaCl 0,9%/normal saline) were injected intraperitoneal (i.p.) to wildtype mice, which were then treated with CXCL1 or CXCL8 i.v.. After chemokine application, the change in adherent murine neutrophils in the microvasculature of the cremaster muscle was assessed. Both normal saline and Ladarixin treated mice showed a significant increase in acute arrest of neutrophils following CXCL1 or CXCL8 injection, which indicates no impacting role of Ladarixin at this stage of recruitment. In a similar *in-vivo* cremaster muscle model, the chemokines CXCL1 or CXCL8 were injected intrascrotally (i.s.) to induce tissue inflammation in the cremaster muscle. The results also showed no significant difference in rolling or adherent cells, when comparing NaCl and Ladarixin treated mice. In additional tests with a similar CXCR1/2 inhibitor (DF2755A), first indications came to light that compounds like Ladarixin might impair not neutrophil rolling and adhesion, but the subsequent step of transmigration. To further assess this hypothesis, the previously generated cremaster muscle whole mount samples with i.p. NaCl or Ladarixin pretreatment and CXCL1 as intrascrotal stimulus were stained with Giemsa and the amount of perivascular leukocytes was counted. Interestingly, the numbers of perivascular leukocytes in the Ladarixin samples were significantly lower than in NaCl controls. As neutrophils make up the largest part of leukocytes, we could conclude an impairing role for Ladarixin in CXCL1-initiated neutrophil transmigration[52]. To complete testing the different steps of neutrophil recruitment, an *in-vitro* chemotaxis assay was performed using a CellDirector2D-Gradientech-device. A chemotactic gradient was implemented with CXCL8. Here, no difference in chemotactic abilities between Ladarixin or phosphate-buffered saline (PBS) treated murine neutrophils could be determined (unpublished data). This leaves the stage of transmigration as main Ladarixin target with the need for further assessment. Transmigration was shown to be dependent on mobilization of the intracellularly stored neutrophil elastase, as well as the docking integrins VLA-3 and VLA-6 to the neutrophil's surface via CXCR1/2 signaling[30,31,35,38–41,48]. Following this lead, an already established translocation assay[31,35] was modified towards Ladarixin, after previous results with the similar substance DF2755A displayed reduced NE, VLA-3 and VLA-6 vesicle relocation to the cell surface after compound incubation, compared to NaCl controls[52]. Tying on to our previous lab findings, this thesis presents those new results and the succeeding experiments and gives further insight into the role of Ladarixin in neutrophil transmigration.

# 2 AIM OF THE STUDY

Neutrophils are an indispensable part of our immune defense. But from their multiple capabilities, also damaging mechanisms can arise. Research in this area has identified many diseases where especially our focus of interest, the CXCR1/2-chemokine interaction, is pathophysiologically relevant. The neutrophils' CXCR1/2 receptors, activated by their corresponding chemokines, play an important role in neutrophil activation for subsequent firm adhesion, transmigration and chemotaxis, as well as the induction of their defense abilities, like granule exocytosis. After blockade or removal of the CXCR1/2 receptor or their ligands, different studies showed a great reduction in neutrophil emigration, which was, in some cases, accompanied by disease stabilization or even recovery. This thesis is focused on one of those receptor blockers: the small molecule inhibitor Ladarixin. Previous work in our lab elucidated its influence on neutrophil transmigration and found an impairing effect on this step of the recruitment cascade. The mechanisms behind this transmigration inhibition are still unclear.

Because there are indicators leading towards the vascular basement membrane as the responsible obstacle, this project is centered on the influence of Ladarixin on the serine protease neutrophil elastase, whose function is degrading the basement membrane. This protease is stored in neutrophil azurophilic granules and mobilized to the cell surface upon activation via CXCR1/2 stimulation by their respective ligands. The aim is to clarify Ladarixin's effects on neutrophil elastase localization, function and distribution with different *in-vitro* and *in-vivo* experiments. This will further enlighten the impact of Ladarixin on the human body and the mechanisms behind overcoming the basement membrane in general, which will eventually result in a more detailed understanding of neutrophil transmigration and new therapy options.

# 3 MATERIALS AND METHODS

## *3.1 Laboratory Animals*

All experiments were conducted with wildtype mice, using the C57Bl/6-strain. They were delivered by Charles River Laboratories (Sulzfeld, Germany) and held in the Core Facility Animal Models (CAM) at the Biomedical Center (BMC) München-Martinsried, Germany, under IVC (individually ventilated cages) conditions. All performed experiments were authorized by the Regierung von Oberbayern (AZ 55.2-1-54-2531-122/12 and 55.2-2532.Vet_02-17-102)

## *3.2 Buffers, Solutions and Reagents*

| | |
|---|---|
| Aqua ad injectabilia (Ampuwa) | Fresenius Kabi, Bad Homburg vor der Höhe, Germany |
| BD FACS Lysing Solution | BD, Franklin Lakes, NJ, USA |
| Bovine serum albumin solution (BSA) | Capricorn Scientific, Ebsdorfergrund, Germany |
| Casein from bovine milk | Sigma Aldrich, St. Louis, MO, USA |
| Dako Antibody Diluent with Background Reducing Components | Agilent, Santa Clara, CA, USA |
| EasySep Mouse Neutrophil Enrichment Kit with Recommended Medium | Stemcell Technologies, Vancouver, Canada 100ml PBS + 200µl EDTA 0,5M + 2ml FCS |
| Fetal bovine serum (FBS) Superior | Sigma Aldrich, St. Louis, MO, USA |
| Hank's buffered salt solution (HBSS) with or without Phenolred | Apotheke Klinikum der Universität München, München, Germany |
| HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) | Sigma Aldrich, St. Louis, MO, USA |
| Isotonic NaCl 0,9% (in the following indicated as NaCl or normal saline) | Fresenius Kabi, Bad Homburg vor der Höhe, Germany |
| Ladarixin | Dompé Farmaceutici S.p.A, Milano, Italy |

| | |
|---|---|
| N-formyl-methionyl-leucyl-phenylalanine (fMLP) | Sigma Aldrich, St. Louis, MO, USA |
| Paraformaldehyde (PFA) | Carl Roth, Karlsruhe, Germany |
| Phosphate-buffered saline (PBS) Medium 148mmol/l $Na^+$ 1,8mmol/l $K^+$ | Apotheke Klinikum der Universität München, München, Germany |
| Percoll | Sigma Aldrich, St. Louis, MO, USA |
| PermaFlour Aqueous Mounting Medium | Thermo Fisher Scientific, Waltham, MA, USA |
| Poly-L-Lysine | Sigma Aldrich, St. Louis, MO, USA |
| ProLong Diamond Antifade Mounting Medium | Invitrogen, Carlsbad, CA, USA |
| Recombinant Mouse CD31/PECAM-1 | R&D Systems, Minneapolis, MN, USA |
| Recombinant Mouse ICAM-1/CD54 | R&D Systems, Minneapolis, MN, USA |
| Recombinant Murine CCL5/RANTES | PeproTech, Cranbury, NJ, USA |
| Recombinant Murine KC/CXCL1 | PeproTech, Cranbury, NJ, USA |
| Titriplex III for EDTA | Merck KGaA, Darmstadt, Germany |
| Triton X-100 | Sigma Aldrich, St. Louis, MO, USA |
| Türk's Solution | Merck KGaA, Darmstadt, Germany |
| Vectashield PLUS Antifade Mounting Medium | Vector Laboratories, Burlingame, CA, USA |

**Table 1: Buffers, solutions and reagents**

## *3.3 Antibodies*

### *3.3.1 Primary Antibodies*

| Name | Color | Isotype and Reactivity | Clone | Manufacturer | Used concentration |
|---|---|---|---|---|---|
| **Anti-Neutrophil Elastase** | _ | IgG Rabbit anti-mouse | Polyclonal | Abcam, Cambridge, UK | 1:100 |

| | | | | | |
|---|---|---|---|---|---|
| **Anti-CD31/PECAM-1** | Alexa Flour 488 | IgG2a, k Rat anti-mouse | MEC13.3 | BioLegend, San Diego, CA, USA | 1:100 |
| **DAPI (4',6-diamidino-2-phenylindole)** | _ | _ | _ | Invitrogen, Carlsbad, CA, USA | 1:5000 |
| **Anti-Ly-6G** | Pacific Blue | IgG2a, k Rat anti-mouse | 1A8 | BioLegend, San Diego, CA, USA | 1:600 |
| **Isotype Ctrl Antibody** | APC | IgG2a, k Rat anti-mouse | RTK2758 | BioLegend, San Diego, CA, USA | 1:100 |
| **Anti-CXCR2/CD182** | APC | IgG2a, k Rat anti-mouse | SA044G4 | BioLegend, San Diego, CA, USA | 1:100 |

**Table 2: Primary antibodies**

### *3.3.2 Secondary Antibodies*

| Reactivity and Isotype | Color | Clone | Manufacturer | Used concentration |
|---|---|---|---|---|
| **Goat anti-rabbit IgG** | Alexa Flour 546 | Polyclonal | Invitrogen, Carlsbad, CA, USA | 1:400 |
| **Goat anti-rat IgG** | Alexa Flour 546 | Polyclonal | Invitrogen, Carlsbad, CA, USA | 1:400 |
| **Donkey anti-rat IgG** | Alexa Flour 488 | Polyclonal | Invitrogen, Carlsbad, CA, USA | 1:400 |
| **Goat anti-rat IgG** | Alexa Flour 647 | Polyclonal | Life Technologies, Carlsbad, CA, USA | 1:100 |

**Table 3: Secondary antibodies**

## *3.4 Materials*

| | |
|---|---|
| Blood collection tubes | BD, Franklin Lakes, NJ, USA |
| Cannula (27G ¾, 0,4mm x 19mm) | BD, Franklin Lakes, NJ, USA |
| Cell strainer (40µm) | Greiner Bio-one, Kremsmünster, Austria |
| Cover Glasses High Precision (24x60mm, 170±5µm) | VWR, Darmstadt, Germany |
| Eight-Well Chamber, removable | Ibidi GmbH, Gräfelfing, Germany |
| Eppendorf Safe-Lock Tubes (1, 2, 5ml) | Eppendorf AG, Hamburg Germany |
| Falcon Tubes (5, 15, 50 ml) | Falcon/Corning, Tamaulipas, Mexico |
| Menzel-Glasses Superfrost Plus | Thermo Fisher Scientific, Waltham, MA, USA |
| Microcaps glass capillaries | Drummond Scientific Company, Broomall, PA, USA |
| Neubauer Improved Counting Chamber | Optik Labor, Görlitz, Germany |
| Pipette Tips | Starlab, Hamburg, Germany |
| Polystyrene Round-Bottom Tube 5ml (FACS) | Falcon/Corning, Tamaulipas, Mexico |
| Surgical Instruments | F.S.T. Fine Science Tools GmbH, Heidelberg, Deutschland |
| Syringes (1, 5, 10, 20ml) | B. Braun, Melsungen, Germany |

**Table 4: Materials**

## *3.5 Technical devices*

| | |
|---|---|
| Balance Sartorius CPA225D-OCE | Sartorius, Göttingen, Germany |
| Centrifuge Heraeus Multifuge X3R | Thermo Fisher Scientific, Waltham, MA, USA |
| Centrifuge Rotina 420R | Hettich, Tuttlingen, Germany |
| CO2 Incubator Galaxy 170S New Brunswick | Eppendorf AG, Hamburg Germany |
| CytoFLEX S | Beckman Coulter, Brea, CA, USA |

| | |
|---|---|
| Idexx ProCyte Dx hematology analyzer | Idexx Europe B.V., Hoofddorp, Netherlands |
| IKA Rocker 3D basic | IKA, Staufen, Germany |
| Magnetic stirrer IKA C-MAG HS7 | IKA, Staufen, Germany |
| Mini Centrifuge Labnet C1301P | Labnet International Inc., Edison, NJ, USA |
| pH-Meter Mettler-Toledo MP220 | Mettler Toledo, Columbus, Ohio, USA |
| Vortexer Clear Line | Benchmark Scientific Inc., Edison, NJ, USA |
| Vortex-Genie 2 | Scientific Industries Inc., New York, USA |

**Table 5: Technical devices**

### *3.5.1 Imaging*

| | | |
|---|---|---|
| **Sample imaging:** upright confocal microscope | Leica SP8X WLL<br>Objectives: HC PL APO 63x/1.40 OIL CS2, WD 0.14 mm (Translocation-Assay)<br>HC PL APO 40x/1.30 OIL CS2, WD 0.24 mm (Cremaster muscle model) | Leica, Wetzlar, Germany |
| **Cell counts** | Olympus CKX41 | Olympus, Tokio, Japan |
| **Cremaster muscle preparation:** Stereo microscope | Olympus SZ61 with KL 1500 LCD | Olympus, Tokio, Japan |

**Table 6: Imaging**

## *3.6 Software*

| | |
|---|---|
| FLOWJO Single Cell Analysis Software v10 | BD, Ashland, OR, USA |
| GraphPad Prism 7.04 | GraphPad Software, San Diego, CA, USA |
| ImageJ 1.53c (Fiji) | National Institutes of Health, USA |
| Leica Application Suite X 3.7.1.21655 | Leica Microsystems, Wetzlar, Germany |
| Microsoft 365 MSO, Version 2105 | Microsoft, Redmond, WA, USA |

**Table 7: Software**

## *3.7 Ladarixin*

Our substance of interest was generously provided by the company Dompé farmaceutici S.p.A. (L'Aquila, Italy). The chemokine receptor inhibitor was diluted in normal saline at different concentrations. For the NE translocation assay a 100nM, 5µM and 50µM concentration was used to determine dose-dependency. The CXCR2 internalization assay was carried out with the 5µM dose. In all mentioned experiments, the isolated murine neutrophils were incubated with Ladarixin for one hour at a temperature of 37°C/5%CO2. For the NE FAST (see *3.11*) cremaster muscle model, 30µg/g mouse were applied via intraperitoneal injection. All concentrations are well within the tested range of effectiveness[52,78].

## *3.8 Isolation of murine Neutrophils*

For obtaining murine neutrophils, C57Bl/6 mice of both genders were sacrificed. Afterwards their cleaned hip, femur, tibia and humerus were flushed through with PBS in order to isolate the murine bone marrow. The following isolation tool was used:

**EasySep Mouse Neutrophil Enrichment Kit:** For applying the right amounts of EasySep cocktails the cells were washed and counted prior to isolation. Following the EasySep protocol murine neutrophils were extracted by negative selection and resuspended in pH adjusted HBSS.

After isolation the aim was a density of either 3,5 million cells per milliliter (translocation assay) or 1 million cells per milliliter (internalization assay). The neutrophil suspension was then incubated with the specific Ladarixin concentration or the same volume of control compound (normal saline) for one hour in a 37°C/5%CO2 incubator.

## *3.9 In-vitro Translocation Assay*

**The day before** cell isolation, the coated 8-well Ibidi removable chambers were prepared and stored overnight (ON), at 4°C in a wet chamber. Three different **coatings** were used for the

three different experiments, all dispersed with a volume of 100µl per well. An exemplary experimental setup for the CXCL1 assay is shown in *Figure 4*.

**CXCL1/KC-Solution:** 2µg/ml PECAM-1, 8µg/ml ICAM-1, 10µg/ml CXCL1, diluted with 0,1% BSA/PBS

**CCL5/RANTES-Solution:** 2µg/ml PECAM-1, 8µg/ml ICAM-1, 10µg/ml CCL5, diluted with 0,1% BSA/PBS

**fMLP-Solution:** fMLP was put in the wells together with the cell suspension in soluble form at 0,1 and 1ng/ml.

**Control:** 100µl 0,1%BSA/PBS

**For the experiment** the coated chambers were incubated with 200µl of prepared Casein-solution per well for one hour at room temperature (RT). After murine neutrophil isolation and incubation with Ladarixin (100nM, 5µM and 50µM) or normal saline (*Figure 4*) as described above, the wells were washed (three times with PBS) and 200µl of corresponding cell solution was carefully pipetted on and incubated for 30 minutes at 37°C/5%CO2. The next steps consist of cell removal, fixation with 2% PFA for 15 minutes at RT, washing, permeabilization with 0,1% Triton X-100 in 2%BSA/PBS for one hour at RT and again washing with PBS.

The following **staining** was performed with a primary and secondary antibody. Antibodies were diluted with Dako Antibody Diluent and put in the wells for one hour at RT in a volume of 100µl, always followed by two washing steps.

**Neutrophil Elastase Staining:** Anti-NE rabbit anti-mouse (Abcam ab68672) 1:100 + Goat anti-rabbit Alexa Flour 546 (Invitrogen A-11010) 1:400

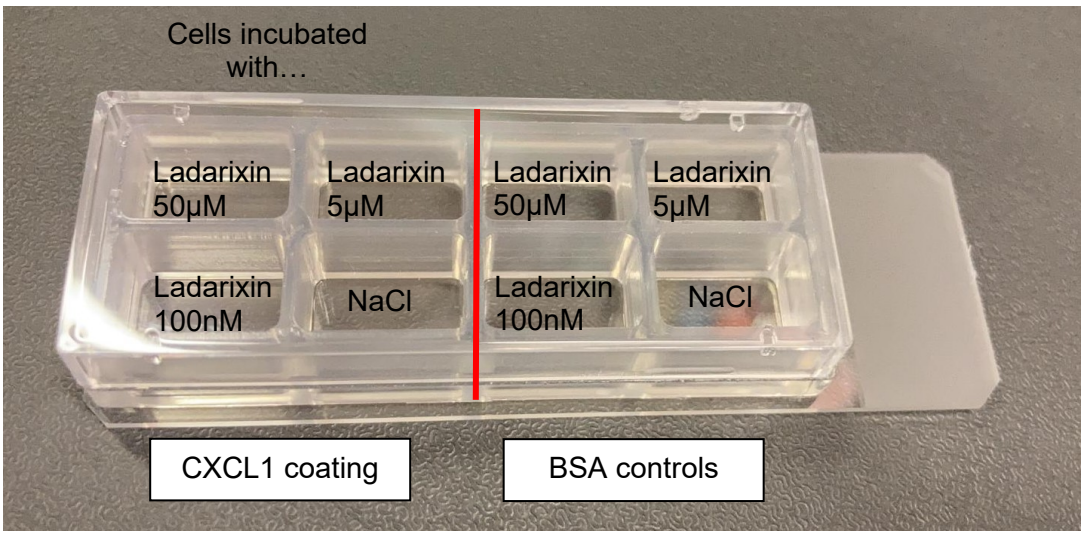


**Figure 4: Example for experimental setup with CXCL1 coating solution**

This picture shows the 8-well Ibidi removable chambers with four coated and four control conditions consisting of three different Ladarixin concentrations and a normal saline incubation.

At the end, DAPI staining (1:5000 in PBS for 5 minutes) was applied, the silicon well separator was removed, and the marked and dry wells were covered by Vectashield PLUS Antifade Mounting Medium. The slide was covered with a high precision cover slip (24x60mm, 170+/-5µm) and sealed with nail polish.

### *3.9.1 Imaging and Data Analysis*

The inverted confocal Leica SP8X WLL microscope with an 63x/1.40 oil objective was used for sample imaging at the BMC Core Facility Bioimaging. The microscope is equipped with a P 0.9 S1 condenser, two excitation lasers (405nm continuous wave, WLL2 470-670nm) and an acousto-optical beam splitter. The settings were as follows: Alexa Flour 546 (excitation 552, emission 562-610), DAPI (excitation 405, emission 415-443). The channels were recorded sequentially using a Hybrid Photo Detector (HyD) for the NE signal and a photomultiplier tube (PMT) Detector for the DAPI signal. 2-5 cells per field of view (FOV) with a zoom set at 2,5, optimized resolution at 880x880 were imaged from top to bottom using Z-stacks with an imaging gap of 0,5µm with the overall aim of 15-20 cells per sample condition (*Figure 5*). The percentage of cells with successful NE translocation to the cell surface was determined with LAS X Software (*Figure 5*) and cross checked by performing intensity profiles: A center line was applied providing the NE intensity values throughout the line. With those, cell surface intensities were compared to intensity values from the cell center by calculating a ratio as shown below in *Figure 6*:

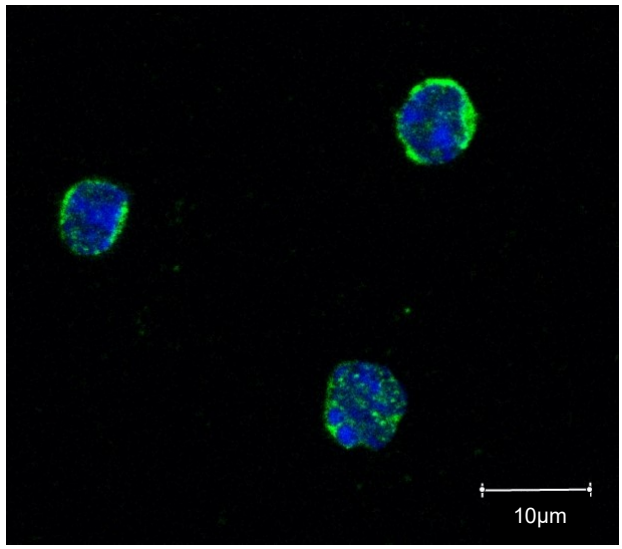


**Figure 5: Representative FOV for NE translocation under CXCL1**

Three murine neutrophils were imaged by confocal microscopy with a DAPI-nucleus staining (blue). The NE staining shown in green was performed using primary anti-NE antibodies and Alexa Flour 546 labeled secondary antibodies. In this representative example for CXCL1 coated, and NaCl pretreated cells, two out of three neutrophils show successful NE ring formation.

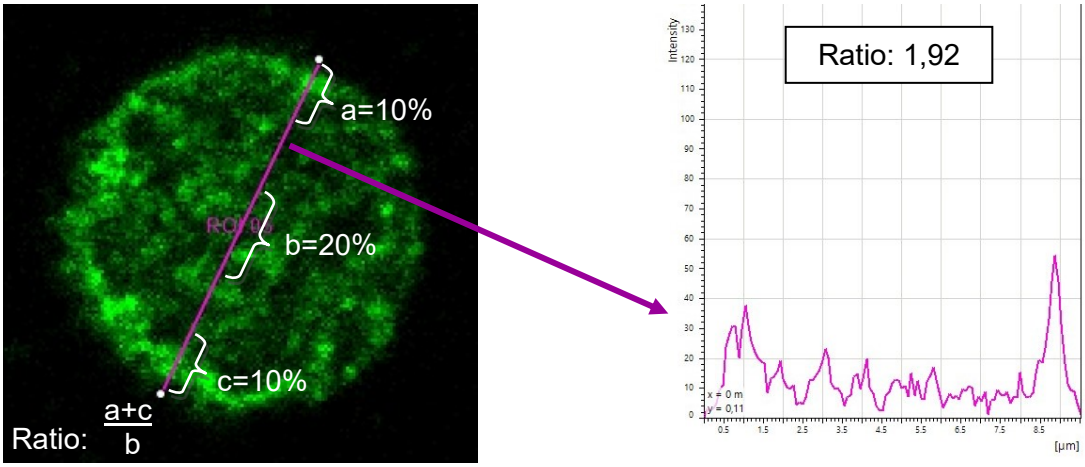


**Figure 6: Intensity profiles – Method**

This figure shows the method for analyzing ring formation by using intensity profiles (right picture) generated with regions of interest (ROIs) from LAS X software. This is shown on an exemplary murine neutrophil stained for neutrophil elastase in green (left picture) with a size of 9,53µm. After applying the center line, the intensities from 10% of both ends of the line (cell surface) were divided by the middle 20% of intensity values. More NE signal on the cell surface results in a ratio >1, more NE signal inside the cell in a ratio <1, while an even distribution gives a ratio around 1.

A successful NE translocation, also described as ring formation, with more NE signal on the cell surface was defined with a ratio ≥1,1.

## *3.10 In-vitro CXCR2 Internalization Assay*

The murine neutrophil isolation was performed by EasySep as explained above (*3.8.*). The isolated neutrophils were diluted in HBSS and incubated with 5µM Ladarixin or normal saline as control at 37°C for 60 minutes. Each condition, comprised of 1ml cell suspension (1 million cells/ml) per FACS (fluorescence activated cell sorting) tube, was then divided and half of it stimulated with CXCL1 at a concentration of 100ng/ml for 10 minutes at 37°C. While to the other halves, only NaCl was added for unstimulated controls. Both groups were stopped with 1,5ml BD FACS Lysing Solution, diluted to 1:10 with demineralized water, before centrifuging the samples. The remaining cell pellets were stained for Ly6G, CXCR2 and the corresponding Isotype controls with a HBSS diluted antibody solution of 100µl per pellet. In the first experiment, also Ly6G and CXCR2 single staining, as well as an unstained sample were prepared to generate the FACS-Template (*Figure 7*). The used antibodies and their concentrations are:

**Isotype-Mastermix:** Pacific Blue anti-mouse Ly-6G 1:600 + APC rat anti-mouse Isotype Ctrl 1:100, diluted with HBSS

**CXCR2-Mastermix:** Pacific Blue anti-mouse Ly-6G 1:600 + APC anti-mouse CXCR2 1:100, diluted with HBSS

After 20 minutes at 4°C, one washing step, and resuspension in 300µl HBSS, the cell suspension was ready for FACS analysis.

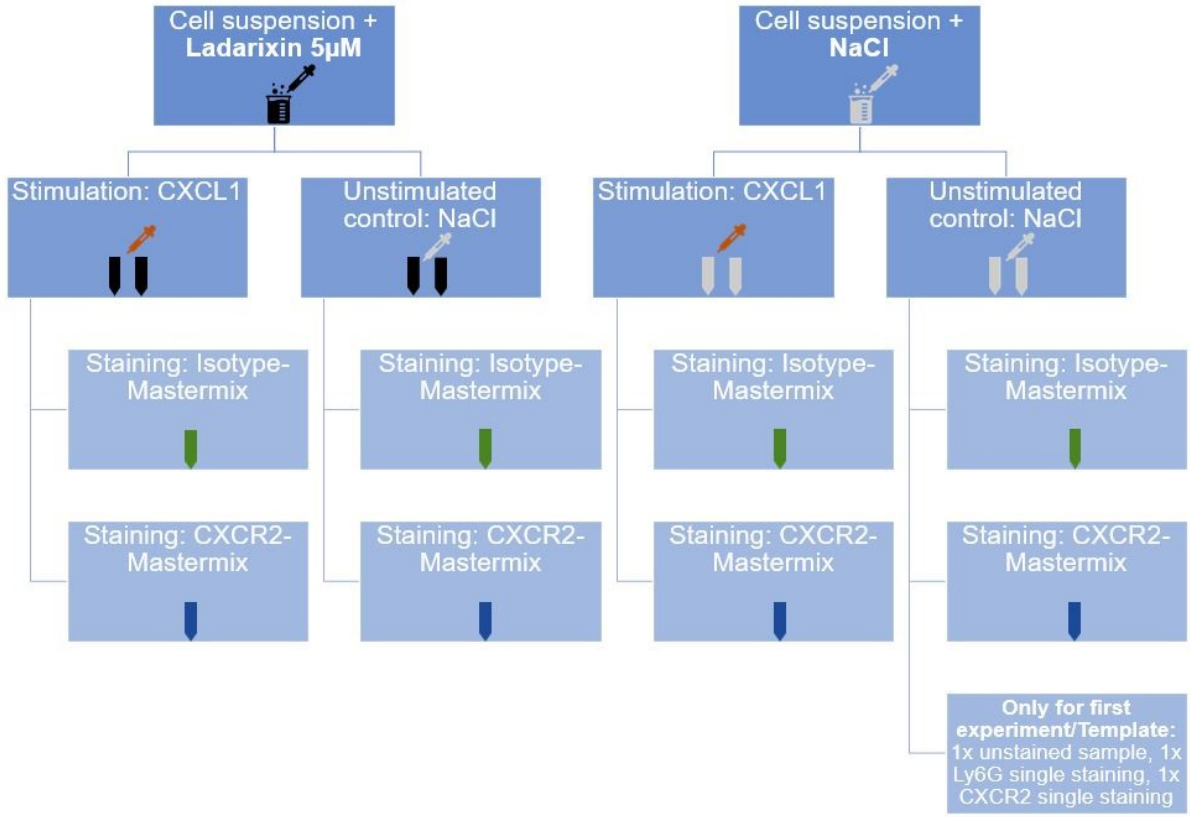


**Figure 7: Overview on the experimental protocol for the CXCR2 internalization assay**

This illustration gives an overview of the different experimental groups and steps in the CXCR2 internalization assay. The **black** pictograms represent the Ladarixin-incubated cell group, the **light grey** pictograms the cells incubated with the same amount of NaCl. The **orange** pipette demonstrates the CXCL1 application to the cell suspension, while the **green** and **blue**-colored tubes mark the different staining. For details, please see the experiment description above.

### *3.10.1 Data Analysis*

Cell cytometry was carried out with the CytoFLEX S. The results were analyzed with FLOWJO Single Cell Analysis Software v10. In a first gating step, the overall cells were selected,

excluding all decayed cells or cell debris. The following strategy consisted of gating for all single cells in the forward side scatter (FSC) and selecting the Ly6G-positive ones, used as a marker for neutrophils. In this population the amount of CXCR2 receptor on the cell surface was assessed with the mean fluorescent intensities from the CXCR2 staining signal. A representative gating process is shown below in *Figure 8*:

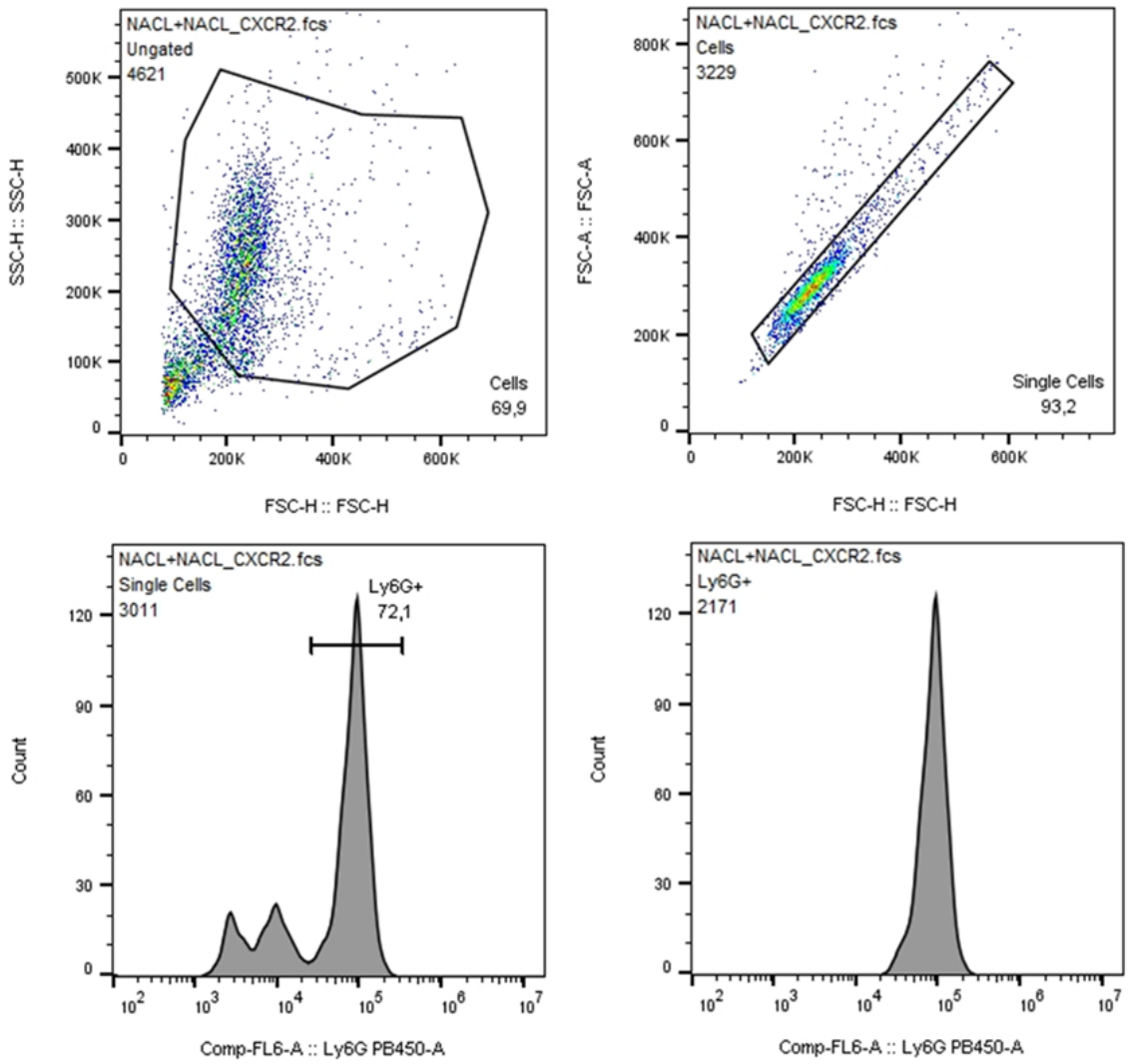


**Figure 8: Exemplary gating process for the FACS CXCR2 internalization assay**

After selection of the cells and single cell gating, the Ly6G+ murine neutrophils were chosen. Their signal for CXCR2 on the cell surface was then analyzed and compared between the different experimental groups. Here a control condition with NaCl incubation and no chemokine stimulation is shown.

For easier comparison and presentation of the data a ratio was applied. In each of the two experimental groups, which means cells incubated with Ladarixin or NaCl, the mean

fluorescent intensity (for the CXCR2 receptor) of the stimulated cells (CXCL1) was divided by the value for unstimulated neutrophils (NaCl). The results give us the percentage of receptors that are still on the cell surface after exposure to the CXCR2 ligand CXCL1 (*Figure 9*). Vice versa, the amount of internalized CXCR2 can be inferred.

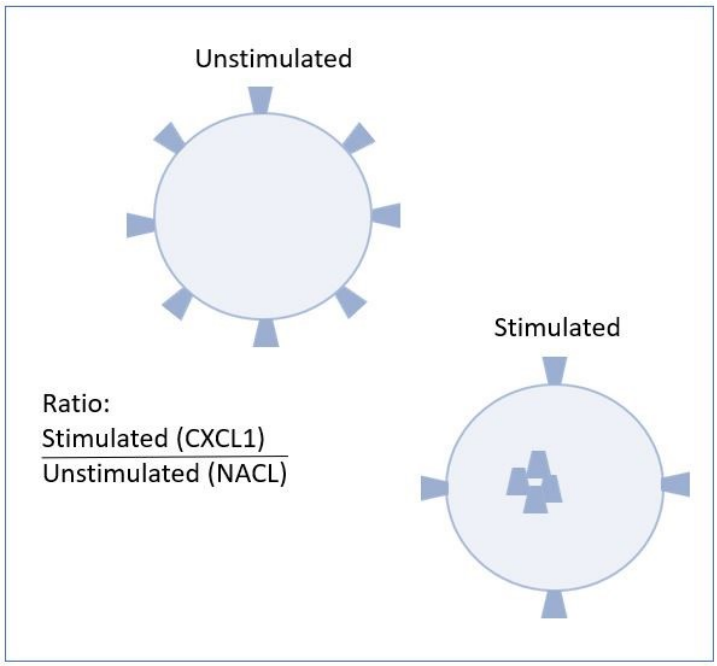


**Figure 9: Internalization ratio calculation - an example**
An unstimulated neutrophil carries CXCR2 receptors on the cell surface (here, 8 receptors). Stimulation with the ligand CXCL1 reduces this amount to 4 remaining receptors. The described ratio would result in a value of 0,5, stating that 50% of the initial CXCR2 receptors remained on the cell surface.
Legend: CXCR2 receptor

## *3.11 In-vivo NE FAST Cremaster Muscle Model*

Male, healthy mice were injected i.p. with 30µg/g Ladarixin from a 2mg/ml solution or the same volume of normal saline as control. After an incubation period of one hour, Neutrophil Elastase 680 FAST Fluorescent Imaging Agent (NE680FAST, FAST for Fluorescent Activatable Sensor Technology, PerkinElmer, Waltham, MA, USA) was applied intrascrotally with 100µl/mouse, representing 4nmol/mouse as suggested by the NE680FAST protocol[140]. Again, after one hour of incubation, either 10µl of 60µg/ml CXCL1/KC, diluted in 200µl NaCl, or 210µl of the control compound NaCl were injected i.s., followed by 2,5 hours of incubation (*Figure 10*). Blood samples were taken for cell counts. The mice were sacrificed directly afterwards. The cremaster muscle was surgically removed under the microscope (*Figure 11*) and transferred into 4% PFA in a spread-out position for one hour, after which it was washed with PBS. 0,5% Triton X-100 in 2% BSA/PBS was applied for two hours, followed by another washing step.

The staining was performed ON with anti-CD31 Alexa Flour 488 (MEC13.3) 1:100 in 2% BSA/PBS at 4°C in a wet chamber, succeeded by three one-hour long incubations with 0,1% Triton X-100 in PBS as well as an additional DAPI staining for 10 minutes. Under the microscope the cremaster muscle was spread out on a Superfrost Plus microscope slide, mounted with PermaFlour Mounting, covered with a high precision cover slip (24x60mm, 170+/-5µm) and compressed with weights for a few hours before the samples were sealed with nail polish.


**Figure 10: Summary of injection steps for the NE FAST cremaster muscle model**

Here, the sequence of injections is described. The process ends with the surgical removal of the cremaster muscle. The red line is marking the cutting line for excision. For further details and the missing experimental steps, see the subsection above.

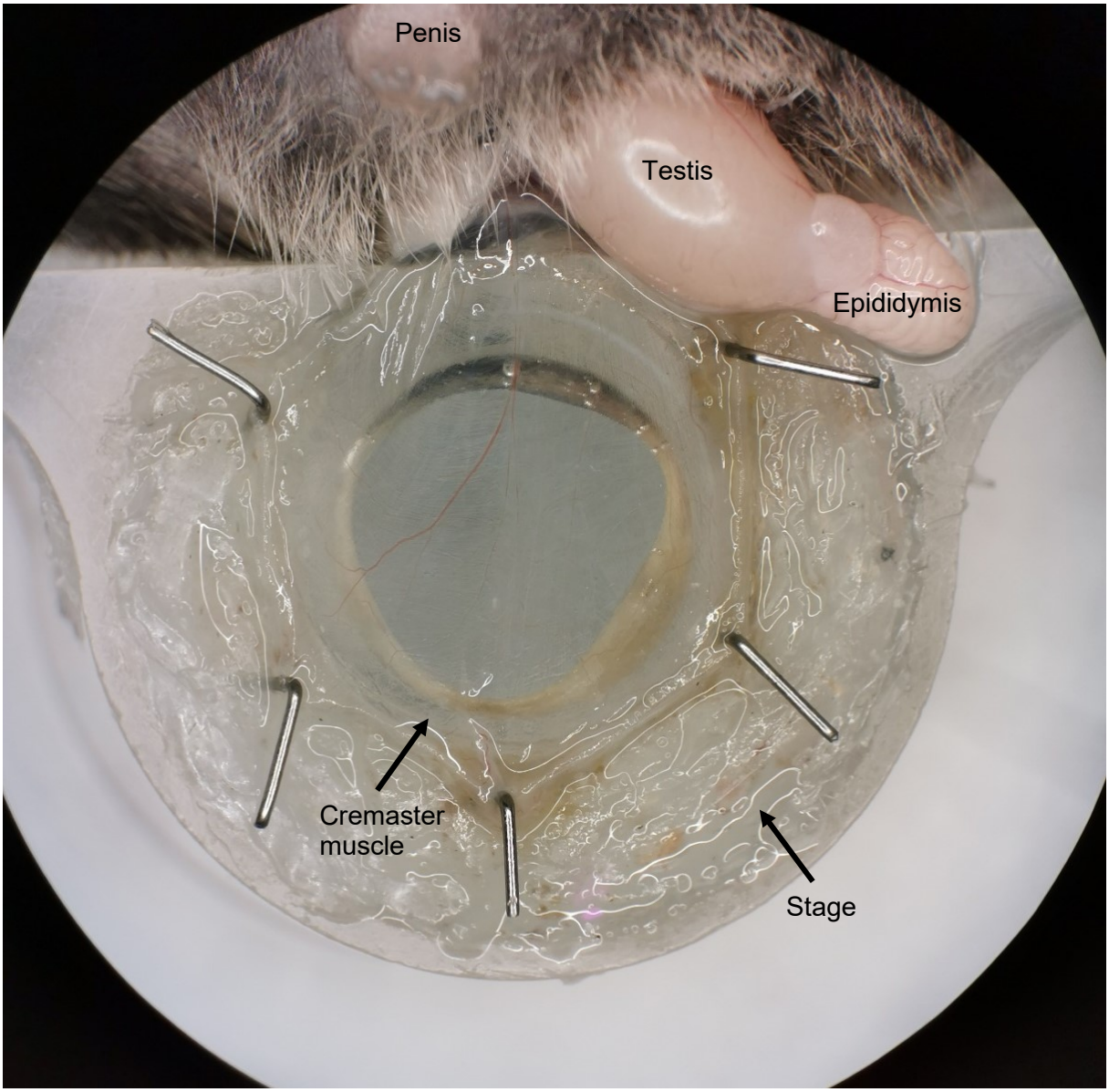


**Figure 11: Example of an exteriorized cremaster muscle**

This figure shows one of two cremaster muscles of a sacrificed mouse pinned open on the experimental stage. The fine vasculature can be seen through the very thin and translucent texture of the cremaster muscle. When surgically opening the scrotum, the cremaster muscle encases the testis and epididymis. After cutting from distal towards the mouse's body, the cremaster muscle opens up and the content, mostly still attached by connective tissue, can be cut from the muscle and pushed to the side, before removing the cremaster muscle at the upper stage end.

### *3.11.1 Imaging and Data Analysis*

Imaging was performed blinded at the BMC Core Facility Bioimaging on the inverted confocal Leica SP8X WLL microscope with a 40x/1.30 oil objective. For further microscope details please see subsection *3.9.1.* and *3.5.1.* The following settings were applied: NE680FAST

(excitation 670nm, emission 690-720mn), Alexa Flour 488 (excitation 488, emission 500-540nm), DAPI (excitation 405nm, emission 415-470nm). For each cremaster muscle sample at least three venous vessels were imaged from upper to lower end with a zoom of 0,75 and an optimized resolution of 4840x4840, using Z-stacks with 2µm between images. The vessel diameters were measured at three subsequent positions to calculate the mean vessel size. Those were on average 25,97µm in vessel diameter. Image analysis was performed blinded with Image J (Fiji) software. To quantify active NE in the perivascular tissue, NE680FAST signals above a tested intensity threshold of 40 were counted at a particle level within five 50µmx50µm squares (ROIs) applied tangentially to the vessel wall. The threshold was empirically chosen to avoid counting background noise as NE activity. This analysis was performed for the middle eight planes of each Z-stack. The mean particle numbers were compared between the different conditions. Exemplary screenshots from the analyzing process are shown below in *Figure 12*.

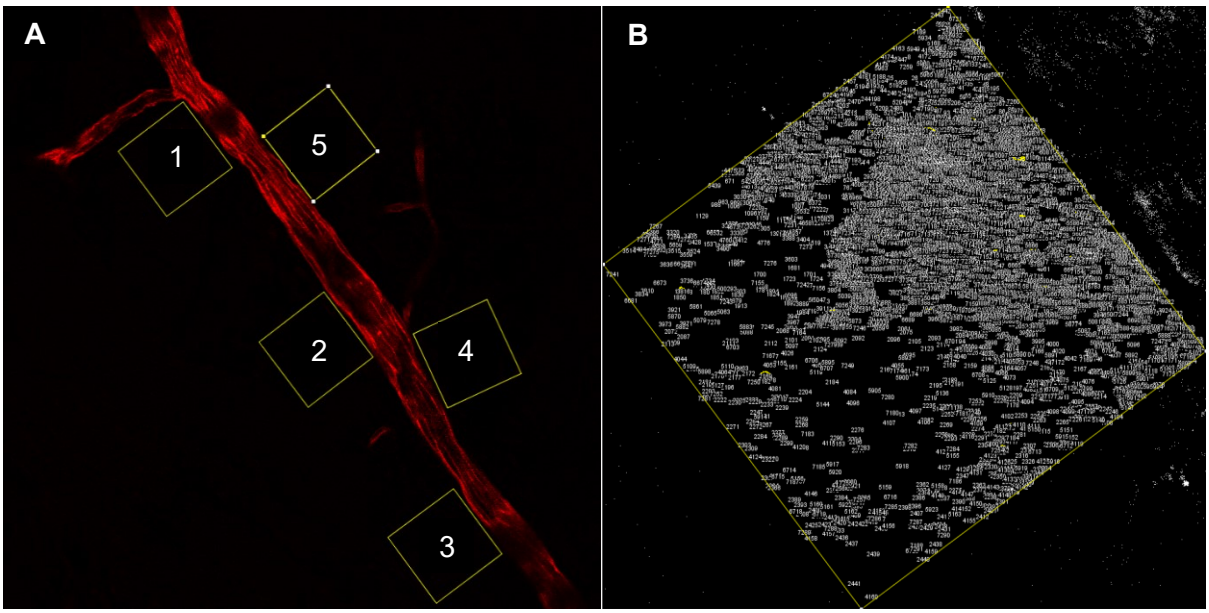


**Figure 12: Analysis of perivascular NE activity**

**A** shows a screenshot from the Image J analysis of a murine venous vessel in the cremaster muscle with a mean diameter of 22,88µm. The tissue was stained with an anti-PECAM-1 antibody to mark the vessel wall, as seen in red. To determine NE activity in the tissue, five 50µmx50µm ROIs were placed next to the vessel wall. The ROI positioning around the vessel was conducted without a visible NE FAST signal. **B** shows the magnification of ROI number one from image A. Within those squares, particles with a NE680FAST signal were counted (marked in white). In this exemplary ROI, a high amount of NE activity can be observed.

## *3.12 Statistical Analysis*

After documenting all data sets in Excel, data analysis was performed with GraphPad Prism. Results for NE translocation were analyzed with a two-way analysis of variance (ANOVA). The generated percentages as well as intensity ratios in all sub-experiments (CXCL1, fMLP or CCL5 as stimulus) were compared individually by Tukey's multiple comparisons test. An unpaired T-Test was used for analysis of CXCR2 internalization, comparing the calculated Ladarixin and control (NaCl) ratio. For analyzing the mean particle results from the NE680FAST cremaster muscle model a two-way ANOVA Šidák's multiple comparisons test was performed, while associated leukocyte cell counts were tested with an ordinary one-way ANOVA (Tukey's multiple comparisons test). In all experiments a p-value <0,05 was considered as statistically significant

# 4 RESULTS

The cumulative results of this thesis are summarized in the tables below. First, the effects of Ladarixin on *in-vitro* neutrophil elastase translocation under different stimuli are shown (*Table 8*). This is followed by the results of the CXCR2 internalization assay (*Table 8*) and Ladarixin's impact on *in-vivo* NE activity and distribution (*Table 9*). All results were generated with C57/Bl-6 mice and will be explained in further detail in the following sections.

| Neutrophil Elastase Translocation | | | | CXCR2 Internalization |
|---|---|---|---|---|
| | **CXCL1** | **fMLP** | **CCL5** | **CXCL1** |
| **Ladarixin** | | | | |
| 100nM | ✘ | – | – | – |
| 5µM | ✘ | ✓ | ✓ | ✓ |
| 50µM | ✘ | – | – | – |
| **Control** | | | | |
| NaCl | ✓ | ✓ | ✓ | ✓ |

**Table 8: Summary of *in-vitro* results**

This table summarizes the different experiments done to determine the effects of Ladarixin on NE translocation to the cell surface under different stimuli as well as effects on chemokine receptor internalization.

Legend: - not investigated ✘ significant inhibiting effect of Ladarixin ✓ no significant effect of Ladarixin

For detailed information please see the corresponding subsections.

| Perivascular Neutrophil Elastase Activity | |
|---|---|
| **Ladarixin** | ✘ |
| **NaCl** | ✓ |

**Table 9: Summary of *in-vivo* results**

This table summarizes the results for fluorescently marked active NE in the cremaster muscle model under

Ladarixin influence or control conditions. Those results were generated after inducing inflammation via CXCL1 injection and observing NE activity in the perivascular tissue.
Legend: × significant inhibiting effect of Ladarixin ✓ high NE activity levels under control conditions
For detailed information please see the corresponding subsections.

## *4.1 In-vitro NE Translocation*

### *4.1.1 Ring induction under Ladarixin with CXCL1 as stimulus*

To continue the preexisting work (see chapter *1.4.*) and corroborate preliminary results[52], an established assay was optimized in a trial phase to show the influence of Ladarixin on the translocation of the protease neutrophil elastase from its vesicle storage to the neutrophil cell surface. This, next to the concurrent relocation of the BM-binding integrins VLA-3 and 6, has been shown to be necessary for proper neutrophil transmigration from the intravasal into the interstitial compartment[30,31,35,39–41]. In a first set of experiments isolated murine neutrophils were put on PECAM-1/ICAM-1/CXCL1-coated wells to mimic inflamed endothelium and induce neutrophil activation, which is subsequently followed by NE ring formation (for experimental setup see *3.9.*). This coating combination has been known from multiple different experiments to initiate a cascade, leading up to NE translocation[31,35,41] When incubated with the control compound NaCl, 66,16 ± 5,72% of neutrophils were able to successfully relocate NE to the cell surface and thereby form a ring. Pretreating of the cells with Ladarixin reduced this to 33,83 ± 2,47% (50µM), 36,56 ± 5,61% (5µM) or 32,05 ± 0,65% (100nM), but with no significant dose-dependent effect. Control coated wells with 0,1%BSA/PBA for each condition generated values between 26,00% and 35,66% (*Figure 13*). To sum up, Ladarixin incubation at all tested doses severely diminished the neutrophil's ability to translocate the protease NE to the cell surface, while NaCl-incubated neutrophils displayed high amounts of successful ring formation (*Figure 14*). This effect tested to be statistically significant with all used Ladarixin doses. Also, the ring inducing effect of the chosen surface coating, as seen with the NaCl-incubated cell group, showed high statistical significance against all BSA control groups (*Figure 13*).

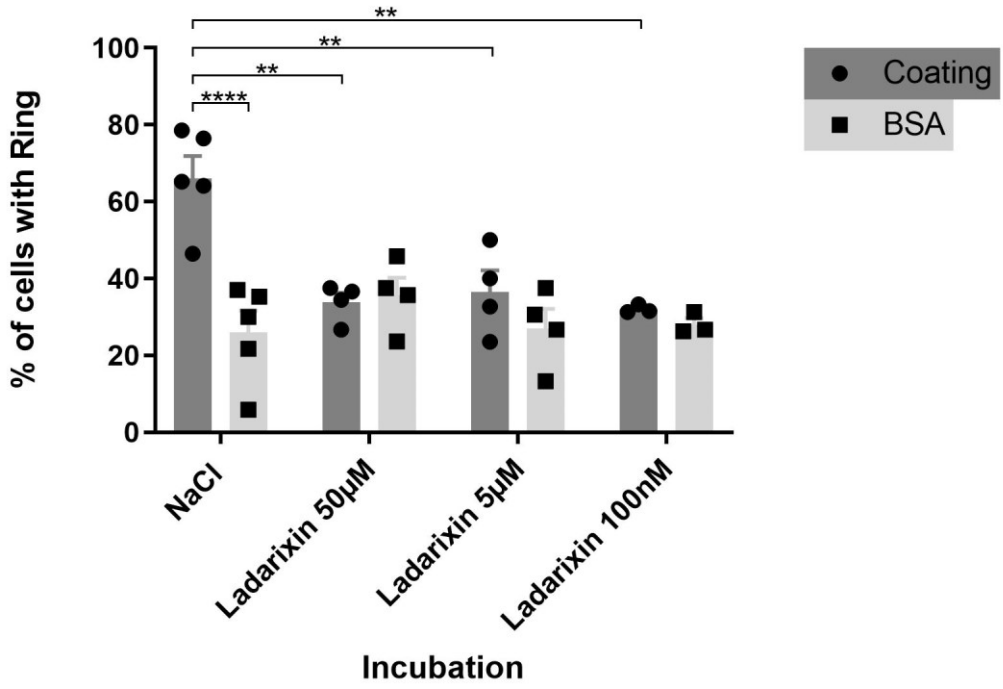


**Figure 13: Ladarixin impairs NE translocation to the cell surface *in-vitro***

The results are shown as percentage of murine neutrophils with successful NE ring formation for coated (PECAM-1/ICAM-1/CXCL1) or control (0,1%BSA/PBS) conditions. n=5, except n(Ladarixin 100nM)=3 with a total of 814 cells, mean ± standard error of the mean (SEM), p<0,05 (*) using two-way ANOVA (Tukey's multiple comparisons test)

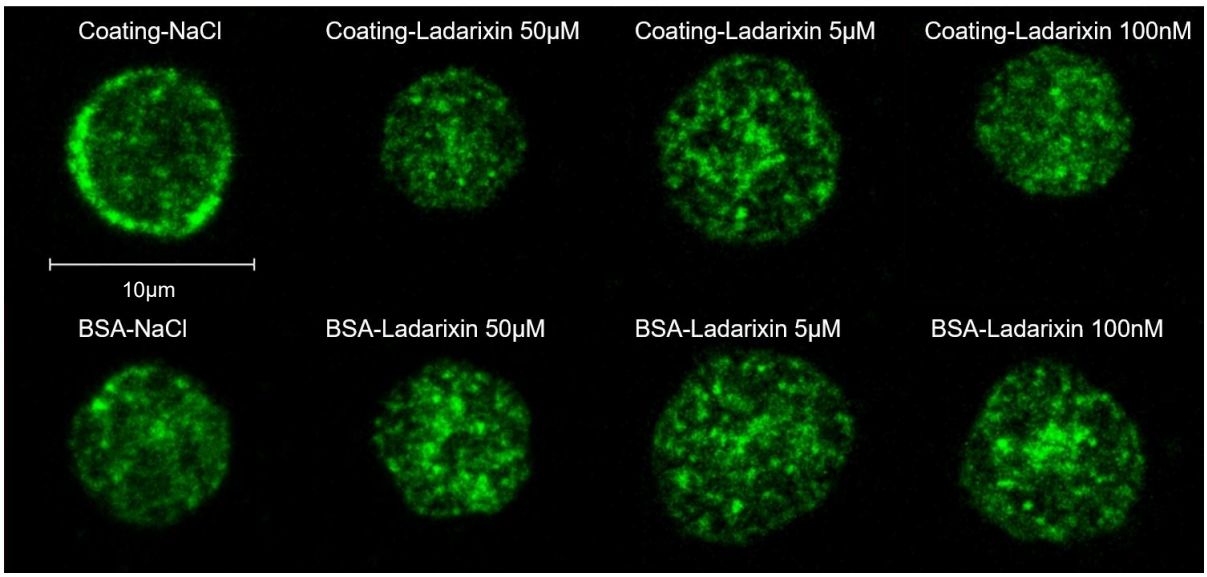


**Figure 14: Representative pictures for NE translocation**

Here, NE distribution in the different coated (PECAM-1/ICAM-1/CXCL1) and control (BSA) groups can be observed. Representative pictures for the coated neutrophils, either treated with NaCl or different Ladarixin concentrations, can be seen in the upper cell row, while the equivalent control conditions make up the lower row. The neutrophil elastase is stained in green by primary and secondary antibodies. The typical NE ring formation is shown on the

coated neutrophil without inhibitor pretreatment (NaCl) in the upper left corner. Imaging was carried out with confocal microscopy. The scalebar is applicable for all displayed cells.

To corroborate those visual results (*Figure 14*), a second analysis method was applied. As described in detail in subsection *3.9.1,* intensity profiles (*Figure 15*) were generated for every analyzed cell to determine the exact ratio between the amount of NE at the cell surface versus intracellularly. Here, higher intensities of the NE staining translate into higher amounts of NE present in the chosen region of interest. The results showed a very similar tendency to those seen above (*Figure 13*). With a mean ratio of 1,69 ± 0,16 the NaCl group showed the highest intensities in the cell surface area and confirmed the before seen high amounts of successful NE translocation. When using Ladarixin, the ratios significantly decreased to means of 0,96 ± 0,12 (50µM), 1,11 ± 0,15 (5µM) and 0,90 ± 0,01 (100nM), also validating the previous results of reduced ring formation. BSA controls remained stable with similar ratios between 0,87 to 0,98 (*Figure 16*).

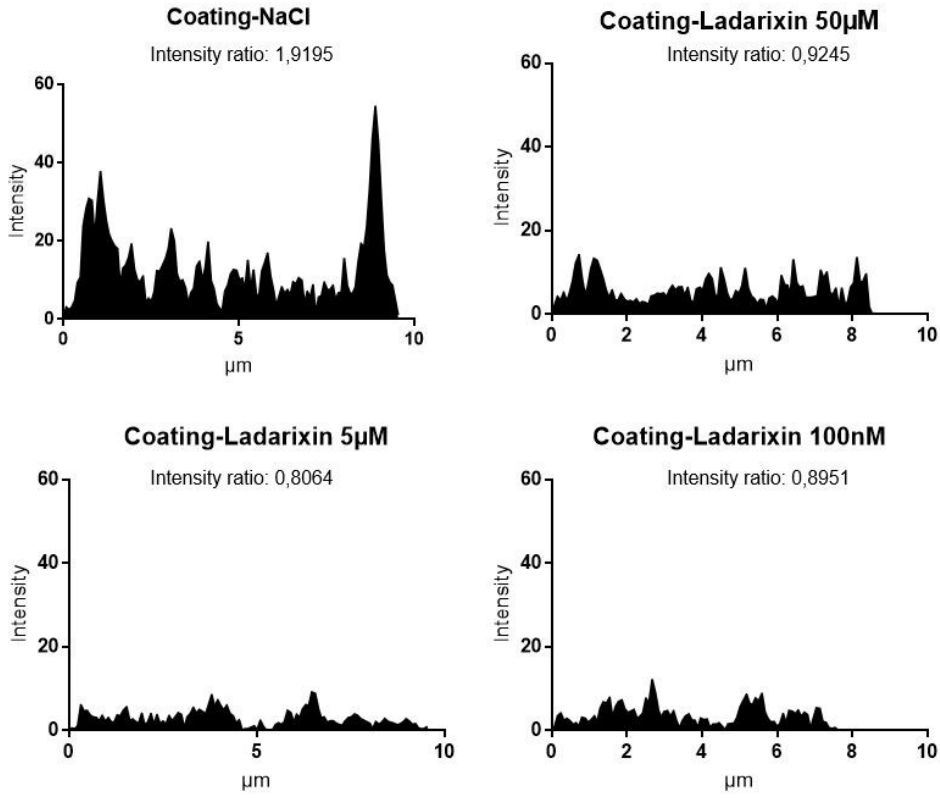


**Figure 15: Representative intensity profiles for NE translocation**

This figure shows some representative intensity profiles for NE distribution from cells coated on PECAM-1/ICAM-1/CXCL1 and incubated with either NaCl or different Ladarixin concentrations. The x-axis indicates the position in the diameter of the analyzed neutrophil. The difference in cellular NE localization and between the corresponding intensity ratios is clearly visible: Neutrophil stimulation by the mentioned coating leads to intracellular NE relocation

to the cell boarders. This process is inhibited by Ladarixin at all tested doses, resulting in a more even NE distribution throughout the cell. BSA controls are comparable with shown Ladarixin conditions.

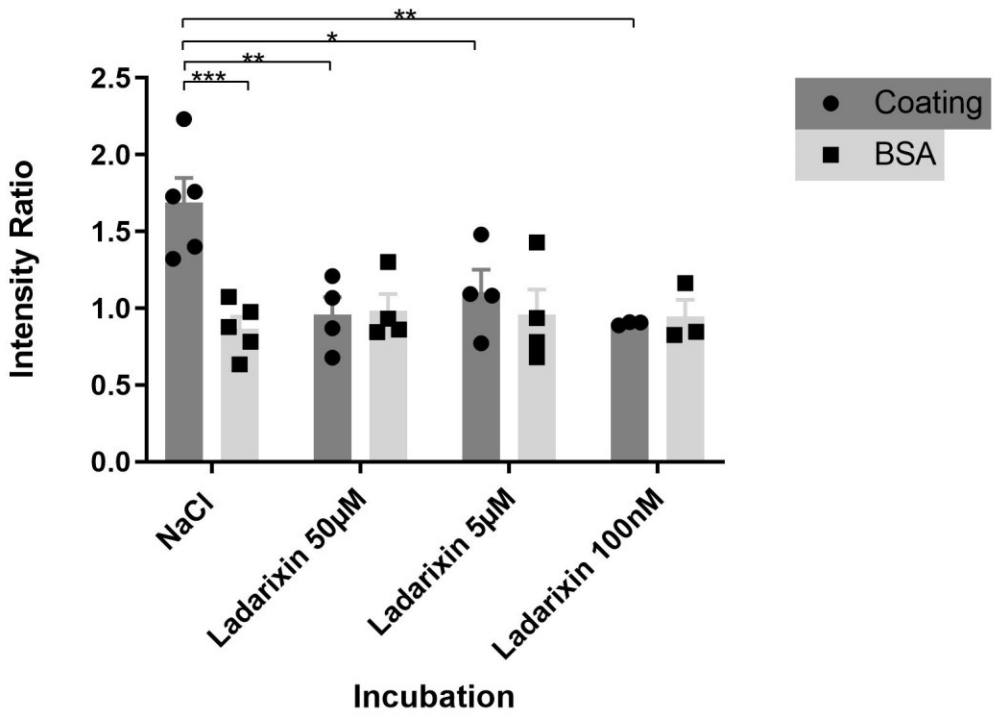


**Figure 16: Intensity ratios – NE translocation to the cell surface**

The results show the mean intensity ratios for each experimental condition. Coating consisted of PECAM-1/ICAM-1/CXCL1, the control coating was performed with 0,1%BSA/PBS. n=5, except n(Ladarixin 100nM)=3, experiments with 814 cells analyzed in total, mean ± SEM, $p<0,05$ (*) using two-way ANOVA (Tukey's multiple comparisons test)

After confirming the inhibiting effects of Ladarixin on NE vesicle relocation after CXCL1-dependent neutrophil activation, the next arising question was, if the observed inhibition was working selectively on CXCR1/2-mediated neutrophil activation and transmigration. To examine this, two different experiments were performed, as described in the following two sections.

### *4.1.2 Ring induction under Ladarixin with fMLP as stimulus*

fMLP is known to be a strong activator and chemoattractant of neutrophils, as it is normally produced by bacteria[59]. By binding to the neutrophil's fPR1, a G-protein coupled receptor, the host innate immune response is directed towards finding and fighting invading pathogens[14]. In

the next set of experiments the coated stimulus CXCL1 was replaced by fMLP to first examine, if this activator could also induce NE translocation to the cell surface, as Owen et al. (1995) has already shown for human neutrophils[141]. In a second step the influence of Ladarixin on the possible fMLP-mediated ring formation was explored. Initial fMLP coating experiments showed no higher amounts of NE ring formation at the cell surface, when compared to BSA controls (data not shown). As fMLP is a small tripeptide[59] and therefore might not be suitable for coating, in the following experiments we switched the strategy from trying to immobilize fMLP with the coating on the well, to pipetting a fMLP solution (soluble fMLP) on top of the pretreated (Ladarixin or NaCl) cells, after transferring them onto the prepared wells. Those had only been coated with PECAM-1 and ICAM-1. After soluble fMLP application, the eight-well chambers with the cell suspension were incubated for 30 minutes at 37°C/5%CO2 according to protocol (Subsection *3.9*). Here, also two different fMLP concentrations were used: 0,1ng/ml and 1ng/ml. When using 0,1ng/ml the generated results were conflicting and inconclusive (data not shown), leading us to the presumption that this concentration might be too low for proper neutrophil activation. While 1ng/ml of soluble fMLP showed quite different results: NaCl pretreated neutrophils, stimulated by PECAM-1/ICAM-1 coating and soluble fMLP, showed a mean percentage of 47,47% ± 3,85% of counted rings in the analyzed cells. Ladarixin was only used at a dosage of 5µM and generated 49,65% ± 6,15% cells with successful NE translocation. BSA controls were lower at mean percentages of 21,05% ± 3,98% (NaCl pretreatment) and 18,30% ± 4,51% with Ladarixin pretreatment (*Figure 17A*). Similar results occurred, when looking at the intensity ratios. With a mean ratio of 1,20 ± 0,10 in the NaCl-incubated and 1,19 ± 0,11 in the Ladarixin-incubated group, no significant effect of Ladarixin on the coated cells could be observed. BSA controls showed, as expected, lower ratios with values of 0,78 ± 0,06 for the NaCl group and 0,76 ± 0,07 in the Ladarixin group (*Figure 17B*). To summarize this data set, the coating combination with soluble fMLP was able to generate a significantly higher number of cells with successful NE translocation to the cell surface, when compared to their BSA controls. This is in accordance with the preexisting knowledge that fMLP does not only induce chemotaxis, but can also initiate further neutrophil activities like ROS production and the respective granule release[14,59]. But in contrast to the CXCL1 results (*4.1.1.*), Ladarixin had no effect in this setting, as the percentage of cells with ring and also the calculated mean intensity ratio over all analyzed cells is very similar between the two experimental groups. This suggests that fMLP-mediated neutrophil activation and transmigration is also dependent on NE vesicle mobilization to the cell surface. But this transmigration induction is not compromised by Ladarixin, suggesting that there is no interference in fMLP-induced NE mobilization by Ladarixin.

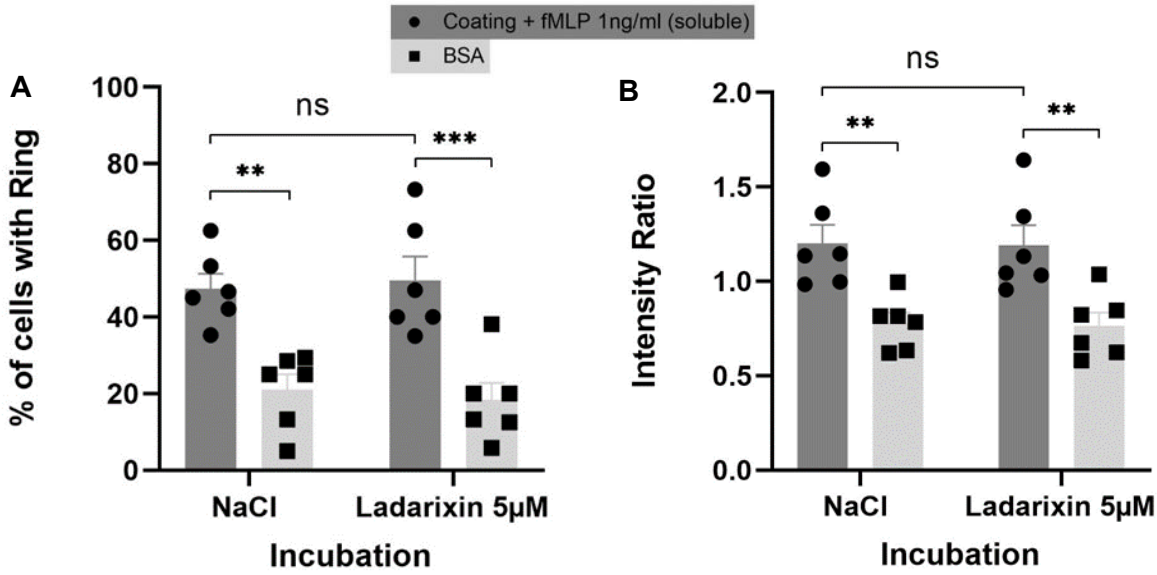


**Figure 17: Ladarixin has no effect on fMLP-mediated NE translocation and transmigration**

**A - Visual analysis:** This figure presents the percentage of murine neutrophils with NE ring formation under PECAM-1/ICAM-1 coating with soluble fMLP at 1ng/ml. Ladarixin or NaCl pretreatments were applied. BSA controls were performed with 0,1%BSA/PBS. **B - Intensity profiles:** The results show the mean intensity ratios per group for all six experiments under different cell incubations (NaCl or Ladarixin) and coating conditions (PECAM-1/ICAM-1/soluble fMLP or 0,1%BSA/PBS). For both figures applies: n=6 per group, total of 414 cells, mean ± SEM, $p<0,05$ (*) using two-way ANOVA (Tukey's multiple comparisons test).

### *4.1.3 Ring induction under Ladarixin with CCL5 as stimulus*

In a next step, another well-known inflammatory mediator was used. This time we chose the chemotactic cytokine CCL5 to further investigate the stimuli needed for NE vesicle transport and subsequently the consequences of applying Ladarixin to this experimental setting. CCL5 is a platelet-derived chemokine and, as fMLP, plays an important part at the site of inflammation by recruiting and activating leukocytes, like neutrophils, to inflamed tissue[5,8,14]. Here, the proclaimed binding targets on neutrophils are the chemokine receptors CCR1, CCR2 and CCR5[76,79]. Although they are not the predominant chemokine receptors on neutrophils, CCR5 has been shown to be upregulated at inflammatory sites[8,14]. With the modified coating, now containing PECAM-1, ICAM-1 and CCL5, we first examined the effects of CCL5 on NE localization and distribution in murine neutrophils. 55,52% ± 3,48% of the NaCl pretreated neutrophils moved NE from vesicle storage to the cell surface when interacting with the immobilized coated factors (*Figure 18A*). Reanalysis using intensity profiles confirmed this direction of high NE translocation capability with a mean of 1,53 ± 0,26 (*Figure 18B*). When

compared to the corresponding BSA control group with 26,00% ± 7,31% and an intensity value of 0,91 ± 0,13, the results show a significant increase in NE ring formation caused by CCL5 coating. The Ladarixin-incubated cells generated an even higher percentage at 63,10% ± 6,86% with a mean intensity ratio of 1,83 ± 0,25 (*Figure 18*). Ladarixin incubation was performed with a concentration of 5µM. After statistical testing those results were again significant against BSA controls, but no significant effect could be shown between the NaCl and Ladarixin pretreated groups, as shown in *Figure 18* below.

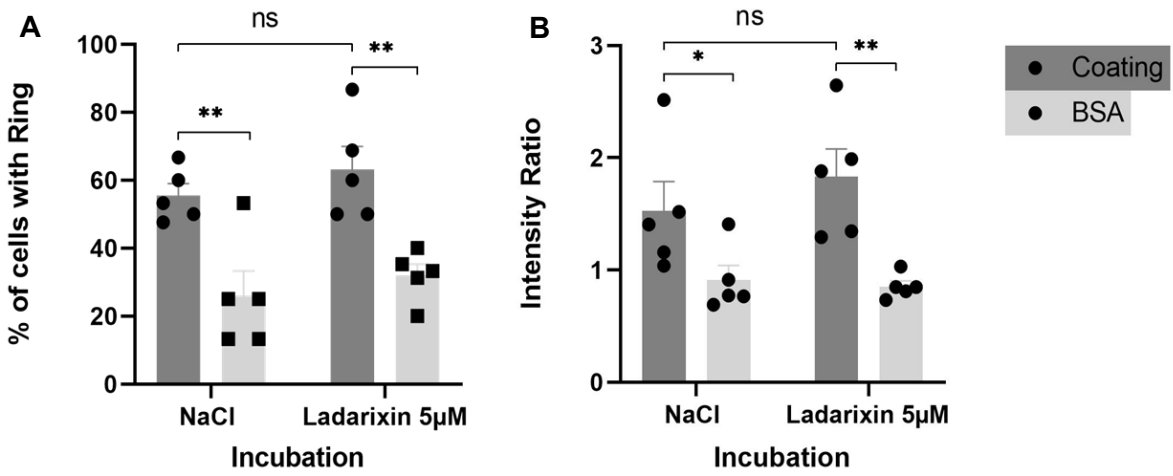


**Figure 18: Ladarixin does not influence CCL5-mediated NE mobilization to the cell surface**

For both sets of results the coating was performed with immobilized PECAM-1/ICAM-1/CCL5, while the control coating consisted of 0,1%BSA/PBS. The murine neutrophils were pretreated with either NaCl or the inhibitor Ladarixin at a concentration of 5µM. n=5 per group, 316 cells in total, mean ± SEM, $p<0,05$ (*) using a two-way ANOVA (Tukey's multiple comparisons test). **A:** Percentage of ring-positive neutrophils after visual analysis. **B:** Mean calculated intensity ratio between cell surface and center.

To sum up, these results are similar to those seen in the fMLP experiments. CCL5 has proven to be a potent activator of NE translocation to the cell border. This process was undisturbed by Ladarixin, reaffirming again Ladarixin's selective mode of inhibition through CXCR1 and 2. Although it had already been shown that Ladarixin has no influence on CC-chemokine- or fMLP-dependent chemotaxis, the effects on intracellular signaling events, like granule mobilization, under those stimuli have not been examined up until today[96].

## *4.2 In-vitro CXCR2 Receptor Internalization*

Although an effect of Ladarixin on NE localization after CXCL1 activation in murine neutrophils became quite clear after the previous experiments, the reasons and mechanisms behind this influence are still unknown. Ladarixin, as well as murine CXCL1, seem to mostly interact with CXCR2 on neutrophils, compared to CXCR1[9,79,95]. Interestingly, Ladarixin is known to reduce the cellular and extracellular effects of chemokine binding to its chemokine receptor, without changing the affinity of this ligand-receptor interaction. This was shown for the analogue human chemokine CXCL8. In contrast to the unaffected receptor interaction, Ladarixin did produce a reduced ligand-mediated G-protein activation downstream of CXCR1/2[78]. Our aim now was to explore the impact of Ladarixin on the amount of CXCR2 receptors on the cell surface, their internalization and recycling in murine neutrophils. Since chemokine receptor internalization is a common and very rapid event after contact to their ligand[82], the goal was to evaluate, if Ladarixin treatment somehow interfered with this specific internalization process. Using FACS (described in subsection *3.10.*), the CXCL1 binding to CXCR2 was examined with regards to such mechanisms. By using neutrophils pretreated with either NaCl or Ladarixin (5µM), subsequently stimulated with CXCL1 or NaCl as control and stained for the CXCR2 receptor, four different experimental groups were analyzed. Between those, the expression of CXCR2 on the surface was compared. *Figure 19* shows a representative FACS plot from one experiment.

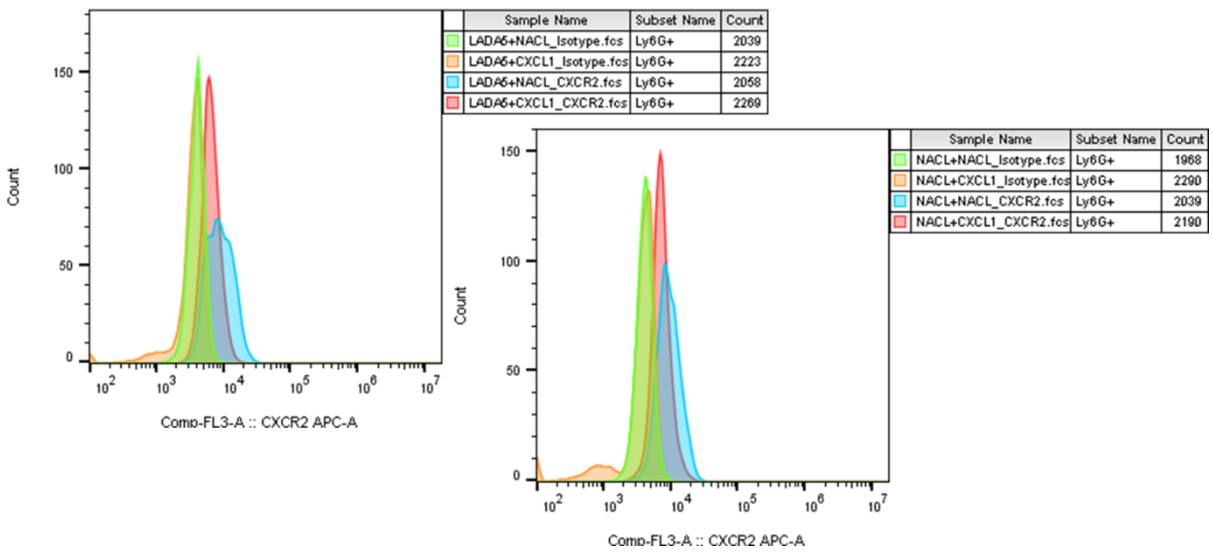


**Figure 19: Representative FACS plot for the CXCR2 internalization assay**

The upper plot shows the CXCR2 results for the Ladarixin-incubated cell group, in the lower plot, neutrophils were only pretreated with NaCl. The green and orange peeks mark the measurements for isotype controls, to which the

results were normalized to. The red color represents the CXCL1-stimulated condition, while blue stands for the NaCl control. For the previous gating process please see section *3.10.1.* The plot shows the results from one representative experiment.

This plot already indicates that in both pretreatment groups (Ladarixin or NaCl) the mean fluorescent intensity for the CXCR2 receptor on the cell surface is higher in the NaCl-incubated cells, than in those stimulated with CXCL1 for 10 minutes. After isotype normalization the exact results confirmed this. For a more approachable data presentation, a ratio was formed. The method is described in detail in subsection *3.10.1*. Dividing the mean fluorescent intensity of the CXCR2 staining from the CXCL1-stimulated neutrophils by the unstimulated/NaCl-incubated ones, resulted in a value of 0,66 ± 0,02 for the NaCl pretreatment group and 0,73 ± 0,05 for the Ladarixin condition (*Figure 20*). This means, regardless of how the murine neutrophils were initially treated, roughly 30% of the CXCR2 receptors were internalized after chemokine stimulation, while about 70% remained on the cell surface. Even though the two results slightly differ, no significance could be found in statistical testing. Since initial CXCR2 recycling takes at least 30 minutes of incubation after ligand removal, any disruption of our results by cell surface re-expression can be excluded[142].

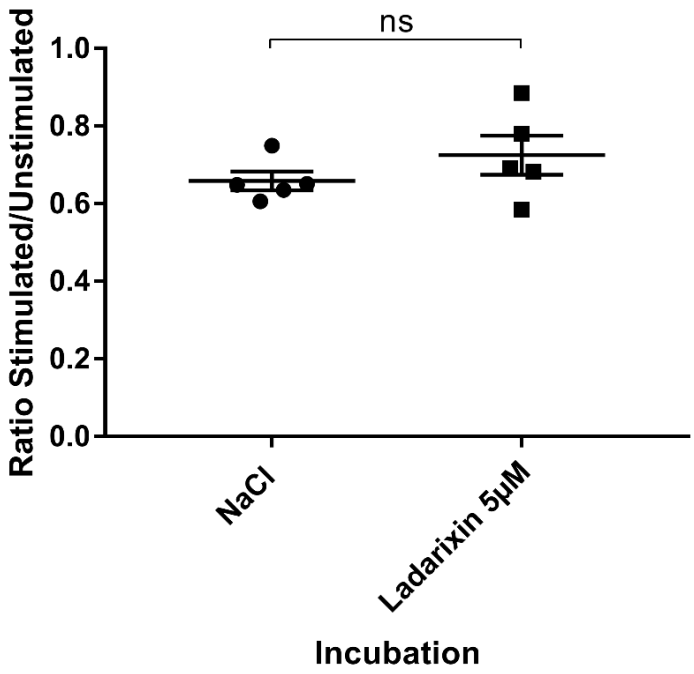


**Figure 20: Ladarixin has no effect on CXCR2 internalization**

This figure presents the ratios of chemokine stimulated (CXCL1) neutrophils against the unstimulated (NaCl) controls for the two experimental groups: NaCl pretreatment or Ladarixin 5µM pretreatment. n=5, mean ± SEM, p=0,27 (ns) using the unpaired t-test.

To sum up, these experiments confirm that Ladarixin does neither affect the amount of CXCR2 on the cell surface, nor the receptor internalization process after chemokine contact. As this would have been one possible cause for the subsequent inhibition of NE translocation, a further downstream influence of Ladarixin in the intracellular signaling process can be assumed.

## *4.3 In-vivo perivascular NE activity*

After focusing on *in-vitro* experiments in the first part, the next step was to further explore the functional implication of NE mobilization for neutrophil transmigration. As already mentioned in the Introduction, this protease is an important player in neutrophil transmigration towards the site of inflammation, more precisely, in overcoming the vascular basement membrane by potential degradation. It has been shown by our lab and other groups that missing NE mobilization to the surface, as seen under Ladarixin, or specific blockade is accompanied by reduced transmigration. In addition to neutrophils stuck in between the layers of the vessel wall, unable to penetrate the basement membrane, even reverse migration back into the blood flow was shown previously[31,32,35,40,41,48,73]. But the corresponding amount, localization, and activity level of the neutrophil elastase in such experimental settings is still unclear. Therefore, an *in-vivo* cremaster muscle model was performed. The experiment details are explained in chapter *3.11.* Briefly, male wildtype mice were pretreated with an initial intraperitoneal Ladarixin injection or NaCl as control, followed by intrascrotal application of the NE 680 FAST Imaging Agent and subsequent intrascrotal CXCL1 or control (normal saline) injection in each group. The chosen NE staining is a special agent with Fluorescent Activatable Sensor Technology (FAST). It is optically silent upon injection and produces a selective fluorescent signal after cleavage by active neutrophil elastase[143]. After the surgical removal of the cremaster muscle and vessel staining, this imaging agent made it possible to quantify NE activity and its distribution in the inflamed cremaster muscle with or without Ladarixin pretreatment. By counting the mean particle numbers with NE680FAST signal in perivascular ROIs, four experimental conditions were compared. With a mean of 2582 ± 886,89 signal-positive particles per ROI, the control mice (NaCl) with CXCL1-induced cremaster muscle inflammation showed the highest amount of active perivascular NE. When CXCL1 stimulation was omitted, those mice only reached a mean value of 566,16 ± 199,63. Looking at the Ladarixin pretreatment group, the results were similarly low. The subgroup stimulated with

CXCL1 generated 424,80 ± 152,08 particles on average, while the NaCl control showed higher numbers with a mean of 904,38 ± 187,80 (*Figure 21*). In this experimental context, also cell counts of each mouse were performed in order to exclude contorted results for NE activity due to diminished or elevated neutrophil counts. Independent from the type of injection, leukocyte differential counts were within the normal range[4,144] with no statistical significant differences (*Table 10*), again confirming that Ladarixin has no effect on neutrophil numbers[95].

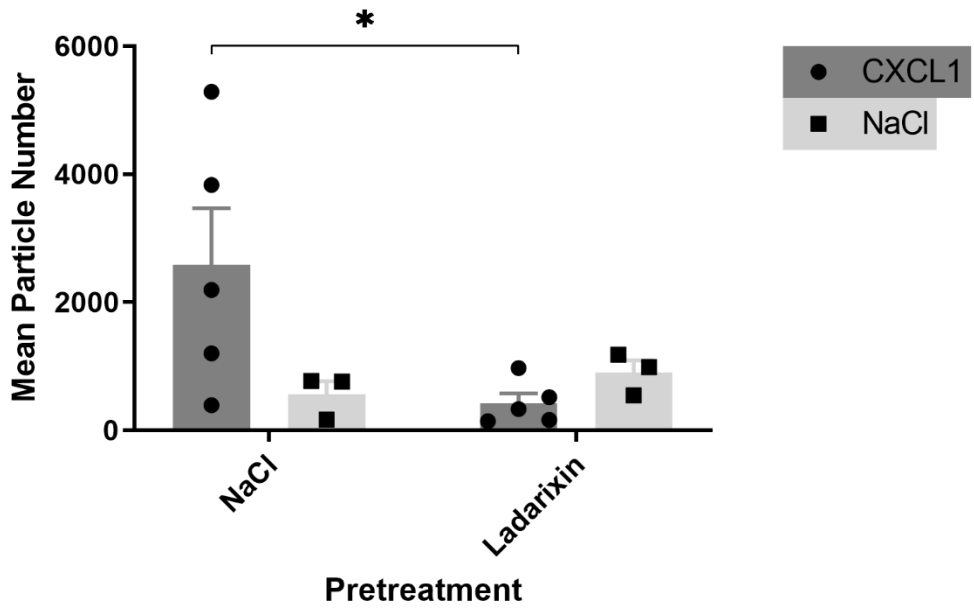


**Figure 21: Ladarixin impairs the perivascular amount of active NE**

Mean particle numbers with fluorescent NE680FAST signal are presented for four different experimental groups, divided into two different pretreatments. n=5 for CXCL1 injection group, n=3 for secondary NaCl injected mice, mean ± SEM, p<0,05 were indicated (*) using a two-way ANOVA (Šidák's multiple comparisons test).

| Ladarixin i.p. | | WBC x10$^{-3}$ | NEU x10$^{-3}$ | LYMPH x10$^{-3}$ | MONO x10$^{-3}$ | EO x10$^{-3}$ | BASO x10$^{-3}$ |
|---|---|---|---|---|---|---|---|
| | CXCL1 i.s. | **6,30** (4,10-9,57) | **2,05** (1,78-2,23) | **4,07** (1,89-7,28) | **0,11** (0,02-0,40) | **0,07** (0,01-0,12) | **0,00** (0,00-0,01) |
| | NaCl i.s. | **5,20** (3,95-6,32) | **0,83** (0,49-1,24) | **4,21** (3,37-4,81) | **0,09** (0,02-0,21) | **0,07** (0,06-0,09) | **0,00** (0,00-0,00) |
| NaCl i.p. | | | | | | | |
| | CXCL1 i.s. | **5,71** (4,54-7,44) | **1,68** (0,64-2,41) | **3,91** (2,78-4,72) | **0,03** (0,02-0,05) | **0,09** (0,07-0,14) | **0,00** (0,00-0,00) |
| | NaCl i.s. | **5,98** (5,10-6,45) | **1,95** (1,53-2,23) | **3,89** (2,81-4,70) | **0,04** (0,02-0,06) | **0,09** (0,03-0,19) | **0,01** (0,00-0,01) |

**Table 10: Murine leukocyte differential counts**

This table shows systemic leukocyte differential counts from the used mice in the cremaster muscle model. The numbers are given as a mean written in bold and the corresponding range for each of the four experimental groups. All counts were assessed with the Idexx ProCyte Dx hematology analyzer and measured in K/µl=x10$^3$/µl. No

significant differences were found, when comparing the four differential treatments per cell type group using an ordinary one-way ANOVA (Tukey's multiple comparisons test, p>0,05, ns, for WBC, NEU, LYPH, MONO, EO and BASO) WBC= White blood cells, NEU= Neutrophils, LYMPH= Lymphocytes, MONO= Monocytes, EO= Eosinophils, BASO= Basophils

These results show a significant decrease in perivascular NE activity in CXCL1-stimulated cremaster muscles, when pretreated with Ladarixin. While NaCl pretreated mice, where the same inflammatory stimulus by CXCL1 was applied, reacted with high NE activity around their cremaster muscle venules, the controls in each pretreatment group (secondary NaCl injection) had low particle numbers due to reduced neutrophil recruitment (*Figure 22*). This supports the preexisting findings on impaired neutrophil transmigration under Ladarixin and closes the missing gap. Because of lower NE mobilization and degranulation, which results in diminished extracellular degradation activity, neutrophils with Ladarixin treatment are unable to overcome the vascular basement membrane and reach the site of inflammation.

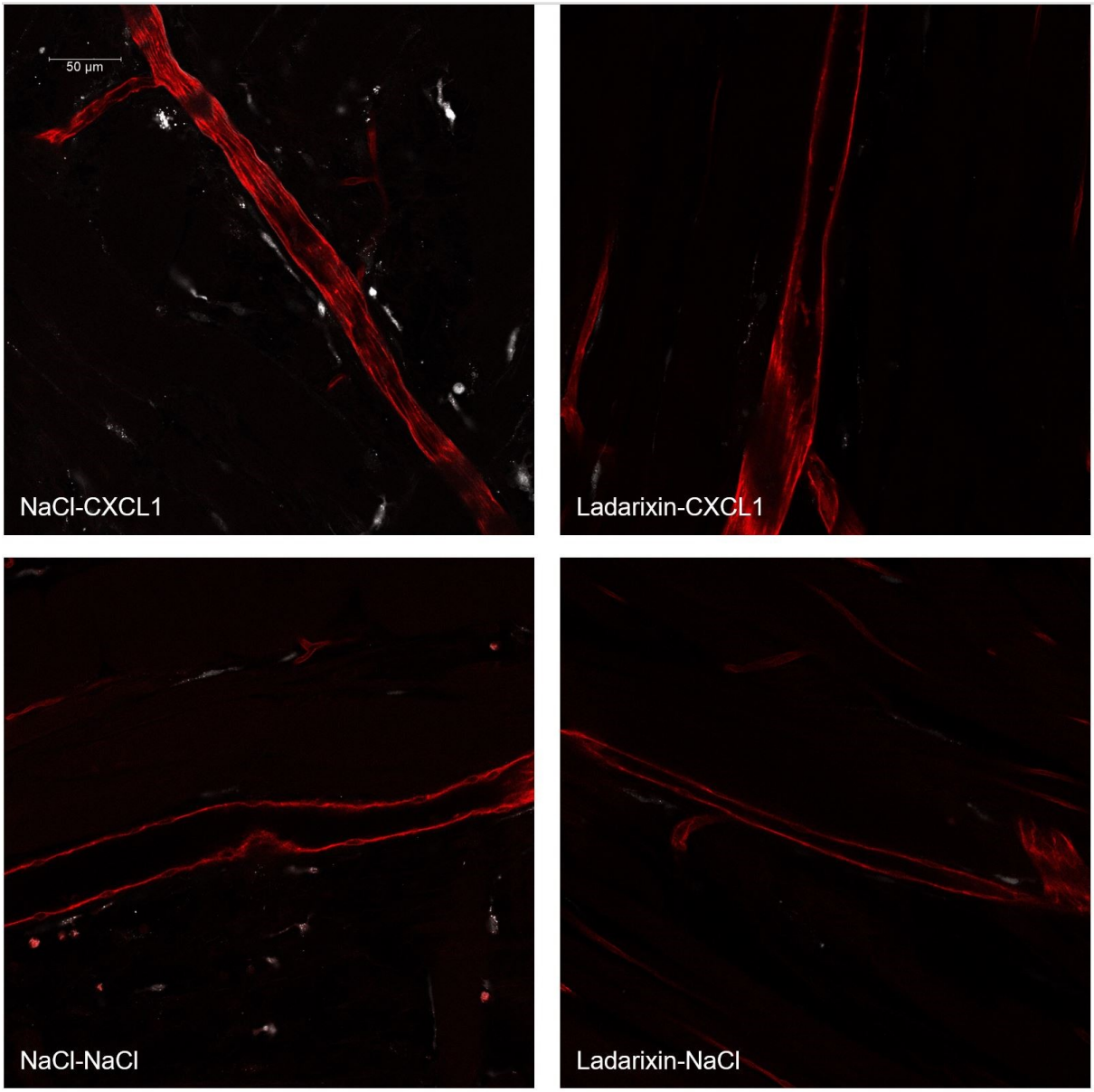


**Figure 22: Representative postcapillary venules and perivascular NE activity**

Our four experimental groups are shown with either inhibitor (on the right) or control i.p. pretreatment (on the left). Each mouse was injected i.s. with CXCL1 or NaCl as control. The PECAM-1 vessel staining is shown in red, the signal for the NE680FAST imaging agent for active NE is presented in white. NE680FAST was applied intrascrotally. Strong perivascular NE activity can be observed in the upper left picture, distributed in spots and stripes, which may even represent preferred neutrophil extravasation sites or tracks. When pretreating with the substance Ladarixin, active NE is almost completely absent both with or without an inflammatory stimulus. The NaCl-NaCl control shows low NE activity. Imaging was performed blinded on a confocal microscope. The 50µm scale bar (upper left) applies for all images.

# 5 DISCUSSION

This thesis focused on the role of Ladarixin (DF2156A), a small molecule, allosteric CXCR1/2 inhibitor[78], and its effect on neutrophil recruitment to an inflammatory site. Specifically, we assessed the impact of this substance on mobilization of integrins (VLA-3 and 6) and the serine protease NE to the cell's surface upon activation by respective chemokine receptors. A step that is necessary for vascular basement membrane penetration, where the ensemble of contact-providing integrins and the degrading protease enables the neutrophil's passage into the inflamed tissue[30,31,35,39–41]. The topic of vascular BM transmigration is still controversial as experimental evidence can be found for both BM penetration hypotheses: non-proteolytical squeezing or degradation (see chapter *1.1.1.* for further details). Those conflicting results mostly stem from different experimental approaches and the difficulty of finding an appropriate and transferrable investigation model or method[22,27,35]. The truth might lay in a mixture of both approaches[48]. It also has to be kept in mind that different organs present themselves with different BM structures, which presumably will require varying strategies of transmigration[9,22,48]. Next to that, the range of experiments performed also noted the importance of different players to be dependent on the used inflammatory stimulus[34,39]. Nevertheless, the involvement of the neutrophil-derived serine protease NE is very probable in view of current data, also from our lab.

In summary, this thesis shows that Ladarixin diminishes the cell surface translocation of neutrophil elastase after CXCR1/2-mediated cell activation by CXCL1, while Ladarixin showed no effect on NE mobilization after neutrophil stimulation by fMLP or CCL5. Ladarixin also showed no effect on rapid CXCR2 receptor internalization after CXCL1 exposure. *In-vivo*, I found reduced perivascular NE activity after Ladarixin application, which is in agreement with decreased amounts of emigrated neutrophils in the presence of Ladarixin.
The experimental setting consisted of murine *in-vitro* and *in-vivo* models. *In-vitro*, isolated murine bone marrow neutrophils were plated on coated 8-well chambers, followed by immunofluorescent staining and assessment by confocal microscopy. In addition, also FACS analysis of those isolated and pretreated neutrophils was conducted. The *in-vivo* method was an established cremaster muscle model with local pretreatment and cremaster muscle imaging, again by confocal microscopy.

*In-vitro* models with coated wells are an often used and a well-established method and, in our context, gave the opportunity to observe the cell's NE distribution on different contact surfaces. Nevertheless, as neutrophils are quite sensitive to their surroundings, the preceding cell isolation and pretreatment steps always leave the chance of premature cell activation[16,145], which would distort the following effects of Ladarixin, as the substance is applied before chemokine activation. Another point is the coating itself. In our initial fMLP experiments we tried to coat this chemotactic peptide directly on the wells. This resulted in NE ring formation similar to BSA controls. As this shows a lack of activation, we assumed that the much smaller fMLP, only consisting of three amino acids[59], could not be immobilized on the well's surface as well as the other coating substances. When applying fMLP soluble on top of the cell solution, high numbers of activated and ring-forming neutrophils could be observed. This shows that every coating has to be tested and it is always unclear how much of the coated compounds stay on the well's surface and really interact with the plated neutrophils. All of the above can lead to uncertain, widespread or even contradicting results, requiring a large *n* number, which was implemented in our experimental setting, accordingly. With a total number of 1544 analyzed neutrophils in the three well-chamber experiments (see section *4.1.*), the results can be viewed as reliable and valid. This is also supported by the two different and independent methods of analysis. As the visual analysis of ring formation is always prone to subjectivity, inter-observer variability and therefore distortions, the second method with analysis of intensity profiles is more objective and was able to confirm the previous results.

Regarding Ladarixin selectivity testing with neutrophil activation by fMLP and CCL5 the results were not surprising, since the previous works of Bertini (2012) and Garau (2006) et al. showed no interactions with other receptors from the CC/CXC family apart from CXCR1 and 2[78,96]. For fMLP it was already shown that Ladarixin has no effect on chemotaxis towards a respective gradient[96]. Also, chemotaxis under Ladarixin has been tested in different settings with many chemokines of the CXCL and CCL families, as wells as other chemoattractants like C5a or fMLP, although there are still many chemokine-receptor combinations missing that should be assessed, also taking into account a murine as well as a human approach[52,78,96]. But apart from those results, effects of Ladarixin on granule mobilization under CCL5 or fMLP stimulation had never been assessed until now and as the results from our lab show a very distinct effect of Ladarixin on venular BM penetration, those further chemokine-receptor combination tests should also be applied to the NE translocation assay. Next to that, initial tests from our lab also showed decreased vesicle mobilization for the integrins VLA-3 and 6, which are known to connect with the basement membrane and thereby help with the process of overcoming it[52]. Those tests were complicated by poor staining and imaging conditions. But optimizing these experiments in the future could show a downstream effect of Ladarixin, not only on azurophilic

granules and their translocation, but also on other granule types occurring in neutrophil granulocytes, as those mentioned integrins are stored in secretory vesicles[35]. Regarding those different granule types, it has to be kept in mind that murine neutrophil granule mobilization is still incompletely understood and therefore any results should not be translated to the human setting prematurely[49].

In an *in-vitro* FACS approach we assessed the influence of Ladarixin on CXCR2 expression on the neutrophil's surface. FACS experiments are, in this context, a rather simple tool with the opportunity to determine the amount of the specific receptor through the fluorescent signal from the inherent staining[145]. In contrast to the experiment above, this approach is less prone to subjectivity. Though, experiences from our lab indicate that chemokine receptors are quite sensitive to any kind of cell manipulation. Too heavy pipetting or again preceding isolation steps can already initiate activation and receptor internalization before Ladarixin is even applied and an effect can be measured. This stresses the need for careful cell handling and an appropriate *n* number. Since our results were quite consistent from the beginning, without any great variance, we can assume that there was either no, or an always consistent receptor interference by cell handling. Nevertheless, a more sparing cell isolation method could have been applied to reduce the possibility of premature CXCR2 internalization. Another interesting approach would have been testing Ladarixin in the context of CXCR1 internalization. Although CXCR2 seems to be the dominant receptor on the cell surface[9] and Ladarixin also showed a higher affinity towards CXCR2[95], an influence on neutrophil extravasation by the compound Ladarixin through CXCR1 cannot be excluded. A study from Rose et al. with human neutrophils from 2004 showed that the amount of agonist-chemokine needed for CXCR1/2 internalization was much higher than for simple downstream signaling[94]. For maximum stimulation (e.g., for chemotaxis or calcium flux) without receptor endocytosis the chemokine concentration needed was 10-times smaller than for endocytosis and in between those concentrations, CXCR1/2 seemed to oscillate between sensitized and desensitized states, allowing restimulation. Such high amounts of chemokines needed for internalization could physiologically occur at the center of inflammation. This suggests that internalization is not necessary, but rather terminating for neutrophil recruitment. Those human tests with CXCL8 and CXCR2, correlating to our experimental setup, discovered a median effective concentration ($EC_{50}$) for internalization of around 20nM CXCL8. When applying the double dose of CXCL8, it took only between 2-5 minutes for 50% receptor clearance. In general, this work also showed CXCR2 to internalize faster at a lower threshold, but also with poorer cell surface recycling, when compared to CXCR1[93,94]. The CXCL1 concentration in our murine approach was much smaller than that used by Rose et al. (2004) to determine the endocytosis threshold[94], but after 10 minutes of stimulation about 30% of CXCR2 were internalized (see

chapter *4.2.*). These findings point in the same direction as those from Feniger-Barish et al. (1999), who also performed CXCR2 internalization tests. Their dose-response graph shows CXCR2 endocytosis rates of around 50% at 100ng/ml of CXCL8 (human approach), which equals the CXCL1 chemokine concentration from our approach. But, as those results were generated with a longer incubation time of 2 hours, compared to the 10 minutes in our assay, these internalization percentages cannot be directly compared to our results[142]. Therefore, discrepancies in internalization rates stem from different incubation times, as well as varying chemokine exposure, where higher chemokine concentrations generally led to higher internalization rates[142]. Also, receptor recycling has an influence on cell surface receptor rates. Initial re-expression of CXCR2 on the cell surface after CXCL8-dependent endocytosis has been shown after 30 minutes without ligand exposure[142]. A distortion of our results by CXCR2 recycling processes can be excluded because of that. Another factor in those observed differences in internalization might be differences in receptor sensitivity between humans and mice. Nevertheless, this does not alter the supposition that Ladarixin's effect is probably independent from CXCR2 endocytosis.

Using the *in-vivo* cremaster muscle model, a method well established in our lab and also in the scientific community[146], made it possible to analyze the thin murine cremaster muscle, which allowed me to observe and measure neutrophil activities in or outside of the vessel under more physiological conditions. In our case the focus was on the neutrophil's NE signal on the cell surface, as well as on released NE. This was assessed mainly in the perivascular compartment. The aim was to see the consequences of NE translocation or impairment thereof in an inflamed tissue setting to deduce Ladarixin's impact on host defense in the mouse. This time a blinded approach was used, where first all samples were prepared, then blinded by an independent person, succeeded by imaging and analysis of all blinded samples. This provided objectivity to an otherwise again very subjective image assessment and prevented biased interpretation. Regarding the "non-inflammatory" control mice, it has to be kept in mind that although they were not intrascrotally injected with an inflammatory chemokine, these mice received a NaCl injection, as well as the other pretreatments, which will also cause minor inflammation. One of those other injections was the NE680FAST imaging agent. Since our focus was more on the perivascular NE activity and it was unclear if the fluorescent agent can adequately pass the vessel wall, we started to inject NE680FAST intrascrotally. The PerkinElmer protocol states that the imaging agent is only optimized for i.v. usage[140], which leaves our approach with unclear staining and imaging conditions. But, as the optically silent reagent needs to be proteolytically activated by NE to be flourescent, its specificity can also be assumed in the tissue. Next to that, we gained a satisfactory fluorescent signal. Since this signal stems from cell surface and released NE, it is not always possible to trace the exact

source. Next to that, NE occurs not only in neutrophils, but also in monocytes, eosinophils and mast cells[65,66]. However, since in this inflammatory setting, neutrophils are the prevailing immune cell group, which was also confirmed by DAPI nucleus staining in the samples, it can be assumed that the main source of the fluorescently located NE stems from the involved neutrophil granulocytes. In our *in-vivo* setting we implemented an intrascrotal inflammation by injecting an amount of 600ng CXCL1 per mouse directly into the scrotum. With a mean volume of both murine testes of around 30µl[147], not including the volume of the surrounding scrotal tissue such as the cremaster muscle, and the assumption of an only localized distribution of the administered chemokine, the result is a highly concentrated amount of CXCL1 in the affected tissue. In an acute murine inflammatory setting, e.g., reaction to an experimentally induced *Aspergillus fumigatus* infection of the lung, the infected tissue showed elevated CXCL1 levels of approximately 2ng/ml[148]. Another example is a murine *Streptococcus agalacitae* infection model, where CXCL1 levels in the hematogenous targeted kidneys and brain reached concentrations of up to 7ng/ml in the first hours after intravenous infection[149]. This shows that the physiological chemokine reaction due to acute infection in the mouse is located at much lower levels than applied in our experimental setting. Those measurements are not in contradiction with our results but state the gap between experimental approaches and physiological reactions.

To sum up, the results for Ladarixin in an inflammatory setting show an impaired NE mobilization, explaining reduced vascular BM transmigration and neutrophil extravasation. But the exact mechanisms behind those findings are still unclear. Previous results showed no influence of the compound on rolling or adhesion *in-vitro* and *in-vivo*[52], which means that the initial neutrophil activation needed for subsequent firm adhesion remains undisturbed, although the chemokine-CXCR1/2 interaction plays a main part in this activation process[13–15]. Ladarixin was discovered to be a non-competitive, allosteric CXCR1/2 inhibitor, acting from a conserved site and not interacting with ligand affinity of the receptor[78]. We have excluded effects on CXCR2 internalization by FACS experiments. This leaves further downstream CXCR2-dependent signaling processes, involved in vesicle mobilization, as suspected target of Ladarixin.

CXCR1/2 are GPCRs which, after activation, signal via PLCβ, IP3 and DAG to induce calcium influx[9,55]. This raise in calcium levels but also the activation of the cytoskeleton reorganizing GTPase Rac2 are important prerequisites for azurophilic granule release, and therefore NE degranulation[1,47,57]. Recent studies from our lab also showed neutrophil SRC family kinases to be involved in this process, as they seem to be triggered by GPCR signaling, colocalize with azurophilic granules and are involved in Rac2 activation, next to some other degranulation

related mechanisms[1,35,58–61]. Last, but not least, it has been reported that azurophilic granules are positive for a GTPase called Rab27a, which, together with its effectors JFC1 and Munc 13-4, helps to bring and fuse the vesicle with the cell membrane[48]. Our lab also recently discovered the involvement of MST1 in the Rab27a-JFC1 axis[31]. All of those known signaling steps are possible ways for Ladarixin to impair NE relocation to the cell surface. This needs to be assessed in further studies. For example, it has been shown that neutrophils from double knock out of the SRC kinases Hck and Fgr could still adhere in response to an inflammatory stimulus, but were not able to degranulate, similar to our results with Ladarixin[58]. But not only the azurophilic granule release has to be further explored. Since in initial tests conducted by my predecessor, Dr. med. Sebastian Sitaru, the mobilization of the integrins VLA-3 and VLA-6 was diminished[52], also the secretory vesicle degranulation seemed to be affected by Ladarixin application. Since their intracellular signaling is still not entirely clear, it can be assumed that Ladarixin either impairs their respective signaling, or a general upstream mechanism leads to this reduced granule/vesicle mobilization. Here, it has to be mentioned that, for example, Rac2 deficiency had no influence on specific and gelatinase granule exocytosis, although they also, like azurophilic granules, need cytoskeletal rearrangements for that process[1]. Next to that, fMLP-dependent primary granule release was shown to be mediated by p38MAPK, which had previously been activated by SFKs[59]. As fMLP initiated NE translocation remained undisturbed by Ladarixin, this signaling pathway can probably be excluded as possible downstream target. Interestingly, deficiency of the neutrophil CCLR2 receptor was found to reduce calcium signaling following CXCL8 stimulation, which subsequently led to diminished NE degranulation in response to CXCL1 and CXCL8, but not fMLP. CCLR2 is an atypical chemokine receptor (ACKR)-similar, 7-transmembrane receptor that forms homodimers and heterodimers with CXCR2 and is suspected to thereby modulate the downstream CXCR2 signaling in inflammatory settings. Although CCLR2 deficiency or blockade also showed reduced neutrophil adhesion, this elucidates that if CCLR2 turns out to be necessary for BM transmigration, also different receptor interactions could turn out to be a target for Ladarixin[150]. But not only receptor dimerization plays a role in regulating the downstream inflammatory response. When looking at new developments, some recent experiments may have identified the macrophage inhibitory factor (MIF) and parts of collagen (containing proline-glycine-proline/PGP-motifs) as new interacting partners of CXCR2 in the context of chemotaxis[151,152], although studies by Chapman et al. (2009) were not able to confirm those findings[107]. Next to that, it has been known for more than a decade that also the chemokine CXCL8 can occur as a monomer and a dimer. Even though our studies were solely carried out with CXCL1 because of the murine setting, it is interesting to know that in human *in-vitro* neutrophil experiments the CXCL8 monomer more effectively induced an intracellular

calcium rise, phosphoinositide hydrolysis, chemotaxis and also exocytosis through CXCR1 and 2, while in terms of receptor phosphorylation, desensitization and internalization, the monomer only showed to be more efficient for CXCR1[82]. Coming back to our substance of interest, Ladarixin, pharmacological testing regarding the CXCL8-CXCR1/2 interaction showed no change in ligand affinity towards the GPCRs after application of the compound[78]. But CXCL8 monomeric or dimeric forms were not considered, which leaves another angle on how Ladarixin could possibly influence the receptor's downstream signaling and thereby granule exocytosis.

My thesis was able to narrow down the probable effect of Ladarixin, but at the same time various new questions behind this effect opened up. When looking at the current clinical trials, but also disease related *in-vivo* and *in-vitro* testing, the importance of further research becomes apparent:
Ladarixin has been tested in a range of disease models: Different *in-vitro* and *in-vivo* experiments, using murine, rat as well as human approaches showed promising anti-inflammatory and thereby disease ameliorating results in the fields of obesity[88] and non-obesity[105] related diabetes, ischemia and reperfusion of the brain[96] ("cerebral infarct") and liver[78] ("liver transplantation") next to a variety of lung disease models like asthma, lung fibrosis or exacerbated influenza[87]. Last, but not least, Ladarixin proved to be anti-proliferative, anti-migratory and anti-angiogenic in a murine melanoma model with respect to tumor cells[95]. Those results led to first human clinical trials, especially in the very promising area of diabetes treatment. A recent phase 2 trial with final results from 2022 on adults with new-onset T1D demonstrated significant benefits for a patient subgroup with low C-peptide and high insulin demands. Promising tendencies towards β-cell preservation and disease deceleration led to a further assessment in the ongoing GLADIATOR phase 3 trial, carried out by Dompé[102–104]. But also the treatment options for obesity related T2D are being explored in an ongoing phase 2 trial, enrolling pre-diabetical adults and also elderly patients[88]. Although the results of those and further trials will have to be further consolidated, the possible treatment options with Ladarixin are already apparent and widespread through the field of inflammatory diseases, as the role of the CXCR1/2 axis in such illnesses becomes more evident. This exciting potential is also supported by minimal side effects during treatment, as safety testing showed Ladarixin to be well-tolerated at all applied dosages[95] and the recently published phase 2 diabetes trial showed adverse events like dyspepsia and headaches in 16% of treated patients[102,103]. In comparison to its predecessors, Ladarixin also shows optimized pharmacokinetic properties like a longer half-life and, for the first time, also the opportunity of oral administration, which holds a great advantage in chronic disease settings[95,96]. Although the possible applications are

multiple and promising, there is still a lot of work to be done. Further trials for different diseases need to be carried out next to exploring the long-term effects of Ladarixin. In current studies as well as in our murine *in-vivo* experiments, Ladarixin did not show an effect on neutrophil counts[95], but since CXCR2, the main target receptor, plays a crucial role in the bone marrow release of neutrophils, the danger of neutropenia through chronic administration needs to be assessed[87]. Next to further exploring the workings of Ladarixin, also predecessors or similar substances should not be lost sight of, since exploring their exact signaling mechanisms might help to shed light on the downstream intracellular effects of Ladarixin. One example is the previously mentioned Reparixin, which, in contrast to Ladarixin, mostly inhibits CXCR1[96]. Interestingly, murine cremaster muscle experiments with Reparixin showed significant CXCR2-mediated neutrophil adhesion deficiencies in response to CXCL1, which is not the case with Ladarixin[52,98]. At the same time Reparixin also inhibited signaling events downstream of CXCR1/2 resulting in impaired elastase release[98,153]. As Reparixin also works in a non-competitive, allosteric manner[153], understanding the differences between those substances regarding adhesion and intracellular signaling as well as the consequences of primarily inhibiting CXCR1 instead of CXCR2 will advance our knowledge especially regarding the potential of such compounds in different diseases and long-term application.

To conclude this thesis, continuing to solve Ladarixin's mechanism of action, will not only further treatment options for multiple acute as well as chronic diseases, but also enlighten the molecular mechanisms of our complex and imperative host defense.

# 6 SUMMARY

Neutrophil granulocytes make up the greatest part of our circulating immune cells in humans and thereby play a crucial role in host defense. In order to reach respective inflammatory sites after injury or infection, free flowing neutrophils respond to differently released and upregulated inflammatory signals, which initiate the so called “recruitment cascade”: After a sequence of tethering, rolling, activation and firm adhesion to the inflamed vascular endothelium, neutrophils start to crawl in order to finally find a suitable spot for transmigration through the layers of endothelium, basement membrane and underlying pericytes to reach their site of action in the inflamed tissue, where they can display a battery of defensive skills. The neutrophil’s chemokine receptors CXCR1 and CXCR2 and their respective ligands were shown to play an important part in neutrophil activation and thereby paving the way for firm adhesion and the following transmigration, as well as initiating the degranulation of intracellularly stored vesicle content. Those vesicles also contain an ensemble of compounds, that was found to be necessary for overcoming the vascular basement membrane: the basement membrane-binding integrins VLA-3 and VLA-6 and a basement membrane-degrading serine protease called neutrophil elastase, which is stored in the neutrophil’s azurophilic granules. But CXCR1/2 and also the mentioned protease neutrophil elastase not only advance our immune defense, they were also shown to play a role in the development and worsening of multiple acute and chronic diseases, like asthma, diabetes, rheumatoid arthritis or sepsis among many others. In this context, a new small molecule, allosteric, and non-competitive CXCR1/2 inhibitor called Ladarixin or DF2156A was developed by Dompé Pharmaceuticals. This substance significantly reduces neutrophil migration into affected tissue and thereby decreases subsequent related tissue damage from released mediators, like neutrophil elastase. Those positive effects were observed in different *in-vivo* and *in-vitro* disease models and phase 2 trials in the field of diabetes. But the exact mechanisms behind the workings of Ladarixin are still unclear, as the compound does not affect the primary adhesion to the endothelium, but the subsequent transmigration. In this thesis we tested the theory that Ladarixin impairs vascular basement membrane transmigration by diminishing neutrophil elastase release from azurophilic granules. For this purpose, we used a murine approach consisting of *in-vitro* NE translocation assays in coated well chambers, analyzed by confocal microscopy. Next, we carried out a CXCR2 internalization protocol, using FACS. This was followed by a final *in-vivo* cremaster muscle model with intravital neutrophil elastase staining, again using confocal microscopy. As chemokine ligand, CXCL1/KC was applied. Our

results show Ladarixin to reduce neutrophil elastase translocation to the cell surface upon CXCL1 stimulation, but not fMLP or CCL5 activation. In the CXCR2 internalization assay, Ladarixin had no effect on the amount of receptor endocytosis after CXCL1 application. Finally, our murine *in-vivo* cremaster muscle model corroborated the primary *in-vitro* results by showing significantly lower perivascular neutrophil elastase activity in Ladarixin pretreated mice with an artificially set inflammation by CXCL1.

Therefore, this thesis was able to show the impairing effect of Ladarixin on neutrophil elastase mobilization from azurophilic granules after neutrophil activation. This is a viable explanation for the observed reduction in neutrophil transmigration. Although this gives new insight into the mechanism behind this new small molecule inhibitor, the exact point, where Ladarixin interferes with downstream signaling and vesicle mobilization, remains to be discovered. This requires further experiments, which will not only enlighten the effects of Ladarixin and open up new treatment options, but will also advance our understanding of neutrophil signaling and immune defense.

# 7 ZUSAMMENFASSUNG

Zirkulierende Immunzellen bei Menschen bestehen zum größten Teil aus Neutrophilen Granulozyten, wodurch diese eine entscheidende Rolle in unserer Immunabwehr spielen. Um den Ort der Inflammation nach einer Verletzung oder Infektion zu erreichen, reagieren die sich in der Blutbahn befindlichen Neutrophilen auf verschiedene freigesetzte und hochregulierte inflammatorische Signale, welche eine Rekrutierungs-Kaskade in Gang setzen: Nach einer Abfolge von Anhaften, Rollen, Aktivierung und fester Adhäsion an das entzündete vaskuläre Endothel, beginnen die Neutrophilen zu kriechen, um einen passenden Ort für ihre Transmigration durch die Schichten der Basalmembran und darunterliegender Perizyten zu finden. Dadurch erreichen sie ihren Einsatzort im entzündeten Gewebe, wo sie eine ganze Batterie an Verteidigungsfähigkeiten zu Tage legen. Die Chemokin-Rezeptoren der Neutrophilen, CXCR1 und CXCR2, und ihre zugehörigen Liganden spielen eine wichtige Rolle in der Neutrophilen Aktivierung und machen damit den Weg frei für die feste Adhäsion und die darauffolgende Transmigration, wie auch die Einleitung der Degranulation von intrazellulär gespeichertem Vesikelinhalt. Diese Vesikel beinhalten ein Ensemble an Stoffen, die notwendig sind, um die Basalmembran zu durchdringen: Die Basalmembran-bindenden Integrine VLA-3 und VLA-6 und eine die Basalmembran-degradierende Serinprotease namens Neutrophilen Elastase, welche in den azurophilen Granula des Neutrophilen gespeichert ist. Aber CXCR1/2 und die genannte Neutrophilen Elastase helfen nicht nur bei der Immunabwehr, es wurde auch gezeigt, dass sie eine Rolle bei der Entstehung und auch der Verschlechterung von multiplen akuten und chronischen Erkrankungen spielen, wie zum Beispiel Asthma, Diabetes, Rheumatoider Arthritis oder Sepsis, neben vielen weiteren. In diesem Kontext entwickelte die Pharmafirma Dompé einen neuen, allosterisch und nicht-kompetitiv wirkenden „small-molecule“ CXCR1/2-Inhibitor namens Ladarixin oder auch DF2156A. Diese Substanz reduziert signifikant die Neutrophilen Migration in betroffenes Gewebe und vermindert dadurch die nachfolgende und damit verbundene Gewebszerstörung durch freigesetzte Mediatoren, wie der Neutrophilen Elastase. Diese positiven Effekte wurden in verschiedenen *in-vitro* und *in-vivo* Krankheitsmodellen wie auch einer Phase-2-Studie im Bereich Diabetes beobachtet. Die genauen Mechanismen hinter der Wirkungsweise von Ladarixin sind jedoch immer noch unklar, da die Substanz die primäre feste Adhäsion am Endothel nicht beeinflusst, die nachfolgende Transmigration jedoch schon. In dieser Arbeit wurde die Hypothese getestet, dass Ladarixin die Migration durch die Basalmembran über die verminderte Freisetzung der Neutrophilen Elastase aus den azurophilen Granula von

Neutrophilen beeinträchtigt. Um diese Hypothese zu testen, verwendeten wir eine experimentelle Herangehensweise. Diese bestand zunächst aus *in-vitro* Neutrophilen-Elastase-Translokations-Tests in beschichteten und gekammerten Objektträgern, welche mittels Konfokalmikroskopie analysiert wurden. Als nächstes führten wir ein CXCR2-Internalisierungs-Protokoll mit FACS-Analyse durch. Gefolgt von einem finalen *in-vivo* Cremaster-Muskel-Modell in der Maus mit intravitaler Färbung der Neutrophilen Elastase, bei welchem wiederum konfokale Mikroskopie angewandt wurde. Als Chemokin-Ligand wurde durchgehend CXCL1/KC verwendet. Unsere Ergebnisse zeigen, dass Ladarixin die Translokation der Neutrophilen Elastase zur Zelloberfläche nach einer CXCL1 Stimulation reduziert, nach einer Aktivierung mit fMLP oder CCL5 war dies allerdings nicht der Fall. In den CXCR2-Internalisierungs-Tests zeigte sich, dass Ladarixin keinen Effekt auf die Rezeptor-Endozytose nach CXCL1 Stimulation hat. Schließlich konnten in unserem *in-vivo* Cremaster-Muskel-Modell in der Maus die primären *in-vitro* Ergebnisse bestätigt werden. Es zeigte sich auch hier eine signifikant geringere perivaskuläre Aktivität der Neutrophilen Elastase in mit Ladarixin vorbehandelten Mäusen, bei denen eine artifizielle Inflammation mittels CXCL1 gesetzt wurde.

In Zusammenfassung konnte diese Arbeit den beeinträchtigenden Effekt von Ladarixin auf die Mobilisation der Neutrophilen Elastase aus den azurophilen Granula nach Neutrophilen-Aktivierung aufzeigen. Dies stellt eine valide Erklärung für die entdeckte, fehlgeschlagene Transmigration dar. Obwohl uns dies neue Einblicke in die Wirkmechanismen dieses neuen „small-molecule“ Inhibitors gibt, der genaue Punkt, an dem Ladarixin die intrazelluläre Signalkaskade und damit die Vesikel Mobilisation stört, bleibt ungeklärt. Dies erfordert weitere Experimente, welche aber nicht nur die Effekte von Ladarixin aufklären und neue Behandlungsoptionen erschließen werden, sondern auch unser Verständnis über die Signalkaskaden und Immunabwehr durch Neutrophile Granulozyten voranbringen werden.

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# 9 ABBREVIATION LIST

| | |
|---|---|
| **(m)KC** | (murine) Keratinocyte chemoattractant (=CXCL1) |
| **ACKR** | Atypical chemokine receptor |
| **AKT** | Protein kinase B |
| **ALDH** | Aldehyde dehydrogenase |
| **ANOVA** | Analysis of variance |
| **APL** | Acute promyelocytic leukemia |
| **ARDS** | Acute respiratory distress syndrome |
| **BASO** | Basophils |
| **BM** | Basement membrane |
| **BMC** | Biomedical center |
| **BSA** | Bovine serum albumin |
| **CAM** | Core Facility Animal Models |
| **CD** | Cluster of differentiation |
| **CF** | Cystic fibrosis |
| **COPD** | Chronic obstructive pulmonary disease |
| **COVID-19** | Coronavirus disease 2019 |
| **DAG** | Diacylglycerol |
| **DAMP** | Danger associated molecular pattern |
| **DAPI** | 4',6-diamidino-2-phenylindole |
| **DPP1** | Dipeptidyl peptidase 1 |
| **$EC_{50}$** | Median effective concentration |
| **ECM** | Extracellular matrix |
| **EO** | Eosinophils |
| **ER** | Endoplasmatic Reticulum |
| **ESAM** | Endothelial cell-selective adhesion molecule |
| **FACS** | Fluorescence activated cell sorting |
| **FBS** | Fetal bovine serum |
| **$FEV_1$** | Forced expiratory volume in 1 second |
| **fMLP** | N-formyl-methionyl-leucyl-phenylalanine |
| **FOV** | Field of view |
| **fPR(1)** | Formyl peptide receptor (1) |
| **FSC** | Forward side scatter |

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|---|---|
| **GAG** | Glycosaminoglycan |
| **G-CSF** | Granulocyte-colony stimulating factor |
| **GDP** | Guanosine diphosphate |
| **GEF** | Guanosine exchange factor |
| **GEK** | G-protein coupled receptor kinase |
| **GIMP** | Gem interacting protein |
| **GLUT** | Glucose transporter |
| **GPCR** | G-protein coupled receptor |
| **GSDMD** | Gasdermin-D |
| **GSK** | GlaxoSmithKline |
| **GTP** | Guanosine triphosphate |
| **HBSS** | Hanks' balanced salt solution |
| **HEPES** | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| **HUVEC** | Human umbilical vein endothelial cells |
| **HyD** | Hybrid Photo Detector |
| **i.p.** | Intraperitoneal |
| **i.s.** | Intrascrotal |
| **i.v.** | Intravenous |
| **I/R** | Ischemia/Reperfusion |
| **$IC_{50}$** | Half-maximal inhibitory concentration |
| **ICAM1/2** | Intercellular adhesion molecule 1/2 |
| **IL** | Interleukin |
| **IP3** | Inositoltriphophate |
| **IRS** | Insulin receptor substrate |
| **IVC** | Individually ventilated cages |
| **JAM** | Junctional adhesion molecule |
| **JFC1** | Synaptotagmin-like protein 1 |
| **LER** | Low expression region |
| **LFA1** | Lymphocyte function-associated antigen 1 |
| **LPS** | Lipopolysaccharide |
| **LYMPH** | Lymphocytes |
| **MAC1** | Macrophage-1-antigen |
| **MDSC** | Myeloid derived suppressor cell |
| **MIF** | Macrophage inhibitory factor |
| **MMP** | Matrix metalloproteinase |
| **MNEI** | Monocyte/neutrophil elastase inhibitor |

| | |
|---|---|
| **MONO** | Monocytes |
| **MPO** | Myeloperoxidase |
| **MST1** | Mammalian sterile 20-like kinase 1 (=STK4) |
| **NACL** | NaCl 0,9%/normal saline |
| **NADPH** | Nicotinamide adenine dinucleotide phosphate |
| **NE** | Neutrophil elastase |
| **NE(680)FAST** | Neutrophil Elastase (680) Fluorescent Activatable Sensor Technology |
| **NET** | Neutrophil extracellular trap |
| **NEU** | Neutrophils |
| **NF-kB** | Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells |
| **NGAL** | Neutrophil gelatinase-associated lipocalin |
| **NOD** | Non-obese diabetic |
| **ON** | Overnight |
| **P38MAPK** | P38 mitogen-activated protein kinase |
| **PAMP** | Pathogen associated molecular pattern |
| **PBS** | Phosphate-buffered saline |
| **PECAM-1** | Platelet endothelial cell adhesion molecule-1 |
| **PFA** | Paraformaldehyde |
| **PGP** | Proline-glycine-proline |
| **PI3K** | Phosphoinositide 3-kinase |
| **PI6/9** | Proteinase inhibitor 6/9 |
| **PLCß** | Phospholipase Cß |
| **PLD** | Phospholipase D |
| **PMN** | Polymorphonuclear neutrophils |
| **PMT** | Photomultiplier tube |
| **PRR** | Pathogen recognition receptor |
| **PSGL-1** | P-selectin glycoprotein ligand-1 |
| **PTK** | Protein tyrosine kinase |
| **PVR** | Poliovirus receptor |
| **RA** | Rheumatoid arthritis |
| **ROI** | Region of interest |
| **ROS** | Reactive oxygen species |
| **RT** | Room temperature |
| **SCN** | Severe congenital neutropenia |
| **SEM** | Standard error of the mean |
| **SFK** | SRC family kinase |

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|---|---|
| **SLPI** | Secretory leukocyte proteinase inhibitor |
| **SNARE** | Soluble N-ethylmaleimide-sensitive-factor attachment receptor |
| **STK4** | Serine threonine kinase 4 (=MST1) |
| **SV** | Secretory vesicle |
| **T1D** | Type 1 diabetes |
| **T2D** | Type 2 diabetes |
| **TEM** | Transendothelial migration |
| **TM** | Transmembrane region |
| **TNF-α** | Tumor necrosis factor-α |
| **UC** | Ulcerative colitis |
| **VLA-3/6** | Very late antigen-3/6 |
| **WBC** | White blood cells |
| **α1-PI** | α1-Proteinase inhibitor/α1-Antitrypsin |

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# 11 APPENDIX

## *11.1 List of Figures*



## *11.2 List of Tables*

# 12 AFFIDAVIT

LMU LUDWIG-MAXIMILIANS-UNIVERSITÄT MÜNCHEN

Dekanat Medizinische Fakultät
Promotionsbüro

## Affidavit

Budke, Agnes Margarethe
Surname, first name

I hereby declare, that the submitted thesis entitled

**The Effects of Ladarixin in Leukocyte Recruitment**

is my own work. I have only used the sources indicated and have not made unauthorized 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 dissertation presented here has not been submitted in the same or similar form to any other institution for the purpose of obtaining an academic degree.

Munich, 17.03.2024
Place, Date

Agnes Buke
Signature doctoral candidate

# 13 PUBLICATIONS

- Sitaru, S., Budke, A., Bertini, R. & Sperandio, M. Therapeutic inhibition of CXCR1/2: where do we stand? *Internal and Emergency Medicine,* 1–18; 10.1007/s11739-023-03309-5 (2023).