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The dual non-competitive CXCR1/2 inhibitor ladarixin impairs neutrophil extravasation without altering intravascular adhesion

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Running heads: Ladarixin impairs basement membrane penetration of neutrophils

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CONFLICT OF INTEREST

M.S. had been a consultant of Dompé Farmaceutici. RB is a pre-clinical pharmacology consultant of Dompé Farmaceutici. P.G.A. A.A. and M.A. are employees of Dompé Farmaceutici.

AUTHOR CONTRIBUTION

MN designed and conducted experiments, analyzed data and wrote the manuscript. SS, AB, AK, GQ, RI, IR, SS, BF, LW and SC acquired and analyzed data. PGA, MA, AA and RB provided their expertise, critical reagents and revised the manuscript. MP and MS designed experiments, analyzed data and wrote the manuscript.

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DATA AVAILABILITY

All data that support the findings of this study are present in the article and are available from the corresponding author upon reasonable request.

ABSTRACT

In many acute and chronic inflammatory disorders, recruitment of neutrophils plays a critical role in preventing disease severity and ensuring survival. On the other hand, neutrophil accumulation during inflammation can also favor disease progression in diseases such as autoimmune disorders, cancer or during ischemia-reperfusion injury. Therefore, blocking neutrophil influx has been considered an interesting therapeutic concept in diseases with overwhelming neutrophil responses. Using several *in vivo* and *in vitro* approaches we investigated the mode of action of ladarixin, a dual non-competitive inhibitor of chemokine receptors CXCR1 and CXCR2, to block neutrophil recruitment during inflammation. Examining neutrophil recruitment both *in vivo* in the mouse cremaster muscle via intravital microscopy and *in vitro* via flow chamber assays, we investigated the biological significance of the functional selectivity of the allosteric inhibitors, showing that ladarixin blocks neutrophil extravasation selectively on the level of vascular basement membrane penetration, a prerequisite for neutrophils to reach the inflamed tissue, without impairing rolling and adhesion to the inflamed endothelium. Mechanistically, ladarixin abolished neutrophil elastase translocation to the neutrophil surface, indispensable for neutrophil vascular basement membrane penetration and extracellular matrix degradation, in a selective and specific CXCR1/2 dependent fashion leading to reduced neutrophil elastase surface activity in mouse and primary human neutrophils. Taken together, the allosteric CXCR1/2 inhibitor ladarixin effectively blocks neutrophil recruitment on the level of neutrophil extravasation without affecting firm adhesion. Clinically, this mode of action has interesting therapeutic potential to prevent neutrophil extravasation in inflammatory diseases including inflammatory bowel disease, psoriasis and other neutrophil driven disorders.

INTRODUCTION

Neutrophils are the predominant circulating immune cells in humans. They are considered the first line of immune defense in the context of acute inflammatory responses during infection or following sterile injury [1]. To fulfill this task, neutrophils have to leave the intravascular compartment and transform from a quiescent, inactive state into an activated and pro-migratory phenotype. This transformation occurs mostly during the local recruitment phase where neutrophils get into close contact with the endothelial lining of inflamed vessels [2]. Through a well-described cascade of adhesion and activation events on the luminal endothelial surface, signals are exchanged between neutrophils and endothelial cells finally leading to the decision of neutrophils to leave the blood microvasculature and transmigrate into the inflamed tissue. One of the key signaling events for neutrophil extravasation is provided by interactions of the chemokine receptor CXCR2 on the neutrophil surface with CXCR2-binding chemokines including CXCL8 (in humans) and CXCL1 (in mice) typically presented on the luminal surface of inflamed endothelial cells [3, 4]. CXCR2 has been identified as the chemokine receptor responsible to induce firm neutrophil arrest on the inflamed endothelium *in vivo* [5, 6]. Thereafter, postarrest modifications including spreading, adhesion strengthening and crawling prepare neutrophils for trans-endothelial migration and vascular basement membrane penetration to reach the inflamed tissue [7]. Successful penetration of the basement membrane is a chemokine-dependent process that requires the mobilization of critical components such as neutrophil elastase (NE) and β1 integrins (both stored in various intracellular granules) to the neutrophil surface [8].

Binding of the chemokine CXCL8 to its respective chemokine receptors CXCR1 and CXCR2 induces overlapping, as well as distinct outcomes. Overlapping signals include an increase in intracellular calcium signaling, induction of adhesion, chemotaxis toward the gradient and increased reactive oxygen species (ROS) production [9]. Other, less studied functions include a critical and unique role of CXCR1 in bacterial and fungal killing [10, 11]. Of note, CXCR1 expression is restricted to neutrophils, while CXCR2 is also present on other hematopoietic cells such as monocytes and NK cells, as well as on non-hematopoietic cells including pulmonary endothelial and epithelial cells [12].

Considering the multitude of functions exerted by CXCR2 and CXCR1 on neutrophils, inhibiting both receptors have gained significant interest recently as a potential therapeutic approach in acute and chronic inflammatory disorders with overwhelming and unwanted neutrophil recruitment, including infectious diseases, acute lung injury, acute gout, psoriasis, inflammatory bowel disease and many more [1, 12]. ladarixin, formerly known as DF2156A, is a dual CXCR1 and CXCR2 non-competitive allosteric inhibitor interacting with an allosteric site conserved in CXCR1 and CXCR2 and with a prolonged and improved *in vivo* half-life compared to another related dual CXCR1/2 inhibitor compound named Reparixin [13]. ladarixin was demonstrated to attenuate the inflammatory response

and neutrophil recruitment in a rat cerebral and hepatic ischemia reperfusion model [14, 15]. In addition, it showed comparable inhibitory activity in CXCL8-induced migration of CXCR1- and 2-transfected cell lines in a chemotactic migration assay with an IC_{50} of 0.8nM for CXCR2 and an IC_{50} of 0.7nM for CXCR1 [15]. Several disease relevant *in vivo* mouse models have demonstrated that ladarixin successfully reduces neutrophil recruitment, disease severity and progression, including models for type 1 diabetes, aortic abdominal aneurysm, and infectious as well as sterile lung inflammation [16-18]. ladarixin proposed mode of action aligns to its capacity to selectively modulate pathways activated by CXCR1 and CXCR2 while leaving others unaffected [19]. Nevertheless, the capacity of ladarixin in inhibiting distinct CXCR1/2 mediated functions during the recruitment process remained to be characterized. To fill this gap, we investigated the influence of ladarixin on neutrophil recruitment using various CXCR1/2 dependent *in vivo* and *in vitro* approaches. Our results demonstrate that ladarixin significantly impairs CXCR1/2-induced neutrophil extravasation by inhibiting neutrophil elastase translocation to the cell surface and vascular basement membrane penetration, without affecting neutrophil rolling or adhesion.

METHODS

Animals

C57BL/6NCrl wildtype (WT) mice were purchased from Charles River Laboratories (Sulzfeld). Mice were maintained at the core facility animal models at the Biomedical Center, LMU, Planegg-Martinsried, Germany and used for experiments from 8-25 weeks of age. All experiments were approved by the local authorities (Regierung von Oberbayern, AZ 55.2-1-54-2531-122/12 and 55.2-2532.Vet_02-17-102).

Chemokine Receptor Inhibitor

The compound ladarixin was provided by Dompè Farmaceutici S.p.a (L'Aquila, Italy). If not stated otherwise, we used ladarixin at 5 µM for *in vitro* and 30 µg/g body weight for *in vivo* experiments as reported earlier [15].

Intravital microscopy of CXCL8 and trauma induced inflammation in the mouse cremaster muscle

We used two models of intravital microscopy in the mouse cremaster muscle to study leukocyte rolling and adhesion in inflamed postcapillary venules [6]. Briefly, in the first *in vivo* model, intrascrotal injection (i.s.) of recombinant murine (rm) CXCL1 (600ng/mouse, Peprotech) was conducted one hour after intraperitoneal injection (i.p.) of ladarixin or 0.9% NaCl (carrier). Two hours after CXCL1 injection, mice were anesthetized and the carotid artery was cannulated to assess systemic white blood cell counts (ProCyte Dx; IDEXX Laboratories). Thereafter, the cremaster muscle was surgically

exteriorized for intravital microscopy on an OlympusBX51 WI microscope, equipped with a 40x objective (Olympus, 0.8NA, water immersion objective) and a CCD camera (Kappa CF8HS)[20]. During the experiment, the muscle was permanently superfused with a thermo-controlled bicarbonate buffered salt solution and postcapillary venules recorded using VirtualDub software for later off-line analysis. Venular centerline velocity was assessed using a dual photodiode (Circusoft Instrumentation) [21]. In addition, we obtained rolling flux fraction, which is the number of rolling cells normalized to complete leukocyte flux, number of adherent cells/mm^2, leukocyte rolling velocities, vessel diameter and vessel segment length using the generated movies and Fiji software [21]. In the second intravital microscopy model, 2h after i.p. application of either ladarixin, Pertussis toxin (PTx, 4ng/mouse) or 0.9% NaCl (carrier), the cremaster muscle was exteriorized without any prior proinflammatory stimulus. The surgical preparation of the cremaster muscle causes a mild inflammatory response with upregulation of P-selectin on the endothelial luminal surface leading to P-selectin dependent leukocyte rolling accompanied by only a modest number of adherent leukocytes in postcapillary venules [22]. Intra-arterial (i.a.) injection of rhCXCL8 (600ng/mouse, Peprotech) was used to induced firm arrest of rolling neutrophils as previously reported [6]. Microvascular and adhesion relevant parameters (before and after CXCL8 injection) were obtained as described above.

Human Neutrophil Experiments and Flow Chamber Assays

All experimental details regarding human neutrophil isolation, handling, flow chamber assays, and additional methodological procedures are provided in the supplementary methods. Readers are referred to the Supplementary File for detailed protocols and experimental conditions.

Statistics

Data is shown as mean±SEM, cumulative frequencies or representative images as indicated in the figure legends. GraphPad Prism 9 software (GraphPad Software Inc.) and Adobe Illustrator was used to analyze data and generate graphs. Statistical tests were conducted according to the number of groups being compared. For pairwise comparison, an unpaired Student's t-test and for more than two experimental groups, either a one-way or a two-way analysis of variance (ANOVA) with either a Tukey's or a Sidak's *post-hoc* test was performed. P-values <0.05 were considered statistically significant and indicated as follows: *: <0.05, **: <0.01, ***: <0.005.

RESULTS

ladarixin application does not affect neutrophil rolling and neutrophil adhesion in vivo and in vitro

To study the effect of the dual CXCR1/2 inhibitor ladarixin on neutrophil recruitment, we made use of intravital microscopy and an acute and predominantly neutrophil-driven microvascular inflammation model in the mouse cremaster muscle (Fig. 1A). In a first set of experiments, we injected ladarixin or 0.9% NaCl (carrier) into the peritoneal cavity of male WT mice one hour prior to i.s. injection of CXCL1. Two hours after CXCL1 injection, we exteriorized the cremaster muscle and assessed leukocyte rolling, rolling velocity, and leukocyte adhesion in postcapillary cremaster muscle venules. Application of ladarixin did not alter leukocyte rolling (Fig. 1B) or rolling velocity, nor did it affect the number of adherent leukocytes compared to controls (Fig. 1C and D), suggesting that ladarixin does not interfere with chemokine-driven firm leukocyte arrest. In order to exclude that the effect of ladarixin on leukocyte adhesion is masked by CXCL1-induced upregulation of endothelial E-selectin, known to cooperate with CXCR1/2 signaling in mediating firm leukocyte adhesion, we conducted cremaster muscle experiments in unstimulated mice. In this model, the surgical procedure itself induces mild traumatic injury with mobilization of P-selectin to the luminal surface of postcapillary venular endothelial cells within minutes after the cremaster muscle has been exteriorized. This leads to leukocyte rolling without triggering substantial leukocyte adhesion (Fig. 1E). However, intra-arterial injection of CXCL8 (the human homologue of CXCL1 known to induce leukocyte adhesion in the mouse cremaster similar to CXCL1 [6]) resulted in rapid transition from leukocyte rolling to firm adhesion, which was similar in carrier and ladarixin pretreated mice (Fig. 1E) suggesting no role of ladarixin in inducing firm leukocyte adhesion. In contrast, pretreatment of mice with PTx, which blocks $G_{\alpha i}$ coupled signaling and inhibits GPCR induced transition from rolling into firm adhesion (positive control), prevented CXCL8-mediated induction of leukocyte adhesion. These results clearly demonstrate that ladarixin does not interfere with CXCL8- induced leukocyte adhesion *in vivo*.

To test whether ladarixin affects CXCR1/2 induced leukocyte adhesion in human neutrophils, we conducted flow chamber experiments (Fig. 1F) using neutrophils isolated from peripheral blood of healthy volunteers. Neutrophils pretreated with ladarixin or PBS (carrier) were perfused through flow chambers coated with E-selectin, ICAM-1 and CXCL8. In line with our *in vivo* mouse experiments, ladarixin did not affect the number of rolling neutrophils, rolling velocity nor the number of adherent cells (Fig. 1G-I).

Neutrophil extravasation into inflamed tissue is impaired in ladarixin pretreated mice

Next, we wanted to study neutrophil extravasation in CXCL1-stimulated cremaster muscles in mice pretreated with ladarixin. To do this, cremaster muscles were obtained 2h after i.s. injection of CXCL1 and pretreatment with either ladarixin or 0.9% NaCl (carrier) and stained with Giemsa (Fig. 2A). ladarixin pretreatment significantly decreased the number of extravasated leukocytes (Fig. 2B), which was mostly related to a marked reduction in extravasated neutrophils. The number of

eosinophils and mononuclear cells were not significantly affected by ladarixin pretreatment suggesting that ladarixin specifically prevents neutrophil extravasation. Notably, neutrophils remained close to the abluminal part of the vessel wall in ladarixin pretreated mice (Fig. 2B, representative picture), indicating that ladarixin hinders neutrophils to migrate into the inflamed tissue.

Next, we tested whether ladarixin has a potential role in neutrophil chemotaxis. To do this, we conducted an *in vitro* under flow chemotaxis assay using CellDirector chambers (Fig. 2C). Neutrophil tracking was performed via time-lapse microscopy, restricting quantitative migration analysis to the first 10 minutes to capture the early, directed migratory response within a stable gradient environment. Differently to what has been reported previously using diverse chemotaxis assays (14, 15), ladarixin preincubation did not affect the capability of mouse neutrophils to migrate toward the CXCL1 chemotactic gradient during this initial phase. Accordingly, we found no difference in Euclidean distance (Fig. 2D), crawling directionality (Fig. 2E) nor in crawling velocity (Fig. 2F) between carrier and ladarixin pretreated neutrophils.

Finally, we wanted to study whether ladarixin might interfere with the ability of mouse neutrophils to internalize the CXCL1 receptor CXCR2 [23], as this might explain impaired neutrophil transmigration as well. As expected, CXCL1 induced rapid internalization of neutrophil-expressed CXCR2 was not affected by pretreatment of neutrophils with ladarixin (Fig. 2G) excluding that the impairment of neutrophil extravasation by ladarixin might be regulated through modulating CXCR2 internalization.

ladarixin specifically impairs CXCL1 induced neutrophil elastase mobilization

Earlier work from our group and from others have demonstrated that neutrophil elastase (NE) mobilization from intracellular storage granules to the neutrophil surface is critical during neutrophil vascular basement membrane penetration [24-26]. To test whether CXCR1/2 blockade by ladarixin interferes with NE surface translocation, we investigated NE mobilization in murine neutrophils stimulated PECAM-1, ICAM-1 and CXCL1 coated plates with or without ladarixin pretreatment (Fig. 3A). Neutrophil activation resulted in NE translocation to the neutrophil surface membrane, assessed by the appearance of a ring like structure of NE staining on the cellular surface (Fig. 3B and 3C). Pretreatment of neutrophils with various concentrations of ladarixin led to a strong inhibition of NE translocation to the neutrophil surface compared to carrier treated neutrophils. Coating with BSA (control) did not induce any mobilization of NE to the surface. To investigate whether the inhibitory effects of ladarixin on NE mobilization is specific for the CXCR1/2 axis, we performed the same experiments using CCL5 (RANTES) and the formyl peptide fMLF (added as soluble molecule) instead of CXCL1. Both, CCL5 and fMLF led to a significant mobilization of NE to the neutrophil surface which was unaffected by ladarixin pretreatment (Fig. 3D and 3E). These findings demonstrate that ladarixin specifically blocks CXCR1/2 induced NE mobilization to the neutrophil surface.

ladarixin reduces NE activity during neutrophil transendothelial migration in vivo* and *in vitro

To demonstrate that ladarixin-mediated impairment of NE mobilization to the neutrophil surface also occurs during neutrophil recruitment *in vivo*, we performed experiments in CXCL1-stimulated mouse cremaster muscles using NE680FAST, a fluorescence reporter of NE activity. First, we assessed perivascular NE activity. To do this, we i.p. injected 0.9% NaCl (carrier) or ladarixin, followed by i.s. application of NE680FAST. One hour later, CXCL1 or 0.9%NaCl (control) was injected intrascrotally. Two hours after i.s. injection of CXCL1/NaCl, the cremaster muscle was dissected and prepared for assessing perivascular NE activity using confocal microscopy. We found a significant increase in perivascular NE activity in CXCL1-stimulated cremaster muscle of WT mice pretreated with normal saline (carrier), while pretreatment with ladarixin led to a marked and significant drop in perivascular NE activity (Figure 4A and B). This demonstrates that NE activity is severely reduced in inflamed cremaster muscle tissue of ladarixin pretreated mice. Next, we tested whether ladarixin already influences NE activity within inflamed postcapillary venules. For this, 0.9% NaCl (carrier) or ladarixin was injected i.p. followed by i.v. application of NE680FAST and i.s. injection of CXCL1/NaCl as described above. We found significantly reduced activity of NE within inflamed postcapillary cremaster muscle venules of WT mice pretreated with ladarixin compared to intravascular NE activity in carrier treated mice (Fig. 4C and 4D) indicating that mobilization of NE during neutrophil recruitment is already impaired within the intravascular compartment.

In a second set of experiments we wanted to demonstrate the enzymatic activity of NE localized on the neutrophil surface. To do this, we used a system that allowed us to visualize NE digestion by spinning disc confocal microscopy. Mouse neutrophils were isolated, incubated in the absence (carrier) or presence of ladarixin and loaded with a cell tracker to visualize the cells. In addition, laminin was visualized with an Alexa-647 coupled antibody. We observed a high percentage of laminin-digesting cells in the carrier treated group (Fig. 4E and 4F, Supplemental Movie 1). Pretreatment of neutrophils with ladarixin modestly but still significantly reduced the percentage of laminin-digesting neutrophils (Fig. 4E and 4F, Supplemental Movie 2) demonstrating that ladarixin prevents neutrophils to degrade laminin, a prerequisite for successful penetration of the vascular basement membrane.

ladarixin impairs neutrophil elastase mobilization in human primary neutrophils

To demonstrate that CXCR1/2 blockade by ladarixin interferes with NE surface translocation also in the human system, we performed a NE translocation assay and a transmigration assay *in vitro* (Fig. 5A). Similar to our observation using mouse neutrophils (Fig. 3B and 3C), activation of human neutrophils on PECAM-1, ICAM-1 and CXCL8 coated plates induced NE translocation to the cell surface (Fig. 5B and 5C). Interestingly, NE mobilization upon stimulation was more pronounced in

human neutrophils than in murine neutrophils. However, pretreatment of human neutrophils with ladarixin inhibited NE translocation to the cell surface down to the same level as in murine neutrophils.

Finally, we wanted to investigate the capability of human neutrophils to release NE into the supernatant after stimulation with PECAM-1, ICAM-1 and CXCL8 in the presence or absence of ladarixin. Therefore, we adopted a transwell system, coated the filter with PECAM-1, ICAM-1 or left them uncoated (control) and allowed human primary neutrophils to migrate without any gradient (control) or to CXCL8 (Fig. 5D). With this combination of stimuli we wanted to induce migration and simultaneous NE secretion of activated neutrophils. Compared to CXCL8-treated neutrophils, additional pretreatment of neutrophils with ladarixin had a modest but still significant impact in reducing the amount of NE secreted during migration (Fig. 5D). This demonstrates that ladarixin prevents CXCL8-induced NE translocation and secretion in human neutrophils.

DISCUSSION

Neutrophil recruitment is considered a critical event during the inflammatory response initiating the influx of immune cells into inflamed tissue to help removing invading microorganisms in case of infection or to clear injured tissue from dead cells and cell debris in case of sterile inflammation [1]. One of the key factors regulating the specificity of leukocyte subset recruitment are chemokines and their respective receptors [27]. CXCR1 and CXCR2 are the essential receptors on neutrophils and bind CXCL1 (mice) and CXCL8 (humans). CXCL1/CXCL8 have been identified as the classical arrest chemokines helping neutrophils to transit from slow rolling into firm adhesion on the inflamed endothelium [5, 28]. In addition, CXCL1 and CXCL8 are the main mediators inducing directional movement of neutrophil toward the site of inflammation [29]. Accordingly, blocking CXCR1 and CXCR2 is an interesting approach to prevent unwanted recruitment of neutrophils [1]. Infiltration of neutrophils into inflamed tissue is a hallmark features of many chronic inflammatory diseases including inflammatory bowel disease (IBD), psoriasis, chronic obstructive pulmonary disease (COPD), and type 1 diabetes. In these disease entities, excessive or dysregulated neutrophil activity contributes to tissue damage, disease progression, and therapeutic resistance [30-32]. ladarixin has shown promising efficacy in preclinical models of type 1 diabetes [16] and is currently in advanced clinical trials for the treatment of inflammatory conditions where neutrophil involvement is central [16, 17, 33]. Our findings provide mechanistic insight into how ladarixin impairs neutrophil extravasation while preserving induction of adhesion, potentially offering a selective anti-inflammatory strategy that reduces tissue injury without compromising immune surveillance. This mode of action could

make ladarixin particularly suitable for diseases requiring long-term modulation of neutrophil activity without full immune suppression.

Recently, we and others have shown that CXCL1 and CXCL8 can trigger transendothelial migration by mediating vascular basement membrane penetration [24-26]. Moreover, we have previously reported that in *Mst-1* deficient neutrophils, selective impairment of neutrophil perivascular basement membrane penetration, but not adhesion efficiency, was sufficient to block neutrophil extravasation into the inflamed tissue [26].

ladarixin, a dual non-competitive allosteric inhibitor of CXCR1 and CXCR2 [15], has been proposed to selectively modulate CXCR1/2 pathways rather than indiscriminately affecting all of them [19]. Here, we show that ladarixin does not interfere with chemokine-induced neutrophil adhesion. Interestingly, although ladarixin did not impair neutrophil adhesion *in vitro* and *in vivo*, the compound exerted a strong and selective inhibitory effect on neutrophil elastase mobilization and secretion on human and murine neutrophils *in vitro* and *in vivo*, an indispensable event for vascular basement membrane penetration and extracellular matrix degradation [24-26]. Of note, PECAM/ICAM-1/CXCL8-induced NE surface mobilization was more pronounced in human than in murine neutrophils, which might be of clinical relevance possibly leading to stronger anti-inflammatory effects of ladarixin in humans. Moreover, ladarixin prevented neutrophils to degrade laminin, a prerequisite for successful penetration of the vascular basement membrane and subsequent extravasation into the inflamed tissue, further confirming that the selective blocking of neutrophil vascular basement membrane penetration is sufficient to impair neutrophils diapedesis. Finally, differently to what previously observed with other chemotactic assays (14, 15), ladarixin did not inhibited CXCL1-induced chemotaxis of mouse neutrophils, as assessed using CellDirector®2D chemotaxis chambers, an under-flow assay optimized to capture early chemotactic responses within the first 10 minutes and under a stable gradient, a discrepancy that may require further investigation. On the other hand, in CellDirector®2D chemotaxis assay neutrophil migration occurs independent of diapedesis component linked to basement membrane penetration and matrix degradation. This implies that ladarixin interferes with distinct CXCR1/2 mediated functions during the recruitment process. Its proposed mode of action aligns with ladarixin's ability to selectively modulate pathways activated by CXCR1 and CXCR2. In fact, ladarixin belongs to a new class of well characterized small molecular weight allosteric inhibitors that, by binding to an allosteric site conserved in the transmembrane region of CXCR1 and CXCR2, affects the efficacy of orthosteric endogenous CXCR1 and CXCR2 agonists without altering CXCL1/8 binding to these receptors [13, 15]. Apart from being a non-competitive inhibitor, ladarixin was demonstrated to act as an allosteric receptor efficacy inhibitor, selectively blocking specific signaling pathways activated by CXCR1 and CXCR2 agonists [19]. Accordingly, ladarixin can be

considered to be 'permissive', as it stabilizes a specific receptor conformation that prevents the activation of certain signaling pathways while leaving others unaffected [19].

One of the hallmarks of chemokine receptor engagement is its rapid internalization following chemokine binding, leading to reduced availability of the chemokine receptor on the cell surface [34]. Inhibition of clathrin-mediated endocytosis of CXCR2 upon CXCL8 binding has been shown to result in defective chemotactic responses [35]. In our study, we tested whether ladarixin treatment might interfere with ligand-induced CXCR2 internalization in primary neutrophils, thereby affecting their chemotactic properties. Consistent with observations made using the related compound Reparixin [19], we found no differences in CXCR2 internalization upon chemokine binding between carrier and ladarixin pretreated neutrophils, excluding a potential impairment of CXCR2 internalization to be causative for reduced extravasation of ladarixin-treated neutrophils. However, it is important to take into account that receptor internalization is a way of receptor desensitization. This is an important event in chemokine clearance and homeostasis in the extracellular microenvironment by mediating chemokine scavenging and resolution of inflammation [36].

Recent work by Kuwano and colleagues has highlighted differential signaling of CXCR2 via Gαi2 and Gαi3 proteins. Notably, genetic loss of Gαi3, but not Gαi2, impaired neutrophil transmigration without affecting firm adhesion [37]. Interestingly, Gαi3, but not Gαi2, deficient neutrophils exhibited reduced Akt phosphorylation upon CXCR2 stimulation. Of note, ladarixin is a potent inhibitor of CXCR1/2-mediated Akt phosphorylation [38], reinforcing that ladarixin is responsible of its selective inhibition of CXCR1/2 mediated neutrophil elastase translocation to the neutrophil surface critical for transendothelial migration. Accordingly, we can speculate that ladarixin might interfere with CXCR2-dependent Gαi3 signaling events and NE translocation leaving CXCR2-dependent Gαi2 signaling and firm adhesion unaffected. However, this has to be further tested and confirmed in future experiments.

Recent insights into neutrophil extravasation have highlighted a role of reverse transendothelial migration (rTEM) and its dual role in both the resolution and systemic propagation of inflammation [39]. While rTEM is dependent on NE activity it is likely that ladarixin also affects rTEM. However, as ladarixin primarily prevents neutrophil extravasation and thereby significantly reduces the number of extravasated cells, we assume that rTEM is of minor relevance in our in vivo setting. Future studies will be necessary to further characterize CXCR1/2 signaling pathways and ladarixin in affecting rTEM using appropriate animal models.

In summary, we show that the dual non-competitive CXCR1/2 inhibitor ladarixin specifically blocks neutrophil extravasation into inflamed tissue by preventing neutrophil elastase translocation to the cell surface and penetration of the vascular basement membrane. At the same time, classical CXCR2-dependent neutrophil functions including adhesion on the inflamed endothelium is not affected.

These findings confirm the unique mode of action of this novel chemical class of allosteric CXCR1 and CXCR2 efficacy inhibitors and highlights their clinical therapeutic potential to prevent neutrophil extravasation in chronic inflammatory diseases such as inflammatory bowel disease, psoriasis and others.

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Figure 1 ***ladarixin application does not affect neutrophil rolling and neutrophil adhesion in vivo and in vitro*** **(A)** Leukocyte rolling and adhesion were investigated by intravital microscopy in CXCL1-stimulated mouse cremaster muscle venules *in vivo*. **(B)** Leukocyte rolling , **(C)** rolling velocity and **(D)** adhesion were determined in CXCL1-stimulated mice pretreated with 0.9% NaCl (carrier) or with ladarixin (Ldx) (n=7 per group, ns: not significant; unpaired t-test; data is presented as mean±SEM). **(E)** Leukocyte adhesion was investigated in exteriorized cremaster muscle venules before and after i.a. injection of CXCL8 into WT mice pretreated with 0.9% NaCl (carrier) , ladarixin (Ldx) or Pertussis toxin (Ptx) (n≥3 per group, **: p≤0.01, ***: p≤0.005, ns: not significant; two-way ANOVA, Sidak's multiple comparison; data is presented as mean±SEM). **(F)** In addition, rolling and adhesion was studied with human neutrophils under flow *in vitro*. **(G)** Rolling, **(H)** rolling velocity and **(I)** adhesion of isolated human neutrophils pretreated with 0.9% NaCl (carrier) or ladarixin (Ldx) were determined in microflow chambers coated with E-selectin, ICAM-1, and CXCL8 (≥4 flow chambers from n=4 individuals per group, ns: not significant; unpaired t-test; data is presented as mean±SEM).

Figure 2 ***Neutrophil extravasation into inflamed tissue is impaired in ladarixin pretreated mice***

(A) Leukocyte extravasation was assessed in Giemsa stained inflamed mouse cremaster muscle tissue. **(B)** Neutrophil chemotaxis was investigated using the CellDirector®2D chemotaxis chamber. **(C)** Extravasation of leukocytes and its various subsets including neutrophils, eosinophils and mononuclear cells were quantified in CXCL1-stimulated cremaster muscles of WT mice pretreated with 0.9% NaCl (carrier) or ladarixin (Ldx, n=6 mice per group, **: p≤0.01, ***: p≤0.005, ns: not significant, unpaired t-test, left panel; two-way ANOVA, Sidak's multiple comparison, middle panel; data is presented as mean±SEM or representative pictures, scale bar: 50µms. **(D)** Euclidean distance, **(E)** crawling directionality and **(F)** and crawling velocity were measured to asses *in vitro* chemotactic behaviour of neutrophils pretreated with PBS (carrier) or ladarixin (Ldx) (n= 4 mice per group, unpaired t-test, ns: not significant, data is presented as mean±SEM). **(G)** CXCL1-induced CXCR2 internalization of mouse neutrophils pretreated with PBS (carrier) or ladarixin (Ldx) was analyzed by flow cytometry (n=5 mice per group, unpaired t-test, ns: not significant; data is presented as CXCR2 mean fluorescence intensity (MFI) ratio between CXCL1-stimulated and unstimulated neutrophils).

Figure 3 l***adarixin specifically impairs CXCL1 induced neutrophil elastase mobilization***

(A) Neutrophil elastase (NE) mobilization to the cellular surface was studied in murine neutrophils pretreated with PBS (carrier) or ladarixin (Ldx) on plates coated with BSA (control) or PECAM-1, ICAM-1 and various proinflammatory factors including **(B)** CXCL1, **(D)** CCL5 and **(E)** fMLF (soluble)

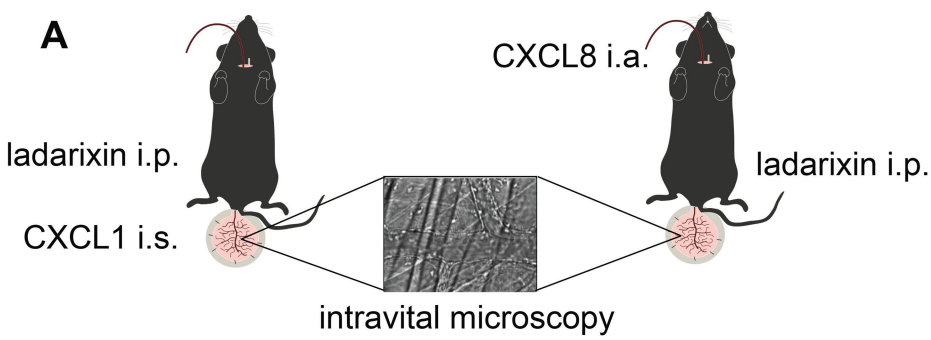
(n≥5 per group, except Ldx 100nm: n=3, *: p≤0.05, ns: not significant; two-way ANOVA, Tukey's multiple comparison, data is presented as NE intensity ratio). **(C)** Representative images illustrating NE mobilization (magenta) to the neutrophil surface in plates coated with PECAM-1, ICAM-1 and CXCL1 or BSA (control). Cells were pretreated with PBS (carrier) or Ladarixin (Ldx) as indicated. Scale bar: 5µm.

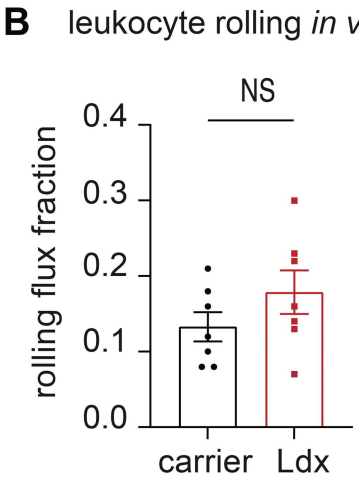
Figure 4 l*adarixin reduces NE activity during neutrophil transendothelial migration in vivo* and *in vitro*

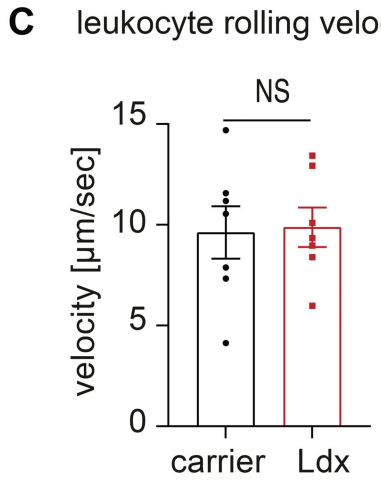
(A-D) Perivascular and intravascular neutrophil elastase (NE) activity was assessed by confocal microscopy in 0.9% NaCl or CXCL1-inflamed cremaster muscles using NE680FAST applied i.s. to monitor perivascular **(A-B)** NE activity or i.v. to assess intravascular **(C-D)** NE activity. NE680FAST application was followed by i.p. injection of ladarixin (Ldx) or 0.9% NaCl (carrier), (n≥3 per group, *: p≤0.05, **: p≤0.01, ns: not significant; two-way ANOVA, Sidak's multiple comparison). Representative images show NE activity (white) in **(B)** CXCL1-stimulated perivascular tissue and in **(D)** CXCL1-stimulated postcapillary venules of the cremaster muscle (vessel indicated in magenta). Scale bar: 20µm. **(E)** Neutrophil elastase activity was assessed by percentage of laminin degrading cells over time *in vitro* in neutrophil pretreated with PBS (carrier) or ladarixin (Ldx, n=3 mice per group, *: p≤0.05, **: p≤0.01, ns: not significant; two-way ANOVA, Sidak's multiple comparison). **(F)** Representative spinning disk confocal micrographs of laminin degradation activity (indicated by black areas, arrows) of neutrophils (green) pretreated with PBS (carrier) or ladarixin (Ldx). Scale bar: 10µm.

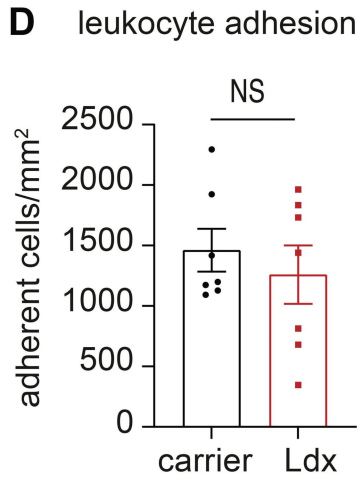
Figure 5 *ladarixin impairs neutrophil elastase mobilization in human primary neutrophils*

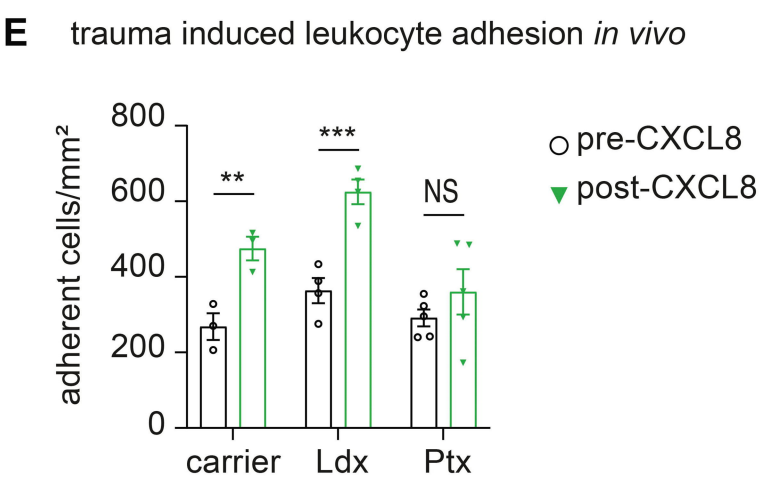
(A) Neutrophil elastase (NE) mobilization to the cellular surface was studied in primary human neutrophils using confocal microscopy or classical transwell system. **(B)** NE translocation to the cellular surface was investigated in neutrophils pretreated with PBC (carrier) or ladarixin (Ldx) on plates coated with 2%BSA (control) or PECAM-1, ICAM-1 and CXCL1 (n=6 per group, *: p≤0.05, **: p≤0.01, ***: p≤0.005, ns: not significant; two-way ANOVA, Sidak's multiple comparison, data is presented as NE intensity ratio). **(C)** Representative images illustrating NE mobilization to the human neutrophil surface in plates coated with PECAM-1, ICAM-1 and CXCL1 or 2%BSA (control). Cells were pretreated with PBS (carrier) or ladarixin (Ldx) as indicated. Scale bar: 5µm. **(D)** NE release was examined in a transwell system, where cells were stimulated with PECAM-1 and ICAM-1 and simultaneously allowed to migrate to a CXCL8 gradient. (n=5 per group, *: p≤0.05, **: p≤0.01, ***: p≤0.005, ns: not significant; two-way ANOVA, Sidak's multiple comparison).

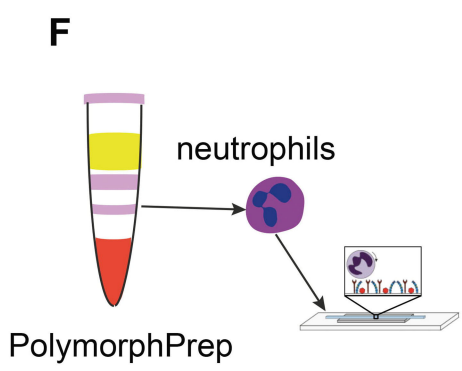

A
CXCL8 i.a.
ladarixin i.p.
ladarixin i.p.
CXCL1 i.s.
intravital microscopy

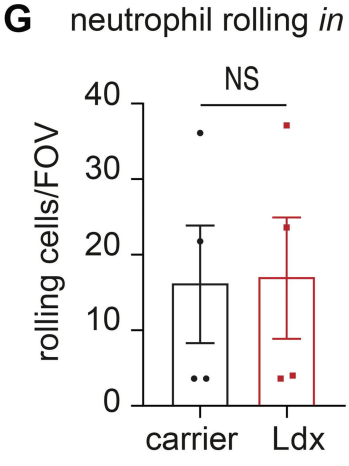

B leukocyte rolling *in vivo*
NS
rolling flux fraction
0.4
0.3
0.2
0.1
0.0
carrier
Ldx

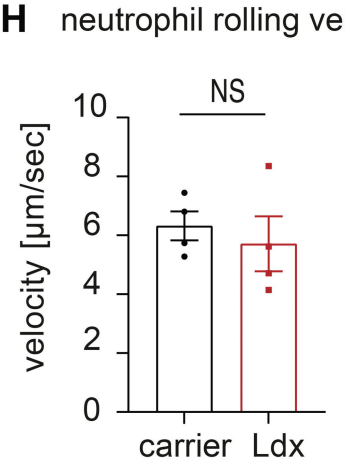

C leukocyte rolling velocity *in vivo*
NS
velocity [µm/sec]
15
10
5
0
carrier
Ldx

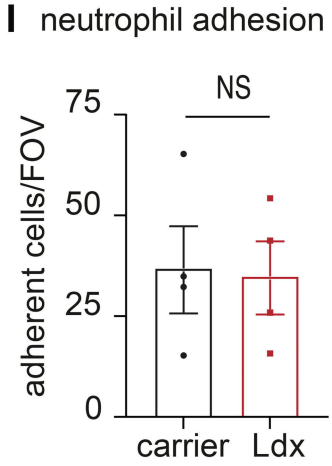

D leukocyte adhesion *in vivo*
NS
adherent cells/mm²
2500
2000
1500
1000
500
0
carrier
Ldx

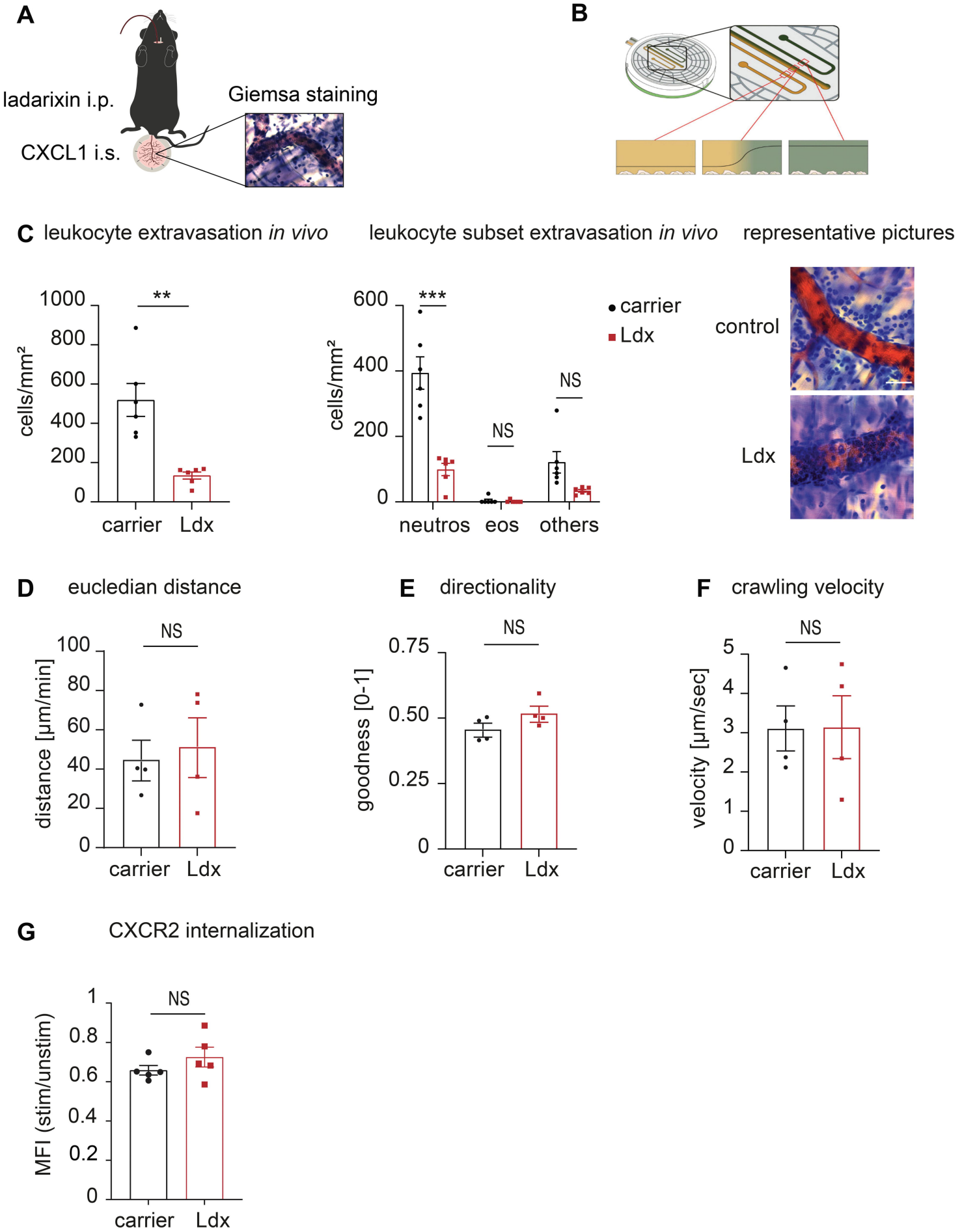

E trauma induced leukocyte adhesion *in vivo*
adherent cells/mm²
800
600
400
200
0
**
***
NS
carrier
Ldx
Ptx
pre-CXCL8
post-CXCL8

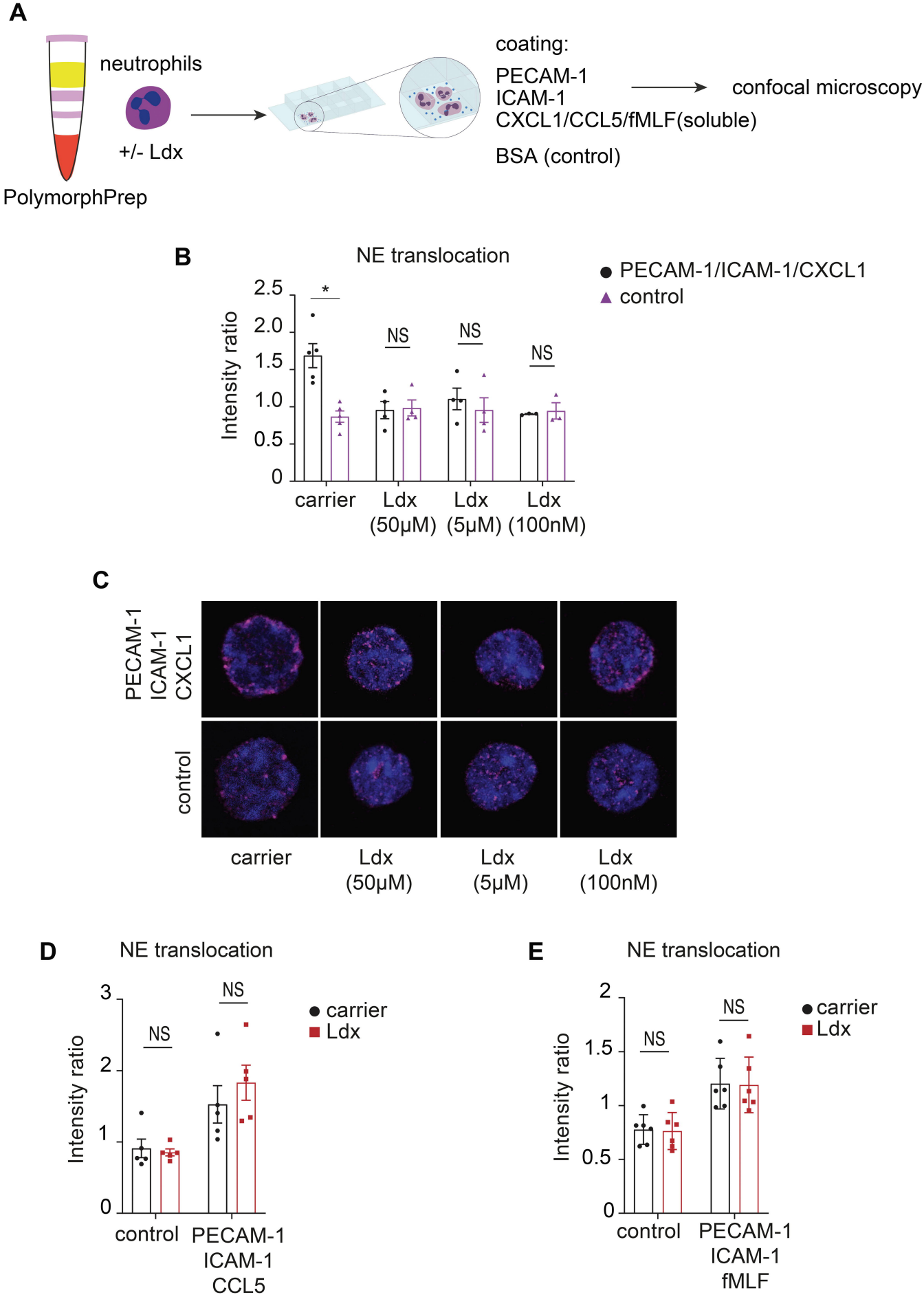

F
neutrophils
PolymorphPrep

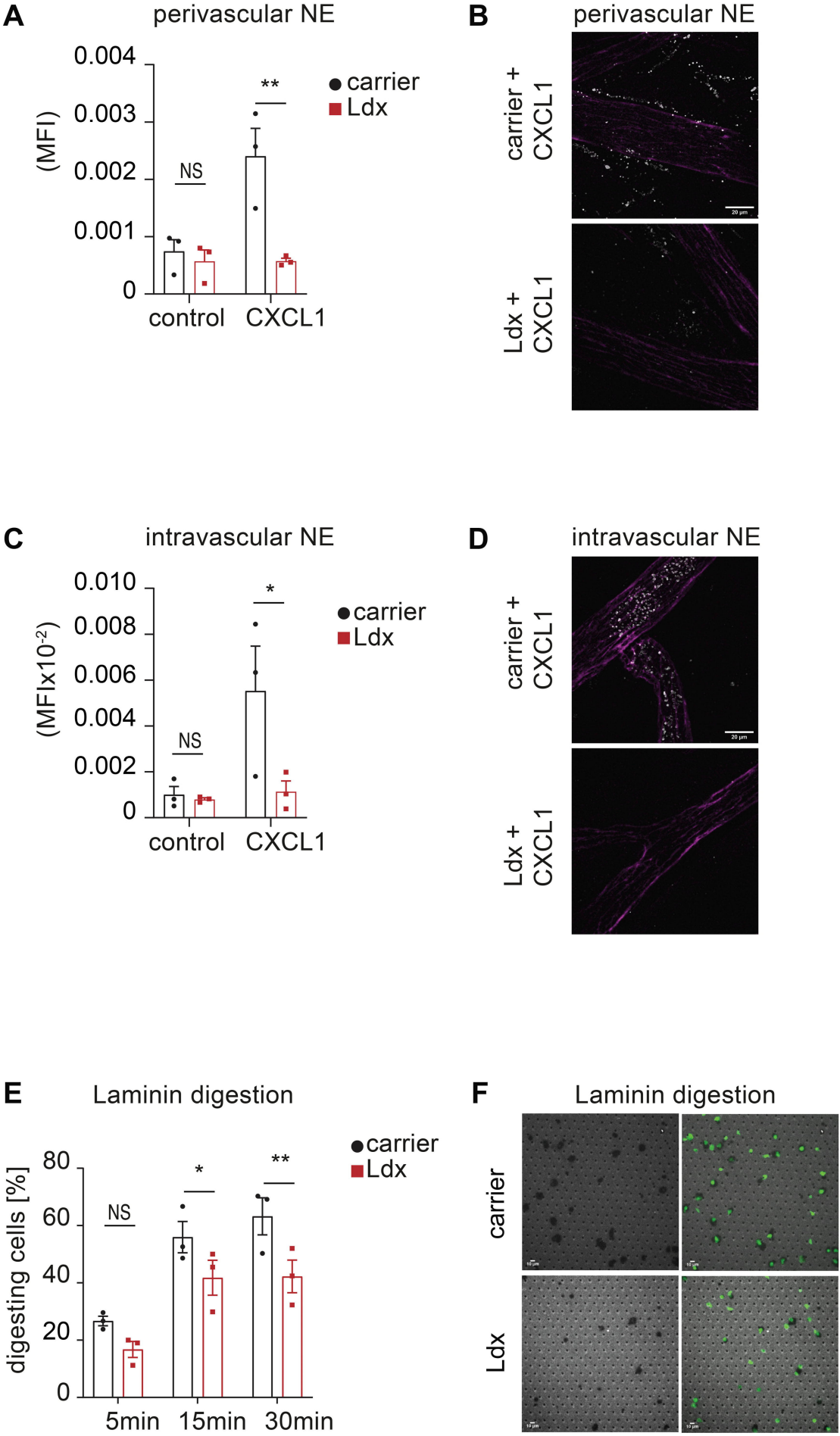

G neutrophil rolling *in vitro*
NS
rolling cells/FOV
40
30
20
10
0
carrier
Ldx

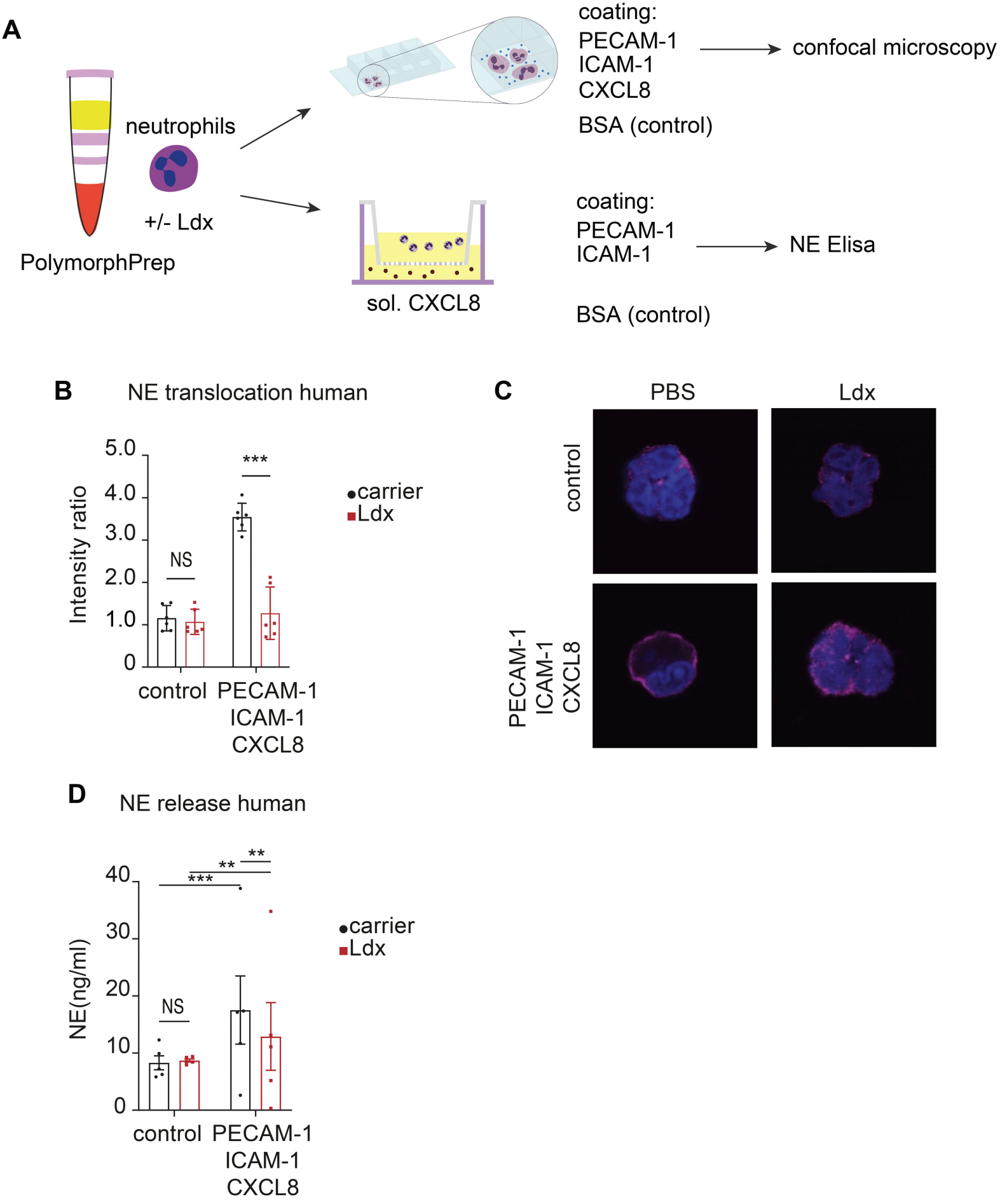

H neutrophil rolling velocity *in vitro*
NS
velocity [µm/sec]
10
8
6
4
2
0
carrier
Ldx


I neutrophil adhesion *in vitro*
NS
adherent cells/FOV
75
50
25
0
carrier
Ldx


A
ladarixin i.p.
CXCL1 i.s.
Giemsa staining
B
C leukocyte extravasation *in vivo*
cells/mm²
1000
800
600
400
200
0
**
carrier
Ldx
leukocyte subset extravasation *in vivo*
600
400
200
0
***
NS
NS
neutros
eos
others
carrier
Ldx
representative pictures
control
Ldx
D eucledian distance
distance [µm/min]
100
80
60
40
20
0
NS
carrier
Ldx
E directionality
goodness [0-1]
0.75
0.50
0.25
0
NS
carrier
Ldx
F crawling velocity
velocity [µm/sec]
5
4
3
2
1
0
NS
carrier
Ldx
G CXCR2 internalization
MFI (stim/unstim)
1
0.8
0.6
0.4
0.2
0
NS
carrier
Ldx


A
neutrophils
+/- Ldx
PolymorphPrep
coating:
PECAM-1
ICAM-1
CXCL1/CCL5/fMLF(soluble)
BSA (control)
confocal microscopy
B
NE translocation
PECAM-1/ICAM-1/CXCL1
control
Intensity ratio
2.5
2.0
1.5
1.0
0.5
0
*
NS
NS
NS
carrier
Ldx (50μM)
Ldx (5μM)
Ldx (100nM)
C
PECAM-1
ICAM-1
CXCL1
control
carrier
Ldx (50μM)
Ldx (5μM)
Ldx (100nM)
D
NE translocation
carrier
Ldx
Intensity ratio
3
2
1
0
NS
NS
control
PECAM-1
ICAM-1
CCL5
E
NE translocation
carrier
Ldx
Intensity ratio
2
1.5
1
0.5
0
NS
NS
control
PECAM-1
ICAM-1
fMLF


A
perivascular NE
0.004
0.003
0.002
0.001
0
(MFI)
NS
**
carrier
Ldx
control
CXCL1
B
perivascular NE
carrier +
CXCL1
Ldx +
CXCL1
C
intravascular NE
0.010
0.008
0.006
0.004
0.002
0
(MFIx10-2)
NS
*
carrier
Ldx
control
CXCL1
D
intravascular NE
carrier +
CXCL1
Ldx +
CXCL1
E
Laminin digestion
80
60
40
20
0
digesting cells [%]
NS
*
**
carrier
Ldx
5min
15min
30min
F
Laminin digestion
carrier
Ldx


A
neutrophils
+/- Ldx
PolymorphPrep
coating:
PECAM-1
ICAM-1
CXCL8
BSA (control)
confocal microscopy
sol. CXCL8
coating:
PECAM-1
ICAM-1
BSA (control)
NE Elisa
B
NE translocation human
Intensity ratio
5.0
4.0
3.0
2.0
1.0
0
***
NS
carrier
Ldx
control
PECAM-1
ICAM-1
CXCL8
C
PBS
Ldx
control
PECAM-1
ICAM-1
CXCL8
D
NE release human
NE(ng/ml)
40
30
20
10
0
**
**
***
NS
carrier
Ldx
control
PECAM-1
ICAM-1
CXCL8

SUPPLEMENTARY FILE

The dual non-competitive CXCR1/2 inhibitor ladarixin impairs neutrophil extravasation without altering intravascular neutrophil adhesion

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[#]MP and MS contributed equally as co-last authors

Supplemental Data

Detailed methods

Neutrophil isolation

Human neutrophils were isolated from peripheral blood of healthy human donors. Blood sampling was approved by the ethical committee of LMU München (Az. 611-15). Polymorphprep (Axis Shield) was added and samples prepared according to the manufacturer's instruction. Alternatively, human neutrophils were isolated by EasySep™ Direct Human Neutrophil Isolation kit (STEMCELL TECHNOLOGIES) according to manufacturer's protocols. After isolation, neutrophils were transferred into pH adjusted (pH 7.4) Hanks's balanced salt solution (HBSS containing 10mM HEPES and 0.25%BSA) at a concentration of $1x10^6$ cells/ml until the experiment was started.

Murine Neutrophils were isolated using a Percoll (Sigma-Aldrich) solutions at 1.08 and 1.11g/ml density or by EasySep™ Direct Mouse Neutrophil Isolation kit (STEMCELL TECHNOLOGIES) according to manufacturer's protocols, respectively from bone marrow cells of WT mice. For some assays, murine

neutrophils were matured overnight using WEHI-3 supernatant [1]. Neutrophils were counted and resuspended in HBSS at $1x10^6$ cells/ml.

Leukocyte extravasation in whole mounts of the CXCL1-stimulated cremaster muscle

To investigate the effect of ladarixin on CXCL1-induced leukocyte extravasation, we injected ladarixin or 0.9% NaCl (carrier) into the peritoneal cavity of mice one hour before intrascrotal injection of rmCXCL1 (600ng/mouse). 2h later, mice were anesthetized, cremaster muscles surgically removed, fixed with 4% paraformaldehyde (4% w/v, Applichem) and stained with Giemsa-solution (Merck). Giemsa-stained whole mounts were then used to assess the number of perivascular neutrophils, eosinophils and mononuclear cells. The microscopic analysis of perivascular leukocytes was carried out at the core facility BioImaging of the Biomedical Center using a Leica DM2500 microscope, equipped with a 100x objective (Leica, 1.4NA, oil immersion) and a Leica DMC2900 CMOS camera.

Flow chamber experiments and chemotaxis

Flow chamber experiments were conducted to assess adhesion of human neutrophils as described previously [2]. Briefly, µSlide $VI^{0.1}$ (ibidi GmbH) microflow chambers were coated with rhE-selectin (5 µg/ml, R&D Systems), rhICAM-1 (4 µg/ml, R&D Systems), and rhCXCL8 (10 µg/ml). Isolated human neutrophils were incubated with ladarixin (5µM) or PBS (carrier) for 30min and then perfused through flow chambers at a shear stress level of 1dyne/cm using a high-precision syringe pump (Harvard Apparatus). Number of adherent leukocytes/FOV were assessed over a time period of 20min. Chemotaxis experiments were conducted with bone marrow murine neutrophils, using CXCL1 and CellDirector®2D chemotaxis chambers (Gradientech). Briefly, neutrophils were matured in RPMI 1640 (Sigma-Aldrich) supplemented with 20% WEHI-3B-conditioned medium overnight at 37°C. Chemotaxis chambers were coated with fibrinogen (100 µg/ml, Innovative Research) for 3h at RT and blocked with 5% casein overnight at 4°C. Subsequently, a CXCL1 gradient (0-10nM) was established within the chamber and mouse neutrophils ($1x10^6$ cells/ml) pretreated with PBS (carrier) or ladarixin for 30min at 37°C were transferred into the chamber. All experiments were conducted and recorded at an inverted microscope (Leica DMi8, x10, 0.3 NA, dry objective) at 37 °C. Euclidean distance, crawling directionality and crawling velocity were assessed over 10min to quantify chemotaxis of mouse neutrophils, as described [2].

CXCR2 internalization

Isolated murine neutrophils were incubated with 5µM ladarixin or PBS (carrier) at 37°C for 1h. Then either stimulation with CXCL1 at a concentration of 100ng/ml for 10min at 37°C or NaCl 0,9% treatment followed. Reactions were stopped with 1,5ml BD FACS Lysing Solution, before centrifuging the samples. Cell pellets were stained for Ly6G, CXCR2 (Pacific Blue rat anti-mouse Ly-6G, clone 1A8, 0,8ng/µl and APC rat anti-mouse CXCR2, clone SA044G4, 2ng/µl, both BioLegend) and the

corresponding Isotype controls measured by flow cytometry (CytoFLEX S) and analyzed using Kaluza Flow Analysis (all Beckman Coulter) or FlowJo software. In each experimental group, a ratio of stimulated (CXCL1) against unstimulated (NaCl 0,9%) was calculated.

Neutrophil elastase mobilization assay

Isolated murine or human neutrophils were pretreated with ladarixin or PBS (carrier) 1h. For murine cells, neutrophil elastase (NE) mobilization from intracellular storage pools to the surface was induced by incubating neutrophils in 2%BSA (control) or rmPECAM-1 (2µg/ml), rmICAM-1 (8µg/ml; both R&D Systems) and rmCXCL1 (10µg/ml, PeproTech) coated wells for 30min at 37°C, as described [3]. For human neutrophils we incubated the cells in 2%BSA (control) or rhPECAM-1 (2µg/ml; R&D Systems), rhICAM-1 (8µg/ml; R&D Systems) and rhCXCL8 (10µg/ml) coated wells for 30 min at 37°C. Thereafter, neutrophils were fixed with 2% PFA, blocked, permeabilized and labeled with rabbit anti-mouse NE antibody (polyclonal; Abcam, 5µg/ml) and goat anti-rabbit Alexa Flour 546 (Invitrogen, polyclonal, 5µg/ml). NE surface mobilization (ring formation) was assessed by confocal microscopy (Leica SP8X System, ×40 or x63, 1.3 NA oil immersion objective) at the core facility BioImaging, Biomedical Center, LMU Munich using Fiji software [4]. Briefly, histograms of NE intensity values were obtained for individual cells by drawing a line throughout the middle of the cell. The intensities from 10% of both histogram sides (cell surface) were divided by the intensities measured in the middle 20% of the histogram. Finally, a ratio indicative for NE surface mobilization was calculated. Values >1, indicate more NE signal on the surface while values <1 indicate more signal inside the cell.

Neutrophil elastase activity in vivo

To test perivascular and intravascular neutrophil elastase (NE) activity *in vivo*, we applied an intravital microscopy model in the CXCL1-stimulated mouse cremaster muscle as described earlier [3]. Briefly, we injected NE680FAST (Perkin-Elmer) i.s. (4nmol/mouse) to monitor perivascular NE activity or i.v. to assess intravascular NE activity. NE680FAST injection was applied one hour after i.p. injection of ladarixin or 0.9% NaCl (carrier). One hour later, 0.9%NaCl (control) or CXCL1 (600ng/mouse) was injected i.s. Two hours after CXCL1/NaCl injection, the cremaster muscle was dissected and fixed with 4% paraformaldehyde, permeabilized and blocked with 0.5% Triton X-100/2% BSA in PBS, and finally stained with rat anti-CD31 antibody Alexa Fluor 488 (MEC13.3; BioLegend, 5µg/ml). Cremaster muscles were then embedded on glass slides in Vectashield®plus (Vector Laboratories) and imaged using confocal microscopy (Leica SP8X system, ×40, 1.3 NA oil immersion objective).

Laminin digestion assay in neutrophils in vitro

Laminin (LN) digestion by neutrophil proteolytic activity was assessed

by live cell imaging and using µ-Slide membrane ibiPore flow chambers (Ibidi) containing a 300 nm thick membrane with 5 µm pores and a subjacent rat-tail collagen gel (1.5mg/mL) loaded with rmCXCL1

(1 ng/ml) as chemoattractant. Coating of the upper compartment was performed with laminin (15µg/ml), rmPECAM-1 (2 µg/ml) and rmICAM-1 (8 µg/ml) as described [3]. For microscopic visualization, laminin was stained using an anti-LN antibody conjugated to Alexa Fluor-647 (Novusbio). Isolated murine neutrophils were pretreated with ladarixin or PBS (carrier) for one hour and stained with CellTracker™ Green CMFDA Dye (Thermo Fisher). Thereafter, neutrophils were added to the upper chamber and laminin digestion was observed using time-lapse spinning-disk confocal microscopy (Zeiss Examiner with a x20/1.0 NA water immersion objective with a scanner unit CSU-X1, Yokogawa Electric Corporation and a EMCCD camera Evolve, Photometrics). 3D images were acquired and percentage of neutrophils with digesting capability analyzed using Fiji software [4].

Neutrophil elastase release from human neutrophils

To quantify neutrophil elastase release from human neutrophils, transwell assays were performed as described above. Shortly, filters (5µm pore size, Corning) were coated with rhPECAM-1 (2µg/ml; R&D Systems) and rhICAM-1 (8µg/ml; R&D Systems) for 2h at RT while human neutrophils were isolated and pretreated with 5µM ladarixin or NaCl control for 30min at 37°C. Then, the lower plate compartment was filled with HBSS buffer alone or HBSS buffer containing 1 ng ml-1 CXCL8 and cells were seeded and incubated for 30min at 37°C. Afterwards, the supernatant from the cell suspension in the lower cell compartment was collected, diluted 1:25 and neutrophil elastase concentration was determined via ELISA (Abcam, ab270204).

Supplementary reference List

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