Aus dem Institut für Kardiovaskuläre Physiologie und Pathophysiologie

im Walter-Brendel-Zentrum für Experimentelle Medizin

Institut der Ludwig-Maximilians-Universität München

The Role of Preimplantation Factor in Leukocyte Trafficking

Dissertation

zum Erwerb des Doktorgrades der Medizin

an der Medizinischen Fakultät der

Ludwigs-Maximilians-Universität München

vorgelegt von

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2024

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Tag der mündlichen Prüfung:	*18.10.2024*

“The glory of medicine is that it is constantly moving forward, that there is always more to learn. The ills of today do not cloud the horizon of tomorrow, but act as a spur to greater effort.”

William James Mayo (1928)

Contents

Abbreviations

Ca^{2+}	Calcium
CCL	C-C motif chemokine ligand
CRAC channel	Ca^{2+} release-activated Ca^{2+} channel
CXCL	C-X-C motif chemokine ligand
DAG	Diacylglycerol
DAPI	4,6-diamidino-2-phenylindole
EDTA	Ethylenediaminetetraacetic acid
ESL-1	E-selectin ligand-1
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
GFP	Green fluorescent protein
GPCR	G-protein coupled receptor
HBSS	Hanks‘ balanced salt solution
HLA	Human leukocyte antigen
ICAM-1	Intercellular adhesion molecule-1
IL	Interleukin
IP_3	Inositol-1,4,5 triphosphate
IP_3R	IP_3 receptor
LFA-1	Leukocyte function-associated antigen-1
Mac-1	Macrophage-1 antigen
M-CSF	Macrophage - colony stimulating factor
MHC	Major histocompatibility complex
MS	Multiple sclerosis
NETs	Neutrophil extracellular traps
NK cell	Natural killer cell
PAP-1	5-(4-Phenoxybutoxy)psoralen
PIF	Preimplantation factor
PIP_2	Phosphatidylinositol 4,5 bisphosphate
PLC	Phospholipase C
PMN	Polymorphonuclear neutrophils
PSGL-1	P-selectin glycoprotein ligand-1
RCF	Relative centrifugal force
rh	Recombinant human
rm	Recombinant mouse
ROS	Reactive oxygen species
SOCE	Store-operated calcium entry
STIM1	Stromal interaction molecule 1
TGF	Transforming growth factor
TNF α	Tumor necrosis factor α
T_{reg} cell	Regulatory T cell
VEGF	Vascular endothelial growth factor
VLA	Very late antigen
WBC	White blood cell
WT	Wildtype

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1 Introduction

1.1 Immunology of pregnancy

Is the maternal immune system a friend or foe of pregnancy? The immunological environment during pregnancy is distinctive, yet it still retains an element of immunological wonder and mystery to some extent.

During pregnancy, the female body undergoes an elaborate shift of anatomical, physiological and immunological functions, allowing close interaction between genetically distinct maternal and fetal tissues. The challenge at the feto-maternal interface is to create an environment in which genetically foreign microchimeric cells of the fetus are tolerated while the ability to defend against external cues is retained.

The intrauterine environment predetermines health and well-being of the offspring. Following fertilization, the zygote evolves further to the blastocyst. This stage marks the point at which the outer cell layer of the blastocyst – called the trophectoderm – engages in the initial physical contact with maternal tissue. It requires a precisely regulated synchronization of hormones and locally produced signaling molecules to facilitate the functional and anatomical modifications in the uterus, allowing the blastocyst to anchor to the endometrium [1]. The cellular transformation of the surrounding uterine stroma triggered by blastocyst implantation is called decidualization [2]. Together, the maternal decidua and the embryo derived trophoblast shape the placenta, fulfilling tasks ranging from nourishment of the embryo to inducing immunological acceptance and hormone production [3]. When the placenta is fully formed, fetal villous trees are bathed in maternal blood allowing maximal exchange of gas, nutrients and waste between mother and child. By term, embryonic trophoblast-covered villi have an epithelial surface of about 13m^2 [4].

Surprisingly, about 30–40% of all decidua cells during early pregnancy are leukocytes, e.g. NK cells, macrophages, T cells and dendritic cells [5]. As pregnancy is associated with a basal inflammatory state, it is not surprising that neutrophils are involved in implantation, ensuring fetal well-being and contributing to parturition. Their pro-inflammatory phenotype in close proximity to the fetus is not without risk. A disturbed

equilibrium between required pathogen defense and exaggerated immune response leads to severe pregnancy-related disorders such as recurrent fetal loss [6], pre-eclampsia [7], or gestational diabetes mellitus [8]. Recent findings suggest that neutrophils are able to recruit and modulate T-cell function at the feto-maternal interface, thus playing a key role in the immunological balancing act [9].

Infiltration of immunocompetent cells into the decidua is a decisive factor for successful pregnancy outcome [2]. Important components orchestrating this interplay are hormones and trophoblast-derived factors [1]. The trophoblast itself contributes to immune cell regulation via different mechanisms:

1. Immune cell recruitment

   Trophoblast-secreted cytokines such as CCL2, CXCL12, CXCL8 and TGFβ promote the recruitment of immune cells to the feto-maternal interface and support trophoblast invasion [10].

2. Immune cell education

   Several cytokines secreted by the trophoblast promote the differentiation of the immune cells attracted to the placenta and decidua. IL-15 and TGFβ cause a conversion from peripheral NK cells to less cytotoxic decidual NK cells [11]. Furthermore, TGFβ induces tolerance-promoting regulatory T cells [12]. Under the influence of trophoblast-secreted IL-10 and M-CSF, macrophages adopt an M2-like phenotype, crucial for tissue remodeling and cytokine production [13].

3. Environmental sensing

   Trophoblast cells express receptors allowing them to respond to signals from both tissue damage (damage-associated molecular patterns, DAMPs) and infection (pathogen-associated molecular patterns, PAMPs).

   Furthermore, the trophoblast diminishes the chemokine gradient directed toward the fetal tissue by internalizing and scavenging proinflammatory chemokines at the interface between the fetus and the mother via the atypical chemokine receptor ACKR2 [14].

The trophoblast secretes a large number of cytokines, chemokines and growth factors that contribute to the process of immune regulation during pregnancy. In addition to the ones named above, the trophoblast also releases a protein called preimplantation factor (PIF).

Preimplantation factor (PIF)

The preimplantation factor (PIF), derived from embryonic sources, is a peptide comprised of 15 amino acids. It is produced by trophoblast cells and steadily discharged into the maternal bloodstream [15]. Physiological PIF serum levels vary throughout pregnancy: PIF secretion begins as early as the two-cell stage and persists throughout pregnancy, peaking in the second trimester with detectable concentrations of 60.7 ± 7.3 nM [16].

The peptide fosters a pro-receptive environment at the feto-maternal interface at the onset of pregnancy by augmenting local progesterone activity and elevating steroid secretion. Adequate levels of PIF in the bloodstream are necessary for embryonic development and successful childbirth [17]. In addition, PIF promotes the expression of pro-tolerogenic HLA molecules in trophoblastic cells and interacts with immune cells [18].

By interacting with immune cells, PIF is able to attenuate the severity of various T cell-driven autoimmune conditions in animal models. Weiss et al. showed that administration of PIF preserved the insulin production in a model for type I diabetes mellitus [19]. Paidas et al. highlighted the pregnancy-induced protection against multiple sclerosis in women, attributing neuro-protective properties to PIF [20]. Recent findings revealed an altered immune response in acute inflammatory scenarios *in vivo* involving the potassium channel $K_v1.3$ as descibed below (chapter 1.3) [21]. These examples of recent publications in this field demonstrate that our comprehension of the molecular mechanisms governing the impact of PIF on the immune system is still in its nascent stages.

1.2 Neutrophil recruitment cascade

Approximately 40-70% of circulating leukocytes in humans are polymorphonuclear neutrophils. Neutrophils are a vital component of the innate immune system with a repertoire of tools for rapid immune responses such as phagocytosis, release of potent bactericidal enzymes by degranulation, production of reactive oxygen species (ROS) and formation of neutrophil extracellular traps (NETS). Beside their primary function in innate immunity, neutrophils also exert modulatory effects on adaptive immunity.

During acute inflammation, tissue-resident macrophages and other cells trigger the release of vast amounts of cytokines. These cytokines, such as tumor necrosis factor alpha (TNF-α) and interleukins, induce activation of both endothelial cells and circulating neutrophils. Neutrophils are recruited to the site of inflammation and locally exert their powerful tools of modulating immune responses, facilitating tissue repair and eradicating microbes.

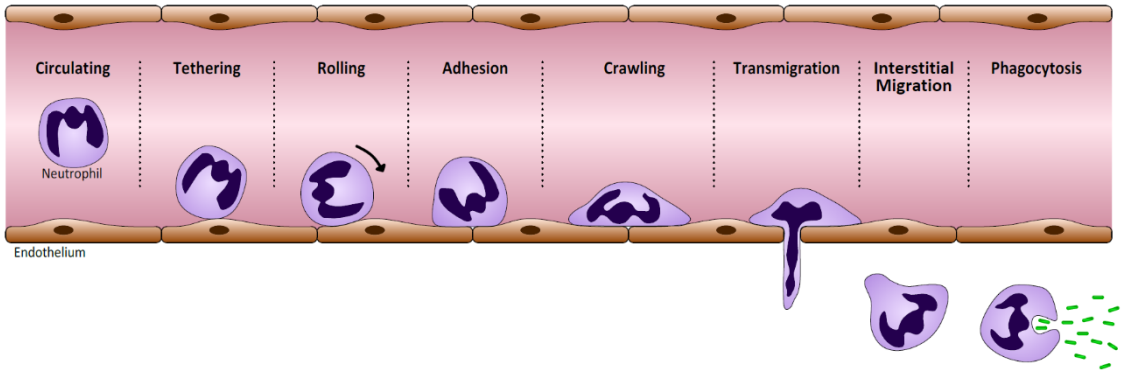


Figure 1: Neutrophil recruitment cascade. Neutrophils follow a well-defined process to leave the intravascular compartment. They are the initial cells to reach sites of inflammation from the systemic circulation, where they execute effector functions such as killing microbes.

Neutrophils are circulating in the vascular system, from where they can rapidly respond to inflammatory signals. In the context of an inflammation, activation of these cells by endogenous or pathogen-specific substances leads to their recruitment from the bloodstream into the affected tissue (Figure 1).

Tethering and rolling

In postcapillary venules, neutrophils are pushed towards the vessel wall by erythrocytes [22]. The initial step of neutrophil recruitment, rolling, is facilitated by low blood flow velocity in postcapillary venules. Further, already adherent neutrophils amplify the tethering process of floating neutrophils [23]. This process is called secondary tethering [24].

Pro-inflammatory stimuli cause an upregulation of P- and E-selectin on the endothelial surface [25] and free-floating neutrophils come into contact with the inflamed endothelium. Selectin ligands on the neutrophil surface such as PSGL-1, CD44 and ESL-1 enable neutrophils to interact then with selectins on the activated endothelium [26]. Shear stress generated by the blood flow causes the selectin – selectin ligand bonds to form at the front of the contact zone between neutrophil and endothelium with short binding duration [27]. The balance of catch and release enables then rolling neutrophils along the vessel walls [28].

Adhesion

During rolling, the interaction of neutrophils with endothelially presented chemokines causes an increasing activation and clustering of integrins on the leukocyte surface (inside-out-signaling). For leukocyte adhesion, the β_2 integrin subfamily plays a major role [29]. β_2 integrins consist of a β_2-chain combined with an α-chain, resulting in heterodimers like LFA-1 (CD11a/CD18, $\alpha_2\beta_2$) or Mac-1 (CD11b/CD18, $\alpha_M\beta_2$) [30]. According to the degree of activation, integrins change their conformation and affinity to their binding partners on the endothelium, intercellular adhesion molecule 1 (ICAM-1) and vascular adhesion molecule 1 (VCAM-1) [31, 32]. Moreover, β_2 integrins can be activated by extracellular S100A8/A9 in an autocrine manner [33]. This process results in the transition from rolling to arrest and subsequent firm adhesion of the neutrophil to the endothelium.

Crawling and Transmigration

To ensure firm adhesion, the cytoskeleton of the neutrophil is subject to some post-arrest modifications [34]. Clustered β_2 integrins bound to their endothelial ligands become adhesion spots, to which the actin cytoskeleton is linked [35]. In order to find a suitable spot for diapedesis, the neutrophil now begins to crawl along the luminal surface of the vessel. The process of crawling is predominantly Mac-1 dependent [36]. Phillipson et al. showed, that neutrophils of Mac-$1^{-/-}$ mice have difficulties finding an appropriate site for transmigration, thus diapedesis is significantly impaired [37].

Once neutrophils have found an appropriate spot for transmigration, they pass through the endothelial layer and vascular basement membrane and migrate into extravascular tissue. Migration through the venular wall is predominantly through the paracellular route and requires an interplay of various molecules, e.g. neutrophil elastase or very late activation leukocyte antigen 3 and 6 (VLA-3, VLA-6) [38].

Phagocytosis

Phagocytosis belongs to the effector functions of neutrophils [39] and targets both pathogens / pathogen components as well as sterile particles [40]. The process of phagocytosis involves the detection of the target particle and the activation of the internalization process. The ingested particle is located in a specialized vacuole called phagosome. Fusion of the phagosome with lysosomes matures the organelle, creates hostile environment and allows potential degradation of the ingested particle. A number of phagocytic receptors are involved in this process, triggering changes of the actin skeleton as well as of the lipid membrane mediated via the induction of various signaling pathways [41]. Phagocytosis as well as other neutrophil effector functions rely on intracellular calcium signaling.

1.3 Intracellular calcium signaling

Calcium plays a key role in intracellular communication processes [42]. Calcium-dependent processes in neutrophils include the transition from rolling to firm adhesion (although some controversy exists in the literature) [43–45] and postarrest modifications during recruitment, chemotaxis [46] and effector functions such as phagocytosis or ROS production [47]. Immune cells regulate their intracellular calcium concentrations mainly via store-operated Ca^{2+} entry (SOCE) (Figure 2) [43].

Store-operated calcium entry (SOCE)

Neutrophils express various cell surface receptors triggering their activation, e.g.:

- G-protein-coupled receptors (GPCR) such as CXCR2 [48]
- Fc γ-receptors [49]
- Adhesion receptors, e.g. PSGL-1, L-selectin [50] or LFA-1 [51]

Upon binding to their ligand, receptors initiate downstream signal transduction pathways, leading to an elevation of intracellular calcium levels. Along the signaling pathway phospholipase C (PLC) converts phosphatidylinositol-4,5 bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol-1,4,5 triphosphate (IP_3), the latter opening receptors in the endoplasmic reticulum (ER) membrane through interacting with the IP_3 receptor (IP_3R) [42, 52]. This allows an efflux of Ca^{2+} from the ER into the cytoplasm, whereupon translocation of the calcium sensor Stromal interaction molecule 1 (STIM1) to Ca^{2+} sensitive Orai1 channels in the plasma membrane is induced [53]. Orai1, belonging to the Ca^{2+} release-activated Ca^{2+} (CRAC) channels, opens due to STIM1-Orai1 interaction. Subsequently, Ca^{2+} enters the cell through the Orai1 channel from the extracellular compartment following both a chemical and electric gradient [50]. Although Orai1 serves as the primary calcium channel in neutrophils, calcium signaling of leukocytes is diverse, with a range of additional ion channels contributing to calcium homeostasis [50, 54].

Voltage-gated potassium channel $K_v1.3$

The voltage-gated channel $K_v1.3$ is among the ion channels regulating calcium flux in leukocytes [55]. Cell membrane depolarization triggers the opening of voltage-activated $K_v1.3$ channel [56], resulting in the efflux of potassium (K^+) out of the cell. As a result of the hyperpolarization generated by the potassium efflux more Ca^{2+} ions enter the cell via the Orai1 channel, causing the intracellular calcium level to rise further [57]. Indeed, keeping the plasma membrane potential stable is an important prerequisite for immune cell activation [50].

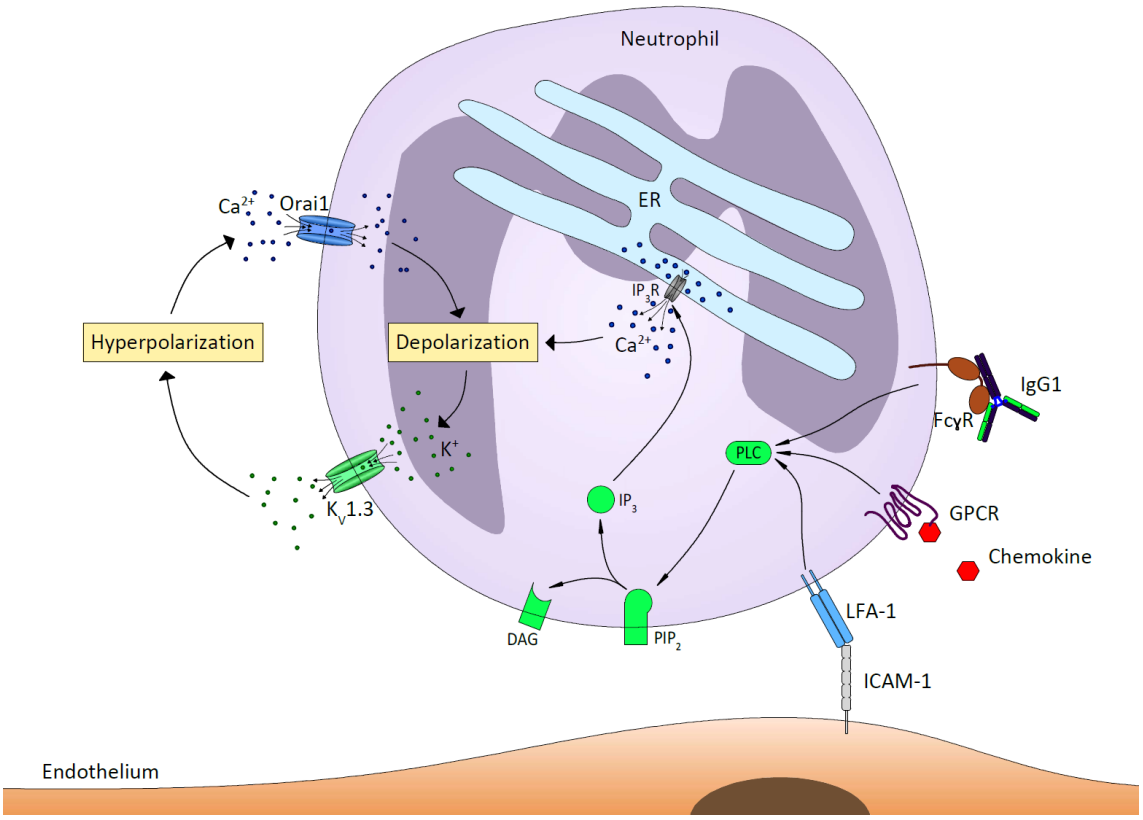


Figure 2: Calcium signaling in neutrophils. Neutrophils get activated via surface receptors like G-protein-coupled receptor (GPCR) CXCR2, Fc γ-receptors or β_2 integrins LFA-1 and MAC-1. The different intracellular signaling pathways result in store-operated calcium entry (SOCE). Cell depolarization triggers the opening of the voltage-gated potassium channel $K_v1.3$ allowing potassium (K^+) to leave the cell. The resulting hyperpolarization stabilizes membrane potential and provides the driving force for Ca^{2+} influx via Orai1 [75].

While the voltage-gated potassium channel $K_v1.3$ has been intensively investigated in T cells [56], the expression and functionality of $K_v1.3$ were only recently discovered in neutrophils [21, 58]. With Ca^{2+} flux being a key regulator in various downstream effector pathways, investigation of $K_v1.3$-dependent neutrophil functions is of interest. Besides the genetic depletion of $K_v1.3$ in mice, applying the $K_v1.3$ inhibitor PAP-1 is a powerful tool to study the role of this ion channel [59]. Moreover, whole-cell patch-clamp experiments revealed inhibitory properties of PIF on $K_v1.3$ in a dose-dependent manner [21].

1.4 Clinical significance of PIF and ion channel $K_v1.3$

Remarkably, the immune tolerance induced by pregnancy is not limited solely to the genetically discordant fetus but extends further than the placenta. This tolerance can encompass self-antigens, leading to a temporary alleviation of autoimmune disorders such as rheumatoid arthritis [60], asthma [61] or multiple sclerosis [62]. Furthermore, de Lima et al. observed a potential decrease in the necessity for immunosuppressive therapy for chronic inflammatory diseases during pregnancy, as evidenced in the case of inflammatory bowel disease [63].

Immune tolerance does not inherently imply diminished immunological responsiveness, but rather the susceptibility of pregnant women to infectious diseases is altered. Findings of clinical and epidemiological studies indicate a heightened vulnerability of pregnant women to malaria-causing parasites [64]; viral infections such as measles, Hepatitis E or herpes simplex appear to occur more frequently and/or with greater severity [65]. Despite being considered a high-risk group during the COVID-19 pandemic, pregnant women and their fetuses exhibit an unimpaired humoral immune response to influenza vaccines [66].

These clinical findings must be seen in the context of the influence of ion channel $K_v1.3$, which is specifically upregulated in effector memory T (T_{EM}) cells [67]. Dysregulated T_{EM} cells are responsible for the pathogenesis of many of the previously mentioned disease patterns [68]. Hence, $K_v1.3$ has been identified as a potential therapeutic molecule,

attenuating the severity of T cell driven autoimmune diseases. So far, data highlighting the role of $K_v1.3$ in rheumatoid arthritis [69], multiple sclerosis [67, 70], psoriasis [71], type I diabetes mellitus [72] and neurodegenerative disorders [73] is available.

Despite the amount of data regarding $K_v1.3$ on T cells, little attention has been paid to neutrophils. Also, to date, there are few explanations for the molecular mechanisms underlying the adaptions of the immune system to pregnancy. This work builds a bridge between the clinical conditions observed in pregnant women and the research on ion channel $K_v1.3$.

2 Aim of the study

PIF is a small peptide detectable in the mother's blood during pregnancy [16]. It has been shown to be an immune modulatory factor that does not only contribute to successful pregnancy outcomes, but also attenuate the cause of several diseases in animal models. Moreover, PIF interacts with the voltage-gated potassium channel $K_v1.3$.

The aim of this project was to characterize the effects of ion channel $K_v1.3$ and its endogenous inhibitor PIF on leukocyte recruitment in the mouse as well as in the human system. *In vitro* experiments were conducted with particular focus on neutrophil adhesion, intraluminal crawling and phagocytosis using (a) a genetic global knockout model of $K_v1.3$, (b) pre-incubation with an ion channel inhibitor and (c) a pregnancy model. A better understanding of the impact of PIF on neutrophil dependent autoinflammatory or autoimmune diseases might open new therapeutic approaches.

Some of the results of my thesis are part of a study published in *Cardiovascular Research* in 2022 (Immler et al., The voltage-gated potassium channel $K_v1.3$ regulates neutrophil recruitment during inflammation [45]).

3 Materials and Methods

3.1 Animals

Charles River Laboratories (Sulzfeld, Germany) provided us for this study with *C57BL/6* mice as wildtype animals. *Kcna3*^{tm1Lys} [74] from Jackson Laboratories do lack expression of K_V1.3 and were backcrossed into *C57BL/6* background and bred in-house. These genetic global knockout mice will be referred to as *Kcna3*$^{-/-}$ in this thesis. Female (non-pregnant or gestation day 14.5) and male mice aged 8-25 weeks were used for experiments. All mice were kept at the Core Facility Animal Models, Biomedical Center Munich, Germany. Animal experiments were approved by the government of Oberbayern (AZ. 55.2-2532. _02-15-229 and _02-18-22).

3.2 Human blood samples

Blood withdrawal from healthy female and male participants received ethical approval from the LMU Munich, Germany (Az. 611-15).

3.3 Compounds

3.3.1 Buffers and solutions

Hanks' balanced salt solution (HBSS)

1 mM $MgCl_2$
1 mM $CaCl_2$
0.1 % Glucose
10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)
0,25 % Bovine serum albumin (BSA)

Adjusted to pH 7.4 using NaOH or HCl.

FACS buffer

1 % Bovine serum albumin (BSA) in Phosphate buffered saline (PBS)

Mouse anesthesia solution

Ketamine 125 mg kg^{-1}
Xylazine 12.5 mg kg^{-1}

To induce narcosis, a volume of 0.1 ml/8g body weight was injected intraperitoneally, every hour 0.03ml/8g body weight of anesthesia was administered to maintain narcosis.

3.3.2 Substances and items

Antigen	Dye	Reactivity	Isotype	Supplier
β_2 integrin activation assay				
KIM127	-	mouse α-human	IgG1	InVivo
mAB24	-	mouse α-human	IgG1	Hycult Biotech
Isotype	-	mouse isotype control	IgG1	BioLegend
PE Goat Anti-mouse	PE	goat α-mouse	Ig	BD Pharmingen
CD15-FITC	FITC	mouse α-human	IgM	BioLegend
CD66abc-PB	PB	mouse α-human	IgG2b	BioLegend
CD11a (≙ α-LFA1)	-	mouse α-human	IgG1 κ	BioLegend
CD11b (≙ α-MAC1)	-	mouse α-human	IgG1 κ	BioLegend
Phagocytosis assay				
CD15	APC	mouse α-human	IgG2a κ	BioLegend
CD66b	PB	mouse α-human	IgM κ	BioLegend
Ly6G	PB	rat α-mouse	IgG2a κ	BioLegend

Table 1 Antibodies

Name	Supplier
PIF	BioIncept LLC.
rmCXCL1	Peprotech
rmE-selectin/Fc	R&D Systems
rmICAM-1/Fc	R&D Systems
CXCL8	Peprotech

Table 2 Recombinant Proteins

Name	Supplier
µ-slides VI 0.1	Ibidi GmbH
BSA	Capricorn Scientific
Casein	Sigma-Aldrich
Catheter tube	Smith medical
DAPI	Invitrogen
DMSO	Sigma-Aldrich
EasySep mouse neutrophil enrichment Kit	STEMCELL Technologies
EDTA	Merck
FCS	Invitrogen
Glass capillaries (0.04x0.4mm)	VitroCom
Hepes	Sigma-Aldrich
Ketamine	Pharmacia GmbH
Lysing solution	BD
Object slides	Diwakar Instruments Company
PAP-1	Sigma-Aldrich
Penicillin	Invitrogen
Percoll	Sigma-Aldrich
pHrodo™ *E. coli* BioParticles ® Phagocytosis Kit	Invitrogen
Poly-L-lysine	Sigma-Aldrich
Polymorphprep	Axi-Shield PoC
RPMI 1640	Sigma-Aldrich
Streptomycine	Invitrogen
Triton X-100	Applichem
Türk's solution	MERCK
Xylazine	Bayer
Polymorphprep	Alera Technologies, Oslo, Norway

Table 3 Other Material

3.3.3 Software

Name	Supplier
VirtualDub (1.9.11)	GNU General Public License
ImageJ [75]	National Institute of Health
MtrackJ (ImageJ plugin) [76]	Eric Meijering
GraphPadPrism 7	Graphpad software
Kaluza (1.5)	Beckman Coulter
Adobe Illustrator (CS6)	Adobe

Table 4 Software

3.4 Leukocyte rolling, adhesion and rolling velocity *in vitro*

3.4.1 Flow chamber preparation

To study leukocyte behavior in a simplified scenario, rectangular glass capillaries (0.04x0.4 mm) were coated with rmE-selectin (20 µg ml^{-1}), rmICAM-1 (15 µg ml^{-1}) and rmCXCL-1 (5 µg ml^{-1}) mimicking inflamed endothelium. The proteins were diluted in PBS containing 0.1% BSA and 10µl of the coating solution was applied to each chamber using a high-precision syringe pump. After 3 hours incubation at room temperature, the capillaries were blocked with 5 % casein to prevent unspecific binding and placed in wet chambers at 4 °C overnight. The next day the chambers were flushed with heparinized HBSS prior to the experiment. This approach, along with the concentrations, had been previously established by the group of Prof. Markus Sperandio [33].

3.4.2 Murine blood withdrawal during the flow chamber experiment

Blood samples were obtained from anesthetized mice using a catheter inserted into the carotid artery. Therefore, mice were intraperitoneally injected with ketamine and xylazine (chapter 3.3.1) at a dosage of 0.1ml per 8g of body weight. Whithin the experiment anesthesia was maintained by adminitration of one third of the previously mentioned dose every hour. After the onset of anesthesia, the mouse was positioned on a heating pad set to 37°C. Subsequently, the trachea was cannulated to stabilize breathing during surgery. A catheter was inserted into the right carotid artery to enable safe intravascular access for blood withdrawal during the experiment. The blood was collected into heparinized tubes. In addition, leukocyte count was analyzed regularly with an IDEXX ProCyte DX hemocytometer (IDEXX Laboratories, ME, US).

3.4.3 Flow chamber microscopy

The heparinized murine whole blood was incubated with 300 nM PIF, 10 nM PAP-1 or vehicle control for 10 minutes at room temperature before perfusion of the capillaries at a calculated shear stress level of 2.7 dyne cm^{-2} using a high precision syringe pump (Harvard apparatus). Experiments were conducted on an Olympus BX51 WI

microscope (20x objective, 0.95 NA, water immersion) and the videos of the flow chambers were captured using VirtualDub recording software.

3.4.4 Data analysis of rolling and adherent leukocytes

Quantification of rolling and adherent leukocytes was carried out by analyzing the recorded movies 6 minutes after the start of blood flow in the glass capillaries. Within the period of 1 minute, it was observed whether a leukocyte stayed adherent on the coated capillary wall (=adherent cell) or moved by more than its diameter (=rolling cell). Therefore, all cells were marked at a specific time point of the video, observed for one minute and assigned to the group of rolling or adherent leukocytes depending on whether they have moved more than one diameter as determined by the mark.

3.4.5 Data analysis of leukocyte slow rolling velocity

The same movies were used to analyze leukocyte slow rolling velocities. Therefore, the rolling cells were tracked with Image J software [75].

3.5 Isolation of human neutrophils

With the consent of the donor, up to 50 ml of blood was drawn from a healthy donor. The blood was collected in heparinized tubes (430 IU of heparin per 50 ml of blood) to prevent clotting. Polymorphprep isolation kit was prepared according to its application sheet and human blood was layered on top of the Polymorphprep solution in a tube at a 1:1 ratio. By centrifugation, the blood components were divided into different layers according to their density. The top layers of plasma and peripheral blood mononuclear cells were extracted before collecting the polymorphonuclear cell layer. The neutrophils underwent two washes with PBS, were subjected to staining with Tuercks solution and subsequently were counted using an improved Neubauer chamber. The neutrophils were diluted at a concentration of $6x10^7$ cells/ml in HBSS buffer and incubated with either compounds or control as indicated.

3.6 Isolation of murine neutrophils

Mouse bones of the lower extremities were cleaned from muscle tissue and bone marrow was harvested by flushing the hip, femur and tibia with PBS. To remove coarse impurities, the cell suspension was rinsed through a cell strainer (grid size 40 µm). The cells were then washed (10 minutes at RCF 300), stained with Tuercks solution and counted using an improved Neubauer chamber.

To isolate murine neutrophils, the EasySep neutrophil enrichment kit was used according to the manufacturer's protocol reaching 88.7 % purity [77]. Overnight, neutrophils were cultured in RPMI 1640 medium containing interleukin 3 (IL-3) to promote their maturation process. Interleukin 3 was derived from the supernatant of the WEHI murine lung endothelial cell line, kindly provided by the group of Prof. Barbara Walzog (BMC, LMU Munich) [78]. The next day, the neutrophils were incubated with compounds or control as indicated.

3.7 β_2 integrin activation

Human neutrophils isolated via Polymorphprep were incubated with either PAP-1 (10nM) or vehicle control for 10 minutes at 37 °C. Subsequently, the cells were stimulated with CXCL8 (10nM) or HBSS buffer (negative control) in the presence of mouse α-human β_2 integrin activation-specific antibodies mAB24 (10 µg μL^{-1}), KIM127 (10 µg μL^{-1}) or isotype control for 5 minutes. The stimulation was halted by adding 900µl of ice-cold FACS lysing solution. Afer a fixation process of 10 minutes the cells were stained with secondary goat α-mouse-PE antibody ($5x10^{-4}$ µg μL^{-1}). In a further step, the cells were stained with mouse α-human CD66abc-PB (5µg μL^{-1}) and CD15-FITC (5µg μL^{-1}) labeled antibodies. Analysis of the activation status of β_2 integrins was performed by flow cytometry, with human neutrophils defined as $CD15^+$ and $CD66b^+$ double-positive cells.

Furthermore, the total surface protein levels of β_2 integrins Mac-1 and LFA-1 were examined. To achieve this, activated neutrophils were labeled with mouse α-human CD11b (5 µg μL^{-1}) or CD11a (5 µg μL^{-1}) antibodies. Sequentially the samples were incubated with secondary goat α-mouse-PE antibody ($5x10^{-4}$ µg μL^{-1}) and additionally

stained with mouse α-human CD66abc-PB (5µg μL^{-1}) and CD15-FITC (5µg μL^{-1}). Total surface protein levels of Mac-1 (CD11b/CD18) and LFA-1 (CD11a/CD18) on human neutrophils ($CD15^+$ and $CD66b^+$) were analyzed by flow cytometry.

3.8 Neutrophil crawling *in vitro*

µ-slides $VI^{0.1}$ by Ibidi were coated as mentioned above with rmE-selectin, rmICAM-1 and rmCXCL-1 for 3 hours at room temperature and blocked with 5 % casein at 4 °C overnight.

Isolated and matured murine neutrophils from *C57BL/6* and *Kcna3*$^{-/-}$ were resuspended in HBSS and added to the channel of the µ-slide. After 3 minutes to allow the cells to settle down, flow was applied at 2 dyne cm^{-2} using a high-precision syringe pump. Microscopy was carried out with a ZEISS AXIOVERT 200 inverse microscope (objective 10x, NA 0.25) and MetaMorph software. Samples were recorded for 20 minutes.

Using the Fiji software MTrackJ [76], crawling neutrophils were tracked over a period of 20 minutes. The Chemotaxis tool plugin allowed the analysis of different parameters to describe the crawling behavior of the cells.

3.9 Phagocytosis

To investigate phagocytosis in neutrophils, we turned to the established and frequently used pHrodo system [79–81]. In this assay, whole blood was incubated with pH-sensitive, fluorescent *E.coli* particles. The pHrodo system assesses phagocytic activity by detecting the acidification of particles as they are engulfed. The particles are conjugated to a dye that enhances its GFP signal in response to the acidic pH of their surroundings, such as within the phagosome.

3.9.1 Phagocytosis in mouse neutrophils

To study murine phagocytosis retro-orbital blood collection was performed. This form of blood collection involves anesthetizing the animal, gently tilting its head to expose the retro-orbital veins, and carefully collecting blood from these veins using a capillary. Whole blood of *C57BL/6* and *Kcna3*$^{-/-}$ mice was incubated with PIF (300 nM), PAP-1 (10 nM) or vehicle control for 10 minutes at 37 °C. The samples were then

incubated with *E. coli* particles (pHrodo Green Phagocytosis Kit, Thermo Fisher) for 30 minutes at 37 °C or at 4 °C as negative control. The *E. coli* particles are conjugated to dyes that dramatically increase in fluorescence as the pH of their environment becomes more acidic upon phagocytation. Further processing was conducted following the manufacturer's protocol and complemented by a staining with rat α-mouse Ly6G-PB (5µg μL^{-1}) antibody. Murine neutrophils were identified as $Ly6G^+$ cells and analyzed using flow cytometry (Beckman Coulter Gallios flow cytometer) and Kaluza Flow analysis Software (Beckman Coulter).

3.9.2 Phagocytosis in human neutrophils

For the human set of experiments, heparinized whole blood from healthy donors was incubated with either PIF (300 nM), PAP-1 (10 nM) or vehicle control for 10 minutes at 37 °C. The further procedure was conducted analogous to the treatment of the murine samples. In order to identify human neutrophils, directly conjugated primary antibodies CD66b-PB and CD15-APC were used for staining and detection (Figure 3).

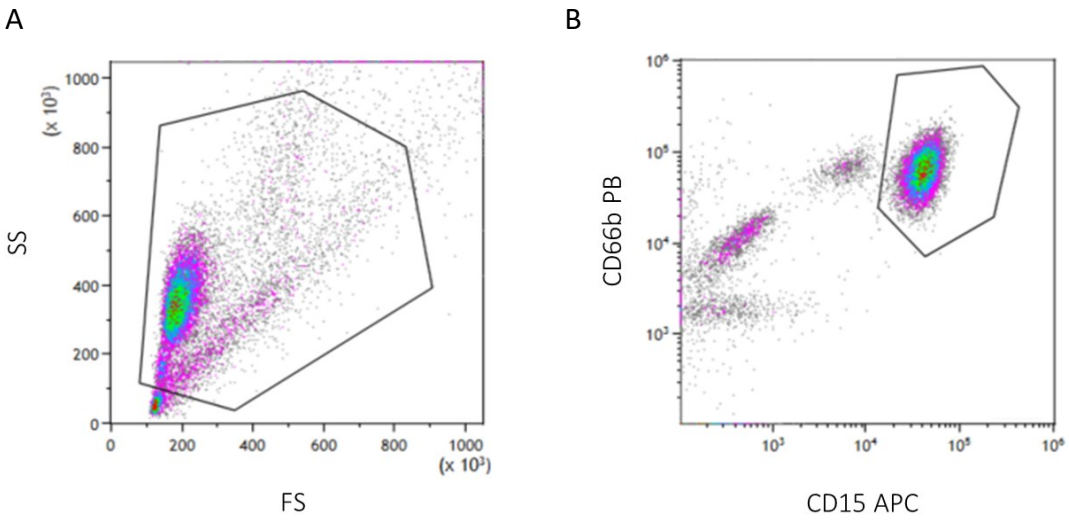


Figure 3: Fluorescence-activated cell sorting (FACS) plot depicting gating specifically on human neutrophils. (A) Forward (FS) and side scatter (SS) to identify the cells. **(B)** The staining and subsequent detection of human neutrophils were achieved using directly conjugated primary antibodies CD66b-PB and CD15-APC, allowing for precise identification and quantification of this cell population.

3.9.3 Confocal microscopy to assess phagocytosis

After incubation of murine and human whole blood with *E. coli* particles for 30 minutes at 37 °C, phagocytosis was stopped by putting the samples on ice. Using the EasySep neutrophil enrichment kit, neutrophils were isolated at 4 °C and seeded on slides pre-treated with 0.1 % poly-L-lysine. The cells were fixed with 2 % PFA, blocked with 2 % BSA, permeabilized with 0.1 % Triton X-100, stained with DAPI and embedded in ProLong Diamond Antifade mounting medium. Phagocytized particles were detected using GFP signal, as mentioned above. Confocal microscopy was performed at the core facility Bioimaging of the Biomedical Center Munich with a Leica SP8X WLL microscope (HC PL APO, 40x/1.30 NA, oil immersion objective). Images were then processed with Fiji software.

3.10 Statistical analysis

Statistical analysis was performed with GraphPad Prism 7 software. For the comparison of two groups, the paired student's t-test was used; if more groups were compared, the 1-way or 2-way analysis of variance (ANOVA) with either Tukey's or Dunnett's post hoc test was used. P-values <0.05 were considered statistically significant. Figures are presented as mean ± SEM; ns.: not significant, *P≤0.05, **P≤0.01, ***P≤0.001 and ****P≤0.0001.

4 Results

4.1 Leukocyte rolling and adhesion *in vitro*

The pregnancy-related peptide PIF has been proposed to alter the function of immune cells. Therefore, we set out to investigate immunomodulatory properties of PIF in scenarios of neutrophil driven acute inflammation with special regard to the effect of PIF on neutrophil recruitment. To elucidate the effects of PIF under controlled conditions, we conducted *in vitro* flow chambers coated with ICAM-1, CXCL1 and E-selectin. A a result of both the coating and cell morphology, the cells analyzed were predominantly neutrophils. However, since the specific cell type was not further characterized, they are referred to as leukocytes.

4.1.1 The effect of PIF on leukocyte rolling and adhesion *in vitro*

To examine the impact of PIF on neutrophil behavior during leukocyte recruitment, we initially compared PIF-treated murine leukocytes with WT control leukocytes in the flow chamber assay.

In this experimental setting, treatment of whole blood with PIF did not affect selectin-dependent rolling of neutrophils in the flow chamber (Figure 4A), but showed a significant decrease in adherent cells per FOV compared to control (Figure 4B).

With a particular focus on rolling, the rolling velocities in the flow chambers were analyzed. After treatment of whole blood with PIF, leukocyte rolling velocity showed an increase compared to WT control (Figure 4C and D).

These results indicate that PIF disrupts leukocyte recruitment by interfering with adhesion and slow rolling.

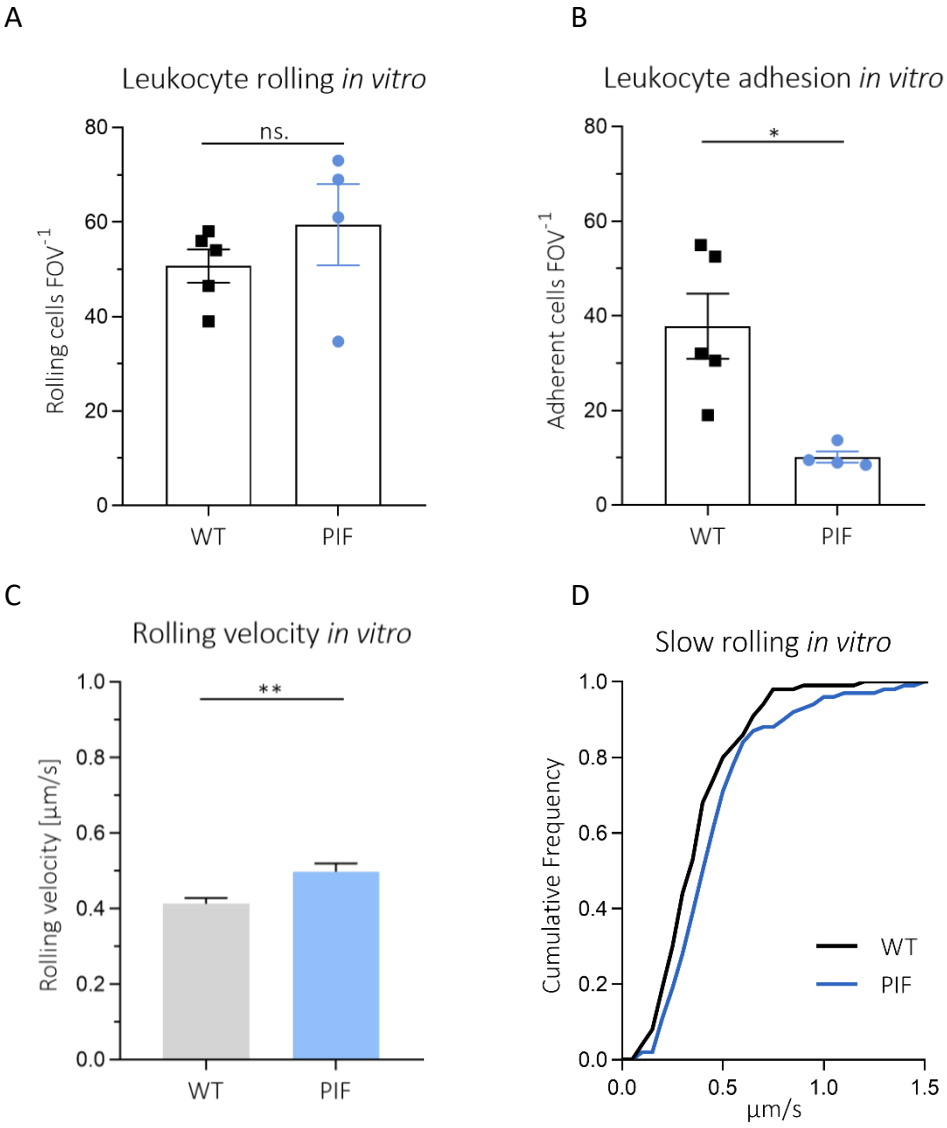


Figure 4: The presence of PIF does not affect leukocyte rolling yet results in a reduced number of adherent leukocytes. Number of **(A)** rolling and **(B)** adherent leukocytes in flow chambers coated with E-selectin, ICAM-1 and CXCL1 and perfused with whole blood of either WT mice or WT mice pre-incubated with PIF [n=4-5 mice per group, unpaired Student's t-test]. **(C)** Rolling velocity (µm/s) of leukocytes was analyzed in the same flow chambers six minutes after the initiation of blood flow in the capillary using ImageJ [n = 130-160 cells of 5 mice per group, unpaired Student's t-test]. **(D)** Cumulative frequency of the rolling velocity [n = 5 mice per group].

4.1.2 Leukocyte rolling and adhesion *in vitro* during pregnancy

In order to compare the behavior of leukocytes observed in the previous experiment to physiologically elevated PIF levels, a second assay was performed where flow chambers

were perfused with the blood of non-pregnant versus pregnant mice. Additionally, blood of some pregnant mice was incubated with synthetically-produced PIF.

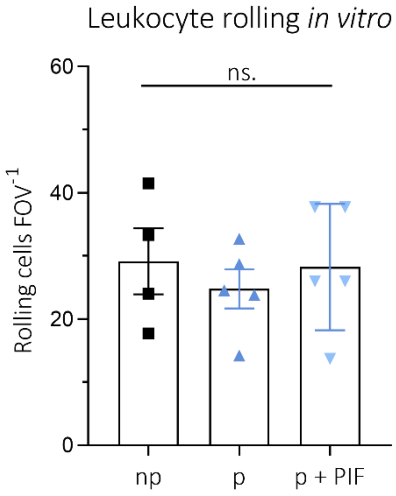
Leukocytes of pregnant mice showed a similar rolling behavior as of non-pregnant mice. PIF-treatment had no influence on the number of rolling cells (Figure 5A).

As shown in Figure 5B, blood samples of pregnant mice showed a significant reduction in the number of leukocytes adhering to the capillary wall. Whereas in the group of non-pregnant mice the number of adherent cells is 22.19 ± 4.89, the assay with the blood of pregnant mice showed 10.23 ± 2.19 adherent cells/FOV. When blood of pregnant mice was incubated with PIF, no additional reduction in the number of adherent cells was observed compared to the pregnant mice group alone, suggesting that the presence of endogenous PIF is already sufficient to reduce leukocyte adhesion as already shown in Figure 4B.

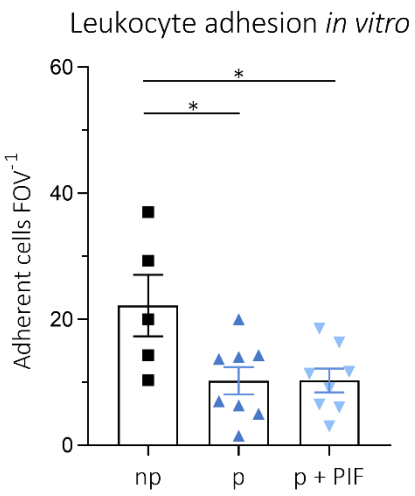
During pregnancy, leukocyte rolling velocity showed an increase compared to non-pregnant mice. The additional application of PIF to the blood of pregnant mice had no further influence on rolling velocity (Figure 5C and D).

As pregnant mice showed a lower number of white blood cells compared to the non-pregnant control group (Figure 5E), we also assessed adhesion efficiency, where the number of adherent cells are normalised to the WBC count, comparing the two groups as shown in Figure 5F.

A



B



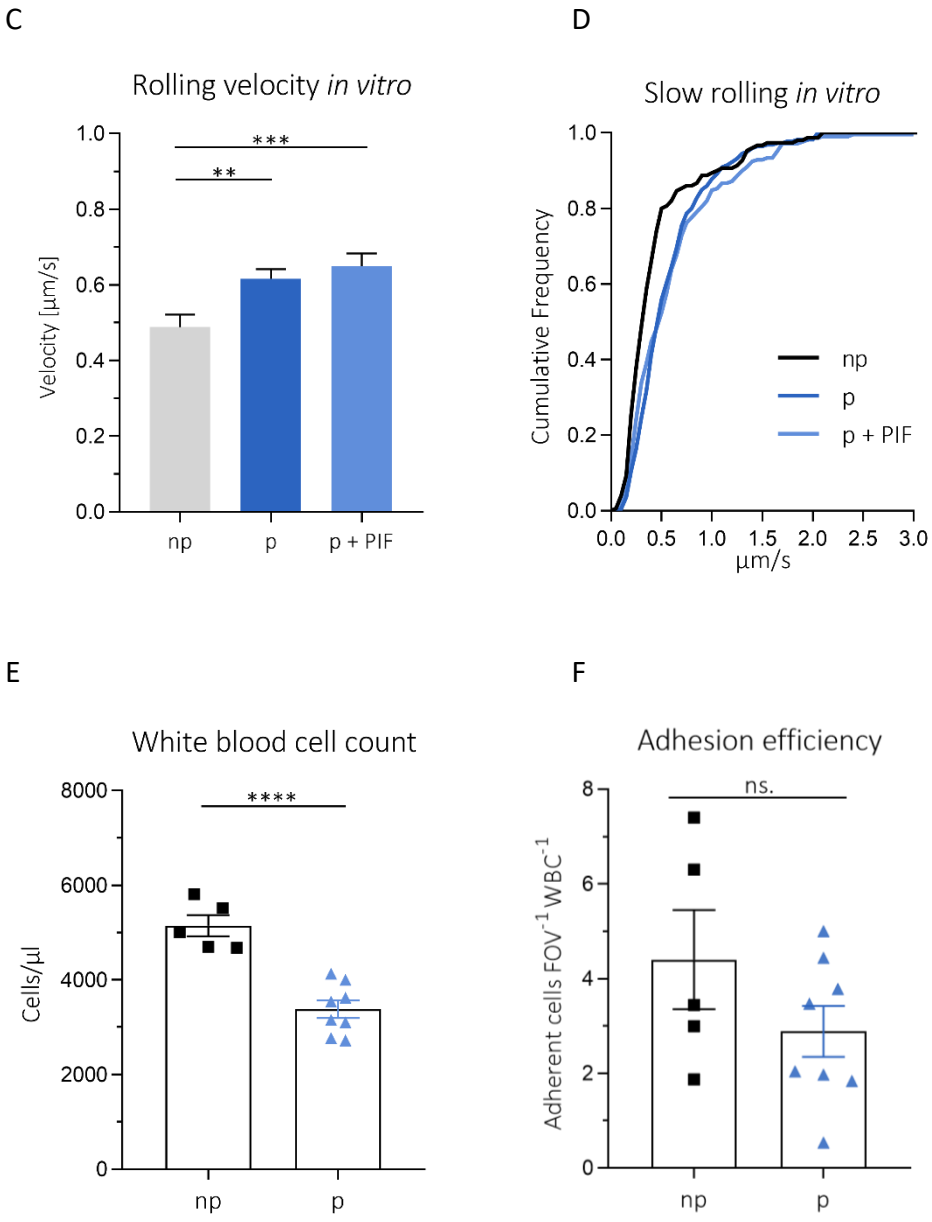


Figure 5: Pregnancy impairs leukocyte adhesion *in vitro*. Number of **(A)** rolling and **(B)** adherent leukocytes in flow chambers coated with E-selectin, ICAM-1 and CXCL1, perfused with whole blood of non-pregnant mice (np), pregnant mice (p) or pregnant mice pre-incubated with PIF (p + PIF) [n = 4-8 mice per group, One-way-ANOVA]. **(C)** Rolling velocity of leukocytes analyzed in the same flow chambers six minutes after the initiation of blood flow in the capillaries using ImageJ is increased during pregnancy [n = 150-220 cells of 5-8 mice per group, One-way-ANOVA]. **(D)** Cumulative frequency of rolling velocity [n = 5-8 mice per group]. **(E)** Overall WBC counts

measured with IDEXX ProCyte DX hemocytometer [n = 5-8 mice, unpaired Student's t-test]. **(F)** Number of adherent leukocytes / WBC count [n = 5-8 mice, One-way-ANOVA].

4.1.3 K_v1.3 alters leukocyte rolling and adhesion *in vitro*

The previous experiments confirmed a link between PIF, respectively pregnancy, and altered leukocyte recruitment. PIF is assumed to interfere with K_v1.3. Since K_v1.3 affects calcium homeostasis in other cells and since it is known that calcium is important in adhesion strengthening, we wanted to see whether K_v1.3 knockout or pharmacological inhibition affects neutrophil adhesion capacity in flow chambers *in vitro* (coating with E-selectin, ICAM-1 and CXCL1).

We hypothesized that PIF might lead to altered recruitment of neutrophils depending on the presence of K_v1.3. Therefore, the impact of a blocked K_v1.3 channel on rolling and adherent leukocytes was examined. *Kcna3*$^{-/-}$ mice were compared with WT animals. As a second study group besides genetic depletion, pharmacological blocking of the K_v1.3 channel was used. Here, murine whole blood of WT animals was incubated with PAP-1, a well-known inhibitor of the K_v1.3 channel.

In line with the previous experimental settings, genetic deletion of K_V1.3 did not affect the number of rolling neutrophils in the flow chamber assay (Figure 6A).

In the same experiment, the impact of a blocked K_v1.3 channel on the number of adherent cells was examined. On average, *Kcna3*$^{-/-}$ mice showed significantly less adherent leukocytes per field of view as WT animals. The treatment of blood with PAP-1 resulted in a reduction of approximately 44% in the the number of adherent leukocytes per field of view. Overall, leukocytes lacking the K_v1.3 channel showed a significantly lower number of adherent cells compared to WT controls (Figure 6B).

The *Kcna3*$^{-/-}$ group as well as the PAP-1 treated group both showed significantly higher rolling velocities compared to the control group (Figure 6C and D).

Kcna3$^{-/-}$ mice exhibited no differences in WBC counts compared to WT mice (Figure 6E). Taken together, these results indicate that K_V1.3 is indispensable for leukocyte adhesion under flow phenocopying the results on neutrophil adhesion in the presence of PIF as depicted in Figure 4.

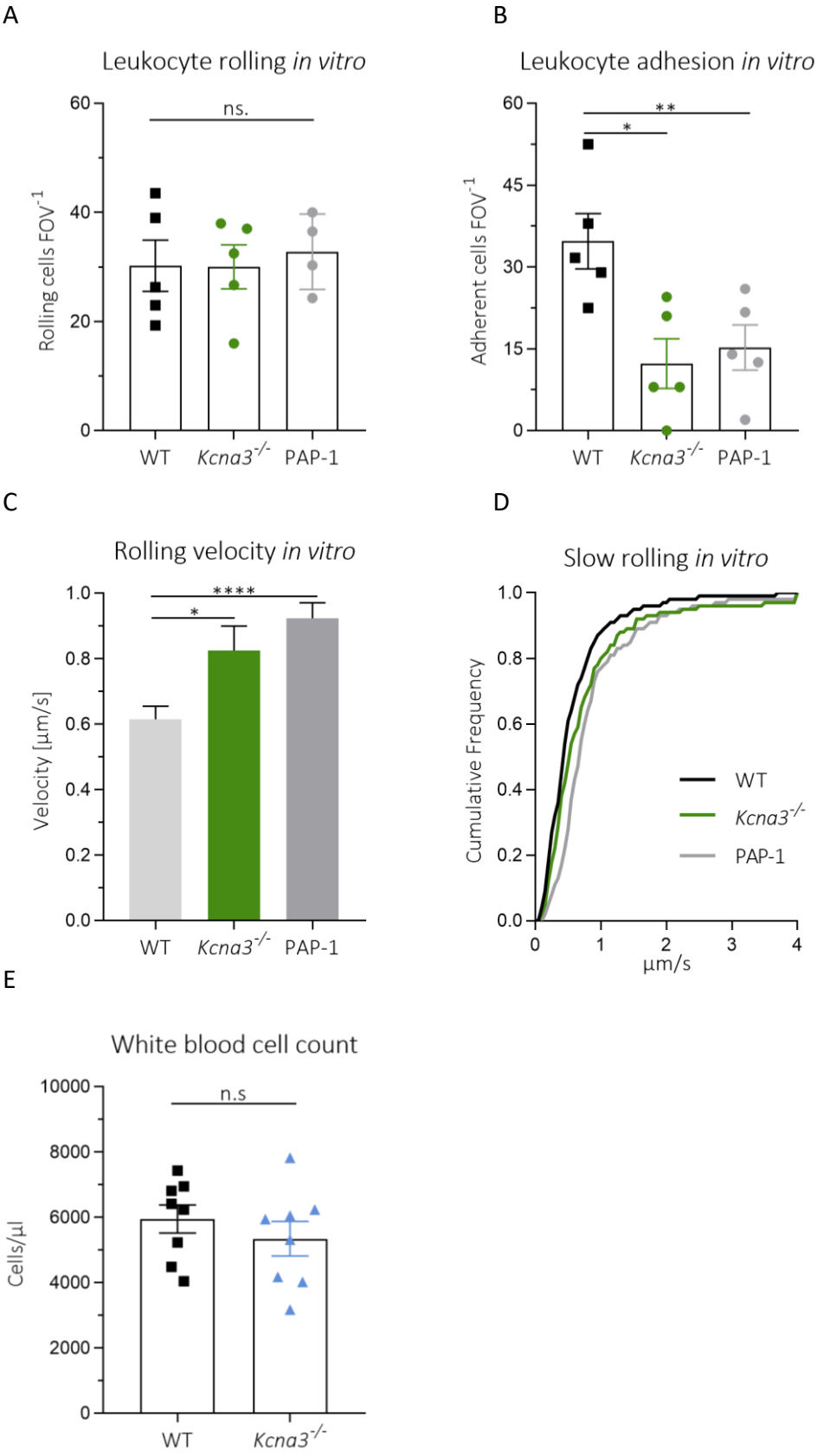


Figure 6: Inhibition of $K_v1.3$ via genetic depletion or pharmacological blocking results in reduced leukocyte adhesion and increased rolling velocity *in vitro*. The number of

(A) rolling and **(B)** adherent leukocytes was quantified in flow chambers coated with E-selectin, ICAM-1 and CXCL-1, perfused with whole blood from either WT mice, *Kcna3*$^{-/-}$ mice or WT mice pre-incubated with PAP-1 [n=5 mice per group, One-way-ANOVA]. **(C)** Rolling velocity of leukocytes analyzed in the same flow chambers six minutes after the initiation of blood flow in the capillaries using ImageJ [n = 280-420 cells of 6-13 mice per group, One-way-ANOVA]. **(D)** Cumulative frequency of the rolling velocity [n = 6-13 mice per group]. **(E)** Overall WBC counts measured with IDEXX ProCyte DX hemocytometer [n=8 mice, unpaired Student's t-test].

4.2 β_2 integrin activation

β_2 integrin activation is important for adequate neutrophil adhesion in the context of leukocyte recruitment. Results of previous experiments suggested that PIF affects leukocyte adhesion and slow rolling via altering β_2 integrin activation.

Building on this knowledge, an experiment was designed to explore whether blocking $K_v1.3$ compromises β_2 integrin activation more broadly. Therefore, human neutrophils were treated with PAP-1 or vehicle control and subsequently stimulated with CXCL8 for 5 minutes. β_2 integrin activation was assessed with activation specific antibodies, including KIM127 (intermediate and high-affinity) and mAB24 (high-affinity), and flow cytometry.

Preincubation with PAP-1 did not alter β_2 CXCL8-induced integrin activation on human neutrophils (Figure 7 A-D), suggesting that $K_v1.3$ activity is not required for β_2 integrin activation. Besides chemokine-mediated activation, the overall surface expression of β_2 integrins on human neutrophils was measured. Human neutrophils showed no change in surface expression of β_2 integrins LFA-1 (CD11a/CD18) or MAC-1 (CD11b/CD18) after pharmacological inhibition of $K_v1.3$ (Figure 7E, F).

These results demonstrate that impaired leukocyte adhesion caused by $K_v1.3$ inhibition is not mediated by altered β_2 integrin activation or surface expression.

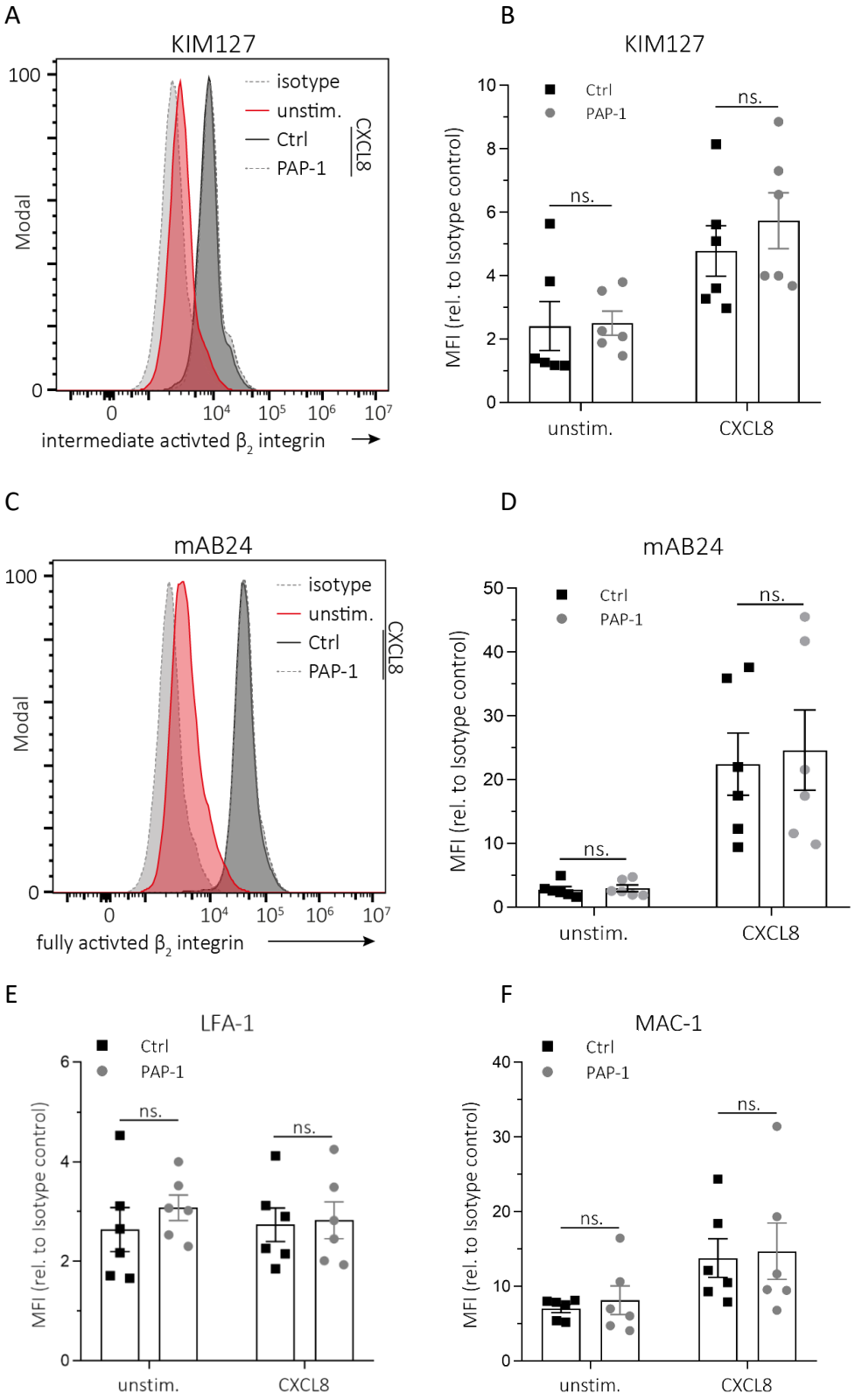


Figure 7: Blocking of $K_v1.3$ with PAP-1 does not alter either the activation state or the expression level of β_2 integrins on human neutrophils. Human neutrophils, identified as $CD15^+$ (mouse α-human, FITC) and $CD66b^+$ (mouse α-human, PB) double-positive cells,

were analyzed for PE signal indicating KIM127, mAB24, CD11a and CD11b, depending on the experimental group. The activation levels of β_2 integrins in **(A, B)** intermediate and fully activated states, as detected by KIM127 (mouse α-human), and **(C, D)** fully activated state, as identified by mAB24 (mouse α-human), were assessed in human neutrophils treated with PAP-1 and vehicle (Ctrl) using flow cytometry upon CXCL8 stimulation. Additionally, **(E)** LFA-1 (mouse α-human CD11a) and **(F)** MAC-1 (mouse α-human CD11b) expression were analyzed in $CD15^+$ and $CD66b^+$ human neutrophils [unstim.= unstimulated, MFI median fluorescence intensity, n = 6, Two-way-ANOVA, Sidak's multiple comparison].

4.3 Crawling *in vitro*

Based on the fact that the altered adhesion behavior of leukocytes cannot be attributed to impaired inside-out signaling, we tried to elucidate the impact of disturbed calcium signaling for post-arrest modifications in neutrophils. Outside-in signaling is particularly important for linking the actin cytoskeleton to activated integrins thereby mediating crawling under flow. The crawling behavior of neutrophils was studied in experiments where isolated murine neutrophils were introduced to E-selectin, ICAM-1 and CXCL1 coated flow chambers, a constant shear stress was applied and the crawling of the cells tracked.

Studying the movement pattern of neutrophils, *Kcna3*$^{-/-}$ cells showed a 'jumping behavior' in comparison to the constant crawling pattern of WT cells (Figure 8A and B). This suggests that *Kcna3*$^{-/-}$ neutrophils did not adhere properly to the surface of the chamber. Analysis of crawling neutrophils over 20 minutes under flow revealed a longer covered crawling distance of *Kcna3*$^{-/-}$ cells compared to WT control (Figure 8C and D). In line, the accumulated distance was significantly longer in *Kcna3*$^{-/-}$ cells compared to the control group (Figure 8E). Moreover, the crawling velocity of *Kcna3*$^{-/-}$ cells was significantly increased (Figure 8F).

In summary, impaired calcium signaling due to genetic deletion of the $K_v1.3$ channel can cause significant dysregulation of crawling and thus post-arrest modifications in neutrophils.

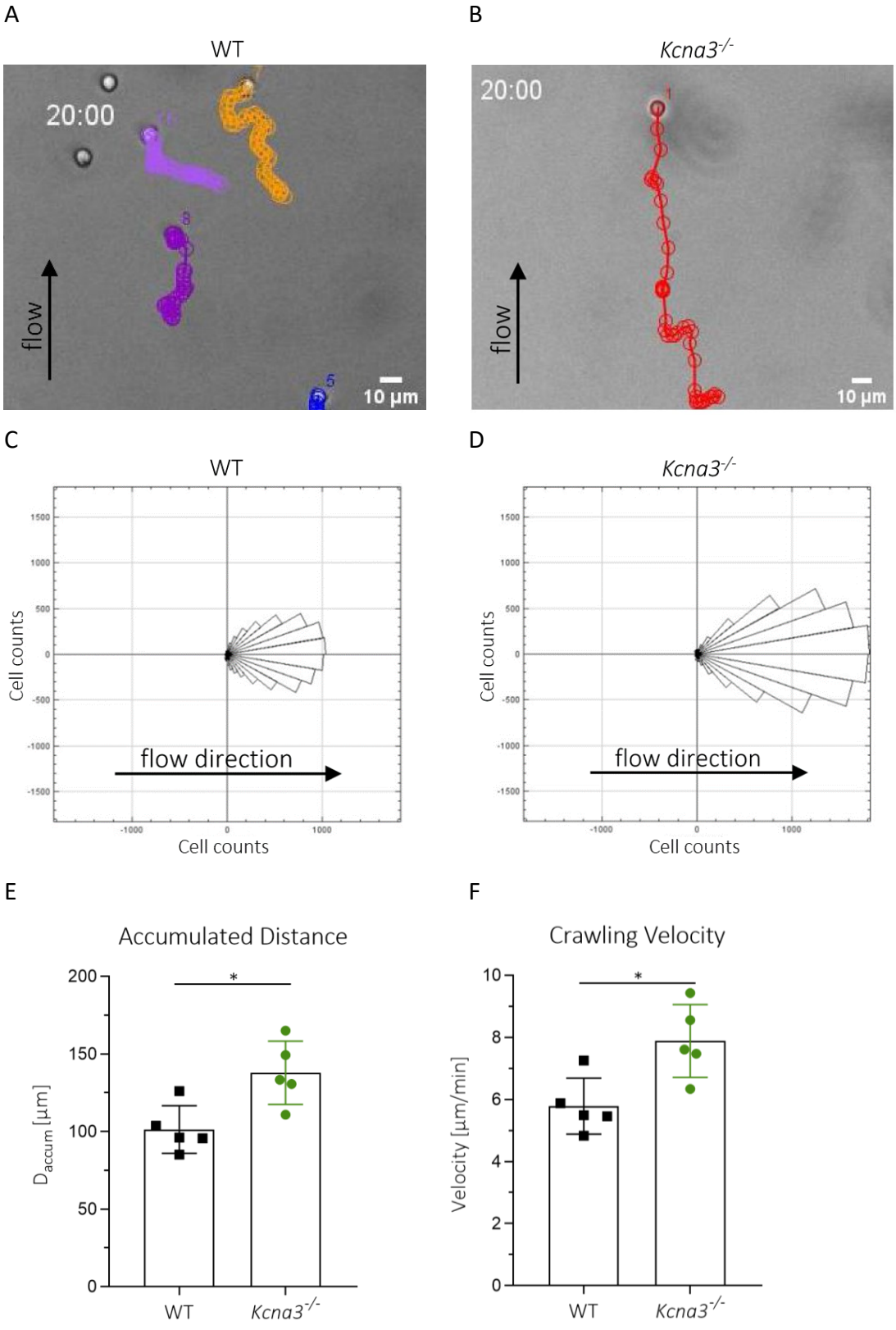


Figure 8: Crawling behavior of bone marrow-derived murine neutrophils under flow *in vitro* is impaired in the absence of $K_v1.3$. Crawling path of **(A)** WT and **(B)** *Kcna3*$^{-/-}$ neutrophils tracked over a period of 20 minutes. *Kcna3*$^{-/-}$ cells covered **(C, D)** a longer

distance, **(E)** a longer accumulated distance and exhibited **(F)** higher velocities compared to WT control [197–272 cells of n = 5 mice per group, unpaired Student's t-test].

4.4 Phagocytosis

An important neutrophil effector function is phagocytosis. Since phagocytosis depends on Ca^{2+} signaling, we hypothesized that blocking $K_v1.3$ results in an impaired phagocytic capacity of neutrophils.

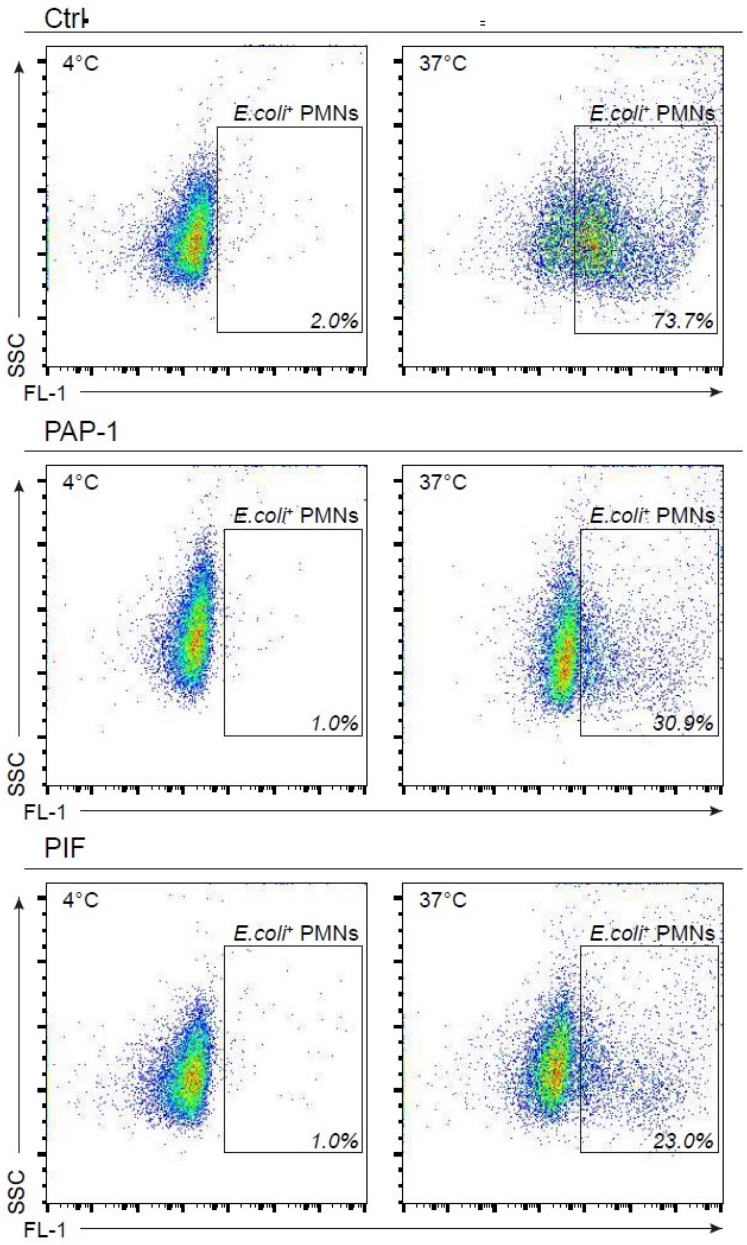


Figure 9: Representative plots during analysis of human neutrophil phagocytic activity via flow cytometry. On the left, most cells of the negative control sample kept at 4 °C

do not show a GFP signal indicating phagocytosed E.coli particles (*E. coli*$^{+}$ PMNs). Positive control samples were kept at 37 °C and the number of phagocytosing neutrophils increased.

To address this, whole blood was incubated with fluorescently labelled *E. coli* particles and subsequently analyzed by flow cytometry. Signal intensities in the green/GFP channel above the negative control (kept at 4 °C without *E. coli* particle uptake) were defined as phagocyting cells as illustrated by representitive plots (Figure 9).

4.4.1 Human Phagocytosis

Quantification of phagocytosis revealed that both, administration of PIF or PAP-1 resulted in a significantly reduced level of phagocytosed *E. coli* particles (Figure 10), suggesting that pharmacological blocking of the $K_v1.3$ channel impairs the phagocytotic ability of human neutrophils.

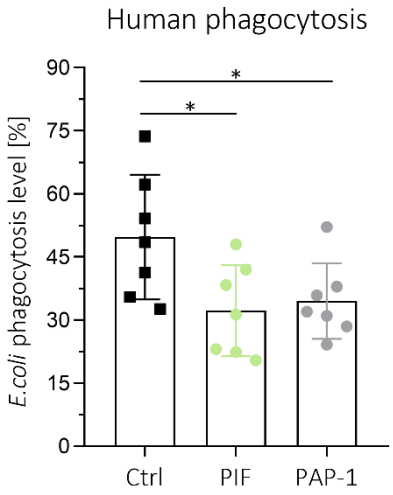


Figure 10: Phagocytic capacity of human neutrophils is reduced after pharmacological inhibition of $K_v1.3$. Quantification of neutrophil phagocytosis in human whole blood pre-incubated with either PIF (300 nM), PAP-1 (10 nM) or saline (Ctrl), using flow cytometry analysis [n = 7 mice per group, One-Way-ANOVA].

4.4.2 Murine Phagocytosis

Next, we investigated whether our observation of impaired phagocytosis in human neutrophils is transferable into a murine setting with *Kcna3*$^{-/-}$ mice.

Experiments involving cells of *Kcna3*$^{-/-}$ mice showed a significant decrease in the number of phagocytosed particles compared to wildtype controls. While 38.3 ± 3.5 % of WT cells were phagocytizing *E. coli* particles, only 26.7 ± 4.0 % of *Kcna3*$^{-/-}$ cells showed phagocytosing behavior. Additional incubation of blood from *Kcna3*$^{-/-}$ mice with PIF did not further decrease the *E.coli* particle phagocytosis level (Figure 11).

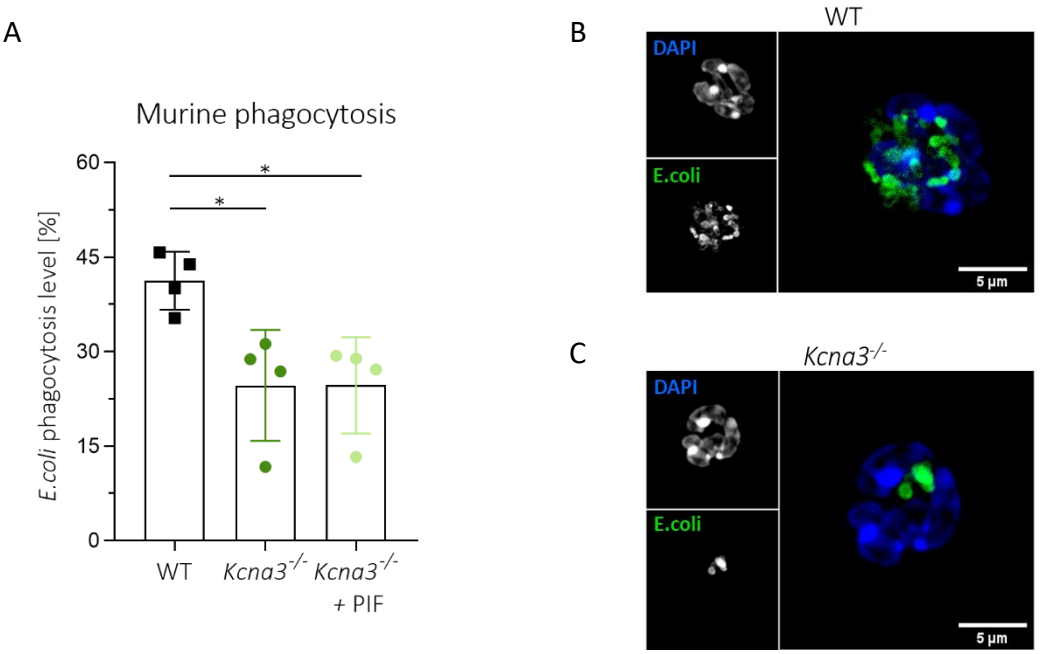


Figure 11: Genetic deletion of K_v1.3 significantly reduces phagocytic activity of murine neutrophils in comparison to WT control. (A) *E.coli* phagocytosis level of murine neutrophils: WT versus *Kcna3*$^{-/-}$ animals as well as PIF treated *Kcna3*$^{-/-}$ mice [n = 4 mice per group, One-Way-ANOVA]. Representative images of murine **(B)** WT and **(C)** *Kcna3*$^{-/-}$ neutrophils phagocytosing fluorescent *E.coli* particles, with cellular nuclei stained with DAPI (blue) and phagocytized particles labeled with GFP signal (green), were captured using confocal microscopy.

To visualize the effect of impaired phagocytotic capacity in *Kcna3*$^{-/-}$ cells, I carried out confocal fluorescence microscopy. The nuclei of the cells were stained with DAPI to facilitate cell identification. The representative image of a neutrophil with genetically depleted $K_v1.3$ showed a reduced number of phagocytosed particles compared to WT control (Figure 11 B, C).

5 Discussion

Immune modulation during pregnancy is mediated by still incompletely understood mechanisms. However, the close proximity of the fetus to the maternal organism requires a precise balance and regulation of immune response during pregnancy.

Preimplantation factor (PIF) is a pregnancy-specific factor that potentially modulates immune cell function in the context of pregnancy. This project examined the effects of PIF on leukocyte recruitment in the context of acute inflammation. Besides complementing data that showed an impact of PIF on neutrophil function, a particular focus was a murine pregnancy model and an *in vitro* system including human neutrophils.

5.1 Pregnancy and leukocyte activation

The modulation of neutrophil recruitment via PIF-mediated inhibition of $K_v1.3$ activity offers novel insights into neutrophil biology. As mentioned before, 30-40% of all decidual cells during early pregnancy are $K_v1.3$ expressing immune cells, actively recruited to the feto-maternal interface in order to promote successful pregnancy. Studies with specific deletion or altering of decidual immune cells report adverse pregnancy outcomes: uterine NK cells, known for aggressive reactions in the rest of the body, show a completely different behavior and are essential for endometrial vascularization [11]. Depletion of decidual dendritic cells leads to inappropriate decidualization and implantation failure [82]. In a murine model for multiple sclerosis, *Kcna3*$^{-/-}$ Foxp3(-) helper T cells exhibit a regulatory phenotype characterized by IL-10 expression, suggesting $K_v1.3$ deletion redirects T cells towards a more pronounced anti-inflammatory profile [83].

While T cells' importance in pregnancy establishment and maintenance is widely recognized, neutrophils' role in gestation has been less studied, despite the fact that it is particularly the granulocyte counts that are increased in the bloodstream of pregnant women. This surge is accompanied by heightened expression of activation markers CD11b, CD11a, and CD54 [84], as well as an increase in neutrophil-derived factors in the

blood [85]. However, recent reports indicate a reduction in various pro-inflammatory functions of neutrophils during pregnancy, which may contribute to maternal acceptance of the fetus [86–88]. This is in line with the work presented here demonstrating impaired neutrophil recruitment and diminished phagocytic activity [86] in circulating neutrophils among pregnant individuals compared to non-pregnant women.

5.2 Leukocyte rolling and adhesion *in vitro*

We hypothesized that the recruitment of neutrophils is modulated by the presence of PIF in blood serum. Weiss et al. showed that administration of PIF led to a decrease in the number of inflammatory cells within the pancreas in a murine model of type I diabetes mellitus. This resulted in the preservation of insulin production and the structural integrity of the islets of Langerhans. The PIF-induced reduction in leukocyte migration prevented diabetes development in more than 90% of recipients, suggesting therapeutic potential in shielding patients from excessive inflammation and an overflow of neutrophil recruitment [19].

This decreased infiltration of immune cells in the presence of PIF is observed in further studies, including a model for experimental autoimmune encephalomyelitis [89] and a model of atherosclerosis [90]. PIF administration successfully attenuated the progression of these diseases, demonstrating an effect of PIF on immune cell function.

The experiments presented in this thesis aimed to elucidate the molecular mechanisms responsible for the PIF-mediated alternations in immune cell recruitment. A particular focus is on the inhibitory effects of PIF on the potassium channel $K_v1.3$, thereby influencing calcium homeostasis.

The pivotal role of $K_v1.3$ in facilitating neutrophil recruitment is strongly substantiated by *in vivo* observations [21]. Genetic deletion of $K_v1.3$ reduced the number of adherent and extravasated cells in murine models of acute microvascular inflammation [45]. The observed reduction in extravasation is, in part, attributed to decreased adhesion and compromised post-arrest modifications. While *in vivo* experiments in the past focused

on the effect of a deleted $K_v1.3$ channel, the results presented in this thesis highlight the effect of PIF on neutrophil recruitment. In line with previous reports, the results demonstrate a significant reduction in leukocyte adhesion and a marked increase in rolling velocity.

The change in number of adherent leukocytes and of rolling velocity in the different study groups with (a) incubation with PIF (b) pregnant mice and (c) PAP-1-treated or $K_v1.3$-deleted cells show similar characteristics. PIF demonstrates dose-dependent inhibition of $K_v1.3$, exhibiting an IC_{50} of 10.2±5nM [21]. With PIF serum levels measured in pregnant women ranging between 50 – 60nM [16], an inhibition of $K_v1.3$ can be expected with physiological PIF serum levels during pregnancy.

The *in vitro* flow chambers help distinguish whether PIF acts on the endothelium or directly on neutrophils. We showcased a direct effect of PIF on neutrophils by observing reduced leukocyte adhesion and increased rolling velocity, both *in vivo* and *in vitro*.

The results of my *in vitro* studies performed in E-selectin, ICAM-1, and CXCL1-coated flow chambers with murine whole blood show no difference in the number of rolling cells. However, PIF – or a blocked $K_v1.3$ channel – has a direct effect on leukocytes in terms of integrin-dependent rolling velocity and number of adherent cells. These steps of the recruitment cascade are mediated by binding of activated LFA-1 integrin on neutrophils to its endothelial counterpart ICAM-1 [91]. Additionally, β_2 integrins play a role in slowing down rolling leukocytes during their recruitment [29]. These findings decisively demonstrate that $K_v1.3$ plays a critical role in mediating β_2 integrin-dependent firm adhesion, which heavily depends on SOCE, while showing no necessity for neutrophil capture and rolling along inflamed endothelium.

An important co-factor for β_2 integrin activation is talin-1 [92]. Integrin expression or activation is not affected by $K_v1.3$ activity. However, talin-1 was identified as a potential interaction partner of PIF [93] and thus provides a possible explanation for the effects observed in the flow chamber experiments.

As blocked or genetically deleted $K_v1.3$ impairs intracellular calcium homeostasis of neutrophils disturbing neutrophil slow rolling and adhesion, PIF also modulates Ca^{2+} signaling (although this I have not formally tested in neutrophils) and this is of clinical

importance, as immunodeficiency-like disorders are described in patients with loss of function mutations in Orai1 or STIM1 who exhibit neutropenia and an increased susceptibility to infections or sepsis [94].

Even though the *in vitro* flow chamber assay is a commonly employed method to investigate leukocyte interactions [95, 96], the susceptibility to errors is high. Possible reasons explaining the high statistic spread are irregular coating, outliers within different chambers perfused with blood of the same individual, premature activation of neutrophils and differences in white blood cell count.

Performing the experiment directly comparing pregnant and non-pregnant mice was a step towards a more physiological setting compared to the pre-incubation of whole blood with pregnancy-related factors or the genetic knock-out mouse model. Confirming these results *in vivo*, e.g. in a mouse-mesentery model, would be a further step. To bring research closer "from bench to bedside", an implementation of an experimental setting with human blood is of interest.

To sum up, we were able to verify that the administration of PIF – corresponding to a blocked $K_v1.3$ channel in the *in vivo* experiments – impairs leukocyte adhesion. We expand the picture of impaired neutrophil recruitment *in vivo* by attributing the effect to neutrophils, independent of endothelial involvement. Through $K_v1.3$ blockade, PIF modulates intracellular calcium homeostasis of neutrophils and consequently their function. This concept can possibly be extended to all immune cells and thus provides us with a potential explanatory approach for immune cell modulation during pregnancy.

5.3 $K_v1.3$ does not affect chemokine-induced integrin inside-out signaling

The first steps during neutrophil recruitment are predominantly mediated by neutrophil expressed LFA-1 binding to endothelial ICAM-1 [91]. Subsequently, Ca^{2+} signaling is required to link LFA-1 cluster sites with the cortical cytoskeleton and enables neutrophils to withstand shear forces and to spread and crawl properly.

However, through a series of experiments conducted on human neutrophils it could be shown that the ion channel $K_v1.3$ does not participate in β_2 integrin inside-out signaling.

The absence of $K_v1.3$ channel activity did not alter chemokine-mediated integrin activation or surface expression. This can also be deduced for the application of PIF which did not alter β_2 integrin activation.

Further experiments conducted in our research group demonstrated reduced paxillin phosphorylation in *Kcna3*$^{-/-}$ cells. This indicates impaired actin-dependent cytoskeletal rearrangement since tyrosine-phosphorylated paxillin is the connecting molecule between the actin cytoskeleton and activated β_2 integrins [97]. Hence, post-arrest modifications, such as efficient outside-in signaling, are impaired on a molecular level in *Kcna3*$^{-/-}$ neutrophils.

5.4 $K_v1.3$-deficienct neutrophils show impaired crawling behavior

Many neutrophil effector functions, including cell migration, are highly dependent on calcium signaling. Modulation of cell motility via $K_v1.3$ was described in microglia cells [98]. Moreover, macrophages [99] and effector memory T cells [100] exhibit decreased migratory behavior when exposed to $K_v1.3$ inhibitors. Neutrophils are less studied, however chemotactic behavior is reduced when potassium efflux via potassium channel $K_{Ca}3.1$ is impaired [101].

Outside-in signaling is particularly important for crawling under flow, with cytoskeleton rearrangements being imperative for firm adhesion, shape change polarization and migration. Ion channel $K_v1.3$ is involved in enabling Ca^{2+} influx supporting cytoskeleton alterations. *In vivo* experiments conducted in a mouse model of acute inflammation in the cremaster muscle revealed that neutrophils were more susceptible to shear forces after inhibition of $K_v1.3$ [21].

Our results confirm that *Kcna3*$^{-/-}$ neutrophils are impaired in their post-arrest modifications. While studying the crawling behavior of leukocytes, an adhesion defect in the PIF group compared to the control group was observed. Due to the adhesion defect, cells temporarily lose contact with the chamber wall and later reattach. These 'jumps' in flow direction differ significantly from the regular crawling motion of control

cells. Along with the observed differences in slow rolling and adhesion, these findings indicate impairment of leukocyte recruitment to sites of inflammation.

Further investigations are necessary to clarify the impact of PIF and $K_v1.3$ on interstitial migration. Attempts to study the chemotaxis of *Kcna3*$^{-/-}$ cells in flow chambers have been initiated.

5.5 PIF impairs neutrophil effector functions

Among various changes the female body undergoes during pregnancy, an increase in the number of neutrophils in the peripheral blood is observed. Compared to non-pregnant women, maternal neutrophils show changed surface molecule expression [102]. Those changes might play a role in adverse pregnancy outcomes such as in preeclampsia, a disorder characterized by an increased systemic inflammatory response or preterm labor, in which neutrophil driven inflammation is a common factor.

In this project we investigated neutrophil effector functions in the presence of PIF as well as of impaired $K_v1.3$ channel function. Both, genetic depletion of $K_v1.3$ in the murine model and incubation with PIF and PAP-1 in the human model, resulted in a reduced phagocytic capacity of neutrophils compared to the control group. Thus, our data is in line with previously published data showing diminished phagocytic capacity of neutrophils from pregnant women [86].

The serum factor PIF – homogeneously distributed in the blood stream of pregnant women – impairs this neutrophil effector function by means of reduced channel activity. Lenzo et al. strengthen this hypothesis by describing reduced phagocytosis in microglia cells with inactivated $K_v1.3$ channel [103].

Besides the importance of calcium, there is evidence that potassium efflux maintains phagosomal NADPH oxidase activity. The potassium channel $K_v1.3$ thus might be of importance in the process of producing reactive oxygen species, consequently killing phagocytized microbes. Further investigations examining the role of $K_v1.3$ on neutrophil effector functions need to clarify this.

5.6 Clinical significance and possible future applications

This work builds a bridge between the clinical conditions observed in pregnant women and the research on ion channel $K_v1.3$. The results of the work presented here show that physiological PIF levels during pregnancy induce the same neutrophil phenotype as deliberate blocking of $K_v1.3$. By conducting experiments on neutrophils, the potential application of $K_v1.3$ blockers was extended to the wide field of inflammatory disorders such as atherosclerosis. Furthermore, it provides an explanation on a molecular basis to previous work attributing therapeutic benefits to PIF, e.g. in mouse models of diabetes mellitus [19], multiple sclerosis [20, 89] or graft-versus-host disease after bone marrow transplantation [104].

In recent years, peptide drugs have become promising candidates opening up new pharmaceutical opportunities. With currently more than 400 peptide drugs under clinical development, peptide therapeutics turned into a leading industry [105]. The concept of using PIF as a specific inhibitor of $K_v1.3$ as a therapeutic drug seems obvious. PIF binds and efficiently inhibits $K_v1.3$ at relatively low concentrations, the endogenous origin of the peptide makes incompatibility symptoms rather unlikely. Furthermore, *Kcna3*$^{-/-}$ mice show a normal WBC count and do not exhibit any obvious immunological abnormalities. In 2018, a phase I study evaluated the safety and tolerability of PIF in patients with autoimmune hepatitis, demonstrating its potential for clinical application [106]. Despite recent advances, there are still major challenges that require further research to establish PIF as a therapeutic drug.

6 Summary

Recruitment of leukocytes to inflammatory tissues plays an important role in innate immune defense. This process proceeds in a strictly regulated sequence of steps and culminates in the targeted execution of effector functions at the site of inflammation.

During pregnancy, the immune system of the mother undergoes several adaptations to ensure immune tolerance to the embryo. PIF, a placenta derived peptide, plays a major role in modulating immune responses in pregnant women. By endogenously blocking the voltage-gated potassium channel $K_v1.3$ in leukocytes, we hypothesized that PIF influences intracellular calcium concentrations concomitant calcium signaling and neutrophil effector functions.

Here, we addressed how the antagonism of $K_v1.3$ by PIF affects neutrophil function during acute inflammatory processes. Therefore, in this project, we studied the effect of PIF and $K_v1.3$, respectively, on neutrophil recruitment using *in vitro* flow chambers and flow cytometry. In summary, the results of the work presented demonstrate that inhibition of $K_v1.3$ impairs neutrophil adhesion to inflamed endothelium: it increases the rolling velocity along a model for inflamed endothelium and alters the crawling behavior of neutrophils. Furthermore, altered calcium signaling of neutrophils due to the absence of $K_v1.3$ activity results in diminished phagocytic capacity, a phenotype also observed during physiological pregnancy.

This work expands our understanding of the mechanisms by which PIF modulates the immune response in an acute inflammatory setting and may also point to possible future pharmacological targets to treat neutrophil-mediated autoimmune or inflammatory diseases.

7 Zusammenfassung

Die Rekrutierung von Leukozyten in entzündetes Gewebe spielt eine wichtige Rolle in der angeborenen Immunabwehr. Der streng regulierte Prozess gipfelt in der gezielten Ausführung von Effektorfunktionen am Entzündungort.

Während der Schwangerschaft unterliegt das Immunsystem der Mutter mehreren Adaptationen, um Immuntoleranz gegenüber dem Embryo zu gewährleisten. Der in der Plazenta sezernierte Prä-Implantationsfaktor (PIF) spielt eine bedeutende Rolle bei dieser Immunmodulation. Durch endogene Blockierung des spannungsabhängigen Kaliumkanals $K_v1.3$ in Leukozyten war unsere Hypothese, dass PIF die intrazelluläre Kalziumkonzentration und die damit einhergehende Signalfunktion beeinflusst.

Entsprechend haben wir uns in dieser Arbeit mit der Frage beschäftigt, ob der Antagonismus von $K_v1.3$ mittels PIF die Funktion von Neutrophilen während akuter Entzündungsprozesse beeinflusst. In diesem Projekt haben wir daher den Effekt von PIF und $K_v1.3$ auf die Rekrutierung von Neutrophilen unter Verwendung von *in-vitro*-Flusskammern und Durchflusszytometrie untersucht. Zusammenfassend lässt sich aus den Ergebnissen dieser Arbeit ableiten, dass die Hemmung von $K_v1.3$ die Adhäsion von Neutrophilen am entzündeten Endothel beeinträchtigt: Sie erhöht die Rollgeschwindigkeit und verändert das Kriechverhalten der Neutrophilen. Darüber hinaus führt die veränderte Ca^{2+}-Signalübertragung von Neutrophilen aufgrund der fehlenden $K_v1.3$-Aktivität zu einer verminderten Phagozytose, ein Phänotyp, der auch während einer physiologischen Schwangerschaft beobachtet wird.

Diese Arbeit erweitert unser Verständnis für die Mechanismen, durch die PIF die Immunantwort in einem akuten entzündlichen Umfeld moduliert, und könnte auch auf mögliche zukünftige Ziele für die pharmakologische Therapie von Neutrophilen-vermittelten Autoimmun- oder entzündlichen Erkrankungen hinweisen.

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Acknowledgments

I would like to express my sincere gratitude to all those who supported and accompanied me on my journey to complete this doctoral thesis.

First, I extend my gratitude to *Prof. Markus Sperandio*, who not only provided me with invaluable academic guidance and inspiration but also remained a patient and supportive mentor. A heartfelt thank you goes to my direct postdoctoral supervisor *Roland Immler*, *PhD*, whose expertise and encouragement have illuminated my path. His insightful guidance and constructive feedback have been invaluable in shaping this research.

I am indebted to the dedicated *labmates in Munich*, who always encouraged me and stood by me for all scientific and technical queries. Their collaboration and friendship have made this path an unforgettable journey. Special thanks to *Matteo* and *Sergi*, who helped perform experiments and offered tremendous technical and emotional support. Furthermore, I would like to thank the *core facility bioimaging* at the Biomedical Center Munich and all the people from the *Integrated Research Training Group* of the SFB914.

To my dear *family* and *friends*, I extend my deepest appreciation for their patience, understanding, and constant belief in my abilities. Their unwavering support has been a driving force in my pursuit of knowledge and personal development. Thank you, *Jan*, for providing me with the illustration

Affidavit

I hereby declare, that the submitted thesis entitled

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