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Mechanismen der Zellmigration bei Entzündungen

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1 Zusammenfassung

Zellmigration ist in vielen Phasen der Entwicklung und des Erwachsenenlebens ein zentraler Prozess. Während einer Entzündung wandern Immunzellen geleitet von chemotaktischen Signalen zum Ort der Verletzung oder Infektion. Zellen können entweder einzeln wandern oder sich im Kontext von Geweben bewegen. Die Bewegung wird durch interne und externe Signale gesteuert, welche komplexe Signaltransduktionskaskaden aktivieren. Diese resultieren in einer hochdynamischen und lokalisierten Umgestaltung des Zytoskeletts und führen zu Zell-Zell- und Zell-Substrat-Interaktionen. Um diese Prozesse zu verstehen, ist es notwendig, die kritischen strukturellen Komponenten des Zytoskeletts, ihre räumlich-zeitliche Dynamik und ihre regulatorischen Signalwege zu identifizieren. Bei der Analyse dieser räumlich-zeitlichen Dynamik spielt die mikroskopische Bildgebung eine immer wichtigere und wirkungsvollere Rolle. In diesem Projekt wurden verschiedene Modellorganismen wie *Dictyostelium discoideum* und Mausmodelle in Kombination mit mikroskopischen Bildgebungstechniken verwendet und neu etabliert, um die verschiedenen Aspekte der Zellmotilität inklusive der Charakterisierung von Chemotaxis und Zytoskelettdynamik von Immunzellen während einer Entzündung zu untersuchen.

2 Summary

Cell migration is an essential process during many stages of development and adult life. During inflammation immune cells migrated to the site of injury or infection following chemotaxis signals. Cell movement is controlled by internal and external signals, which activate complex signal transduction cascades resulting in highly dynamic and localized remodeling of the cytoskeleton, cell-cell and cell-substrate interactions. To understand these processes, it is necessary to identify the critical structural cytoskeletal components, their spatio-temporal dynamics and their regulatory signaling pathways. Microscopic imaging plays an increasingly important and powerful role in the analysis of these spatio-temporal changes over time. In this project a variety of model organisms as *Dictyostelium discoideum* and mouse models combined with advanced microscopic imaging techniques have been used to investigate various aspects of cell motility including chemotaxis and cytoskeleton dynamics during inflammation.

3 Introduction

Directed cell migration is a fundamental property of many cells and plays an essential role during the development of most organisms. It is essential for gastrulation, the process where the endoderm and mesoderm take up their correct topological positions in the embryo, the formation and the wiring of the nervous system, and it plays a major role in the development of organs such as heart, lungs, kidneys and the intestinal tract. When during these processes cell movements are not properly controlled, it results in severe congenital defects and disease. Also in adult life, cell movement continues to play an extremely important role in processes such as wound healing and the functioning of the immune system. Cell migration is a highly complex process that is mediated by dynamic changes in the actin cytoskeleton, and can be modulated by the microtubule system and in some cells through interaction with the intermediate filament systems (Schaedel, Lorenz, Schepers, Klumpp, & Koster, 2021). Dynamic remodeling of the cytoskeleton is responsible for all cell behaviors and has to be precisely regulated in space and time, both in response to external as well as internal signals. It is now generally thought that localized actin polymerization in the front of the cell provides a critical driving force for lamellipodia extension, while contraction of the back of the cell, a myosin-II-dependent process, is necessary for retraction of the rear (Pollard & Borisy, 2003).

Cell movement not only requires the development of forces inside the cell but also requires these forces to be transmitted to the substrate to get traction. Furthermore, interactions with other cells are important and these contacts are mediated by specialized cell-matrix and cell-cell attachment complexes containing integrins and cadherins, respectively (Hynes, 2002; Nelson & Nusse, 2004). The contacts also have to be very dynamic to allow translocation of the cell, which implies that they have to turnover rapidly. Since the cytoskeleton has such a vital function, the regulation of its activity is one of the major tasks that the cell encounters. This involves a very complex regulatory machinery of several hundred proteins, which are the targets for extra- as well as intracellular signaling pathways (Dobereiner, Dubin-Thaler, Giannone, & Sheetz, 2005; Giannone & Sheetz, 2006). Understanding movement of single cells and cells in tissues requires the analysis of these complex processes under normal and perturbed conditions. The perfect

situation would be to measure movement and shape changes of cells and correlate these with the spatio-temporal dynamics of the cytoskeletal elements and the extra- and intracellular signaling pathways controlling these behaviors in isolated cells as well as in cells in tissues. An excellent model to study chemotaxis, cell migration and cytoskeleton dynamics during oriented movement is the amoebae *Dictyostelium discoideum* (*D. discoideum*) (Egelhoff & Spudich, 1991; Egelhoff, Titus, Manstein, Ruppel, & Spudich, 1991). Chemotaxis, the directed movement of cells in a gradient of a chemoattractant, is essential for *D. discoideum* to aggregate during morphogenesis but also for neutrophils to crawl to sites of inflammation and infection, thus directed cell migration is also a major driver in inflammatory responses (Metzemaekers, Gouwy, & Proost, 2020) (Huang & Iglesias, 2014; Petri & Sanz, 2018).

During inflammation immune cells migrated to the site of injury or infection following chemotaxis signals. Cells often migrate in response to specific external signals, including chemical signals and mechanical signals. Errors during this process have serious consequences, including intellectual disability, vascular disease, tumor formation and metastasis. When tissues are damaged, the inflammatory response is initiated, and the immune system becomes mobilized. Leukocytes are the first immune cells recruited to the site of tissue injury or damage via blood vessels and lymphatic system (Leick, Azcutia, Newton, & Luscinskas, 2014).

Leukocyte recruitment is a hallmark feature in inflammatory responses, which involves a sequential series of molecular interaction between the leukocyte and endothelial cells. First, leukocytes in the mainstream of blood flow come into contact with the endothelium and they roll along the endothelial surface via a group of molecules termed the selectins (Springer, 1994). Next, rolling leukocytes are activated by pro-inflammatory molecules presented on the endothelial surface to firmly adhere to the endothelium via integrins. Once adherent, leukocytes emigrate out of the vasculature and respond to directional (chemotactic) stimuli that guide them through the tissue to the inflammatory source (Foxman, Campbell, & Butcher, 1997). The trafficking of leukocytes in the context of inflammation, as cancer metastasis and infection, can be studied in several mouse models. A variety of different model organisms are used to study some aspect of human physiology or disease, but mice are especially useful because they

share mammalian features with humans and suffer from many of the same diseases. A large number of mouse models have been created to target specific human diseases using selective breeding and genetic engineering. Specific transgenic mouse models have been generate to study the dynamics of leukocyte migration and transmigration, combined with high resolution microscopy techniques (Leick et al., 2014).

High-resolution imaging is an excellent method to examine cell movement, migration processes, chemotaxis and gain an underlying of the functional networks and interactions in the cytoskeleton and between migrating cells, since optical sections through biological specimens can be readily collected at high spatial resolution and with high dynamic range and subsecond temporal resolution (Dormann & Weijer, 2006). Through the advent of genetically encoded labels, it is now becoming possible to mark many of the cellular and cytoskeletal components, by incorporating fluorescent protein reports, without interfering with their function (Miyawaki, Nagai, & Mizuno, 2005; Miyawaki, Sawano, & Kogure, 2003). In several systems these molecules can be expressed under the control of their own promoters, combined with fluorescent reports, using knock-in techniques, which ensures that they are expressed at physiological concentrations in the correct cell types and at the right stages of development. This allows visualization of the dynamics of many of the key structural elements of the cytoskeleton, such as actin and actin-binding proteins, myosin, microtubule and associated motor proteins, microtubule binding proteins and intermediate filament components.

The use of fluorescent proteins (FP) and the development of new imaging technologies have revolutionized studies in cell biology. A common application of the FP technique in combination with live-cell imaging is the visualization of the filamentous actin cytoskeleton in order to track cells for instance in chemotactic gradients or to analyze actin cytoskeleton dynamics with high-resolution. Photo-activation is a process characterized by the photo-induced activation of an inert molecule to an active state. Photo-activatable (PA) FPs have been developed to study the dynamic behavior of proteins within live cells and a number of photoactivatable FPs and “photo-convertible” FPs are available like Kaede, EosFP, Dronpa, PA-RFP1, and KikGR (Muller-Taubenberger & **Ishikawa-Ankerhold**, 2013). Taking benefit of FPs and combining it with advanced microscopic imaging methods and fluorescence manipulation techniques, such

as the fluorescence recovery after photobleaching (FRAP), the related fluorescence loss in photobleaching (FLIP), fluorescence localization after photobleaching (FLAP), Förster or fluorescence resonance energy transfer (FRET), fluorescence lifetime imaging microscopy (FLIM) and some of their application in biomedical science were described **(H. C. Ishikawa-Ankerhold**, Ankerhold, & Drummen, 2012; Muller-Taubenberger & **Ishikawa-Ankerhold**, 2013).

Nowadays, several transgenic mouse lines are available to study leukocytes migration *in vivo*, combined with intravital imaging covering a large spectrum of excitation and emission of fluorescent and photo-convertible proteins. This is a great combination of toolsets instrumental to allowing the *in vivo* studies of the immune cell dynamics, using mouse models during inflammatory diseases as cancer and infection, combined with high-resolution intravital imaging approaches (Stackowicz, Jonsson, & Reber, 2019).

Intravital microscopy (IVM) represents a powerful tool to study biological processes in living organisms. IVM offers the unique option to explore highly dynamic cellular processes that cannot be reconstituted *in vitro* or *ex vivo*, or when a link between cellular events and tissue pathophysiology is being pursued (Weigert, Sramkova, Parente, Amornphimoltham, & Masedunskas, 2010). This is the case for studies of inflammatory processes within the microcirculation and in the tissue. Inflammatory changes in the microcirculation, such as margination and rolling as well as transition to adhesion and migration of immune cells can only be visualized by intravital imaging (Pittet & Weissleder, 2011). The transmigrated cells in the tissue and their interaction with other immune cells and pathogens can be triggered and quantified *in vivo*.

Currently IVM is the only method that attains direct and immediate spatio-temporal information at a cellular / subcellular level from the intact mammalian immune system with functional circulation. It has brought particularly significant advances in understanding the biology of neutrophils, tissue resident immune cells and cells of the hematopoietic niche as these cannot easily be studied *ex vivo* due to short life spans, or the difficulty in replicating a complex microenvironment that is essential for functions but is not yet well understood. Multiphoton intravital microscopy is the imaging approach of choice due its advantages of deep infra-red excitation into the organs and reduced scattering of the light, contributing for a high-resolution imaging with less photo-toxicity to the tissue.

In this study, some of the above mentioned techniques have been used, newly established and combined to investigate (**1**) the functional mechanisms of chemotactic cell migration and cytoskeleton changes during cell movement by employing the model of *Dictyostelium* cells in combination with light microscopy; and (**2**) the leukocyte trafficking during inflammation, infection and cancer metastasis using several established mouse disease models with advanced intravital imaging approaches.

4 Results and Discussion

4.1 Models to study chemotaxis cell migration

A major question in understanding cell movement is how external signals modulate the cytoskeletal machinery to result in translocation and movement. One of the mechanisms that cells use to move in a particular direction is to respond to gradients of chemicals, which they can detect through cell surface receptors. If the movement involves direction sensing followed by directed migration up or down the chemical gradient, this process is known as chemotaxis (Affolter & Weijer, 2005). Extracellular signals are detected by membrane receptors that have an extracellular ligand binding domain and an intracellular domain that is involved in signal transduction to signaling pathways to locally modulate the cytoskeleton resulting in directed motion (Devreotes & Horwitz, 2015).

4.2 Chemotaxis in the model of *Dictyostelium discoideum* cells

One of the experimental model systems that is used extensively to study chemotactic cell movement through the use of a variety of imaging techniques is the social amoebae *D. discoideum* (Weijer, 2004). *D. discoideum* cells live as single amoebae in the soil and multiply through binary fission. Starvation triggers a developmental cycle in which hundreds of amoebae aggregate to form a multicellular structure, the slug that migrates away guided by environmental signals such as light and temperature gradients, which direct its migration to the surface of the soil. There the slug transforms into a fruiting body, consisting of a stalk supporting a mass of spores (**Figure 1**) (Dunn et al., 2017; Loomis, 2014). We have triggered the diverse stages of *Dictyosltelium* development by using actin-binding protein expressing fluorescence targets to study motion (H. C. **Ishikawa-Ankerhold**, Gerisch, & Muller-Taubenberger, 2010; H. C. **Ishikawa-Ankerhold** & Muller-Taubenberger, 2019).

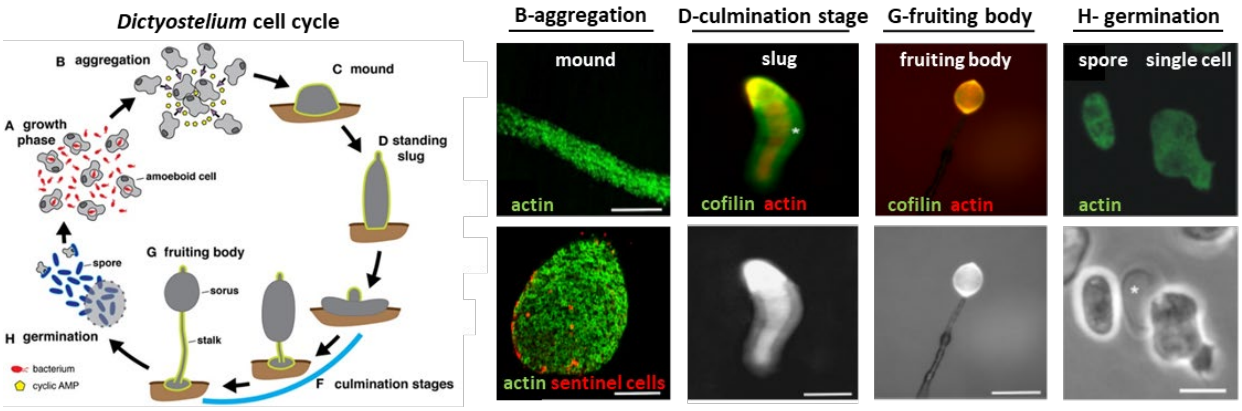

Figure 1. **The *Dictyostelium discoideum* lifecycle includes single cells, multicellular stages and spores**. Cartoon left side: **(A)** at the growth phase of development, amoeboid cells feed on bacteria and replicate. The development cycle starts when resources are restricted, and aggregation occurs when starving cells secrete cyclic AMP to recruit additional cells **(B)**. The aggregating cells organize to form the mound stage, resulting in a multicellular organism, as shown in B at panel on the right side. **(C)** the mound continues to develop into the standing slug **(D-left panel) and (D-right panel)**. Depending on its environment, the standing slug proceeds directly to the culmination stages **(F-left panel)** that ultimately produce the fruiting body (**F-right panel**), which consists of a spore-containing structure, the sorus, held aloft by a stalk of dead cells **(G)**. Spores are released from the sorus and germinate into growing cells **(H). H-right panel,** showing the single cell coming out from the spore. Scale bars are 10 µm. Cartoon modified from (Dunn et al., 2018). Figures from panel right were taken from (**Ishikawa-Ankerhold** et al., 2010 and **Ishikawa-Ankerhold** et al., 2019).

The chemotactic aggregation process is known to result from chemotactic movement in the direction of cAMP waves initiated by the aggregation centre and relayed by surrounding cells outward. These waves trigger the inward movement of the amoebae during the rising phase of the waves, resulting in movement towards the aggregation centre (Weijer, 2004). Under starvation condition, *D. discoideum* become elongate and can sense the gradient of cAMP chemoattractant. The visualization of the cytoskeleton dynamics during chemotaxis with higher-resolution using *D. discoideum* transgenic probes for labelling actin (GFP-LimE) was quantified in wild type and in mutants of actin-binding proteins under cAMP release from a micropipette assay (**Figure 2**) (**Ishikawa-Ankerhold** et al., 2017).

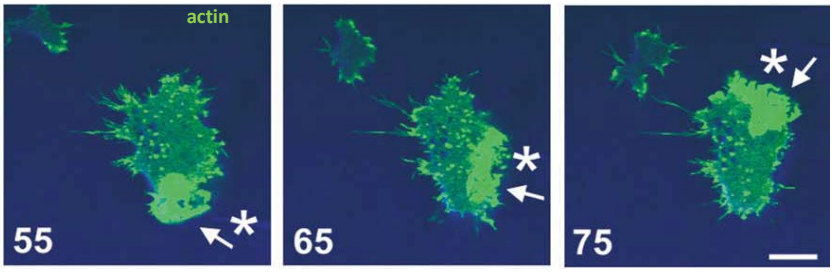

Figure 2. **Actin dynamics in chemotaxis**. Chemotactic responses of starved *Dictyostelium* cell expressing GFP-LimE (marker for actin), to a micropipette (indicated by the asterisks) releasing chemoattractant cAMP. The cell recorded by confocal microscopy shows rapid accumulation of filamentous actin (arrow) at sites nearest to the cAMP stimulus. Time is in seconds. Scale bar, 10 µm. Images were taken from **Ishikawa-Ankerhold** et al., 2017.

Thus, *D. discoideum* is a well-established simple model organism to study cytoskeleton dynamics during chemotaxis cell migration and other processes like cytokinesis, phagocytosis, and morphogenesis. It also has been applied to characterize nuclear and centrosome position during migration (H. **Ishikawa-Ankerhold**, Kroll, Heuvel, Renkawitz, & Muller-Taubenberger, 2022; Kopf & Kiermaier, 2021; Kopf et al., 2020; Renkawitz et al., 2019). It is a valuable model to explore the motile behavior of other fast-moving cells in general such as human neutrophils. In non-muscle cells, the actin cytoskeleton is essential for cellular structure and subcellular organization, and the dynamic regulation of actin assembly and disassembly is a prerequisite for cellular motility. Many diseases have now been associated with abnormalities in cytoskeletal proteins, including several cardiovascular disease syndromes, myopathies, neurodegenerative diseases (e.g. actin rods formation in Alzheimer's disease, Parkinson's disease, and ALS (amyotrophic lateral sclerosis)), cancer (invasion), liver cirrhosis, pulmonary fibrosis, and blistering skin diseases (Ramaekers & Bosman, 2004).

4.3 Actin-cofilin rod formation impairs cell migration

Actin dynamics plays an important role during cell migration and the cooperation between actin assembly and disassembly mediated through actin regulatory proteins is

essential to the maintenance of cellular functions. However, when actin turnover is impaired, it can lead to the formation of actin bundles (called actin rods) found for instance in patients suffering from Alzheimer´s disease. *D. discoideum* can be used as an easily accessible system to investigate the basic principles and the physiological role of actin rod formation during different conditions of cellular stress. Actin rods (also known as actin-cofilin rods) are aggregates consisting mainly of actin and cofilin that are formed in the cytoplasm or even in the nucleus as a result of diverse cellular stress, such as ATP restriction, pH changes and oxidative stress (H. C. **Ishikawa-Ankerhold**, Daszkiewicz, Schleicher, & Muller-Taubenberger, 2017; H. C. **Ishikawa-Ankerhold**, Kurzbach, Kinali, & Muller-Taubenberger, 2021; H. C. **Ishikawa-Ankerhold** & Muller-Taubenberger, 2019). Depending on the type of stress factors, actin rods can arise either inside the nucleus or in the cytoplasm. Actin rod formation is – at least to a certain degree – completely reversible. However, the presence of these bundles in the nucleus or cytoplasm impair migration even under chemotaxis stimulus. It is still unclear what exactly triggers the formation of rods, and what the underlying mechanisms of rod assembly and disassembly are. Furthermore, we have found actin rods in spores. However, it is unknown to what extent nuclear and cytoplasmic rods resemble the actin rods found in spores (**Figure 3**). In this work we also investigated the actin-cofilin rod composition and what are the stress factors inducing rod assembly.

We show that intranuclear rods can be induced by higher concentration of DMSO (10%) and are composed, in addition to actin and cofilin, of a distinct set of other proteins comprising actin-interacting protein 1 (Aip1), coronin (CorA), filactin, and the 34 kDa actin-bundling protein B (AbpB) (H. C. **Ishikawa-Ankerhold** & Muller-Taubenberger, 2019). The constituent proteins are recruited in a finely tuned spatio-temporal pattern during the formation of the rods (**Ishikawa-Ankerhold** et al., 2017). Cytoplasmic rods can be induced experimentally by sodium azid, but they also form in response to a number of different stressors that affect the cellular metabolism such as pH changes (H. C. **Ishikawa-Ankerhold** et al., 2021).

In human cells, nuclear rods are linked to specific forms of myopathies and Huntington’s disease, whereas the appearance of cytoplasmic rods is one of the early hallmarks of certain neurodegenerative diseases (Alzheimer’s disease, Parkinson’s

disease, and ALS (amyotrophic lateral sclerosis)) (Bamburg & Bernstein, 2016; Bamburg et al., 2010; Bernstein, Chen, Boyle, & Bamburg, 2006). However, currently little is known about specific factors that cause actin rod formation and how rod formation is modulated by physiological parameters.

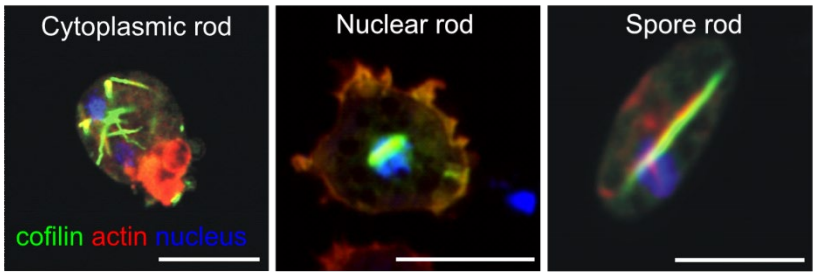

Figure 3. **Actin-cofilin rods formation under stress conditions.** Actin rods in cytoplasm, nuclei and spores. Cytoplasmic rods were induced by treating cells expressing GFP-cofilin and mRFP-actin with 10 mM sodium azide for 1 h at room temperature. Nuclear rods were induced in *Dictyostelium* cells by treatment with 10% DMSO for 30 min at room temperature. Spores generated from *D. discoideum* cells expressing GFP-cofilin and RFP-actin, were fixed with methanol (-20°C) for 15 min and stained with DAPI. All images were taken from **Ishikawa-Ankerhold** et al., 2019. Scale bars, 10 µm.

4.4 TIRF Microscopy to study cell migration

Total internal reflection fluorescence (TIRF) microscopy is a fluorescence technique that selectively excites fluorescently labeled structures within an evanescent field extending only about 150 nm from the substrate into the substrate-near surface of the cell. In *Dictyostelium* cells, the membrane-anchored actin filament network has a thickness of 100-200 nm, and thus almost its entire structure is amenable to visualization by TIRF (Bretschneider et al., 2004). Therefore, TIRF microscopy is an excellent tool and has being used to analyze the actin organization during migration with high resolution close to the substrate (**Figure 4**) (H. C. **Ishikawa-Ankerhold** & Muller-Taubenberger, 2019). By using this microscopic method, we could visualize the fine actin remodeling closer to the substrate and identified proteins of the SCAR-complex as a drivers of actin waves assembly during migration.

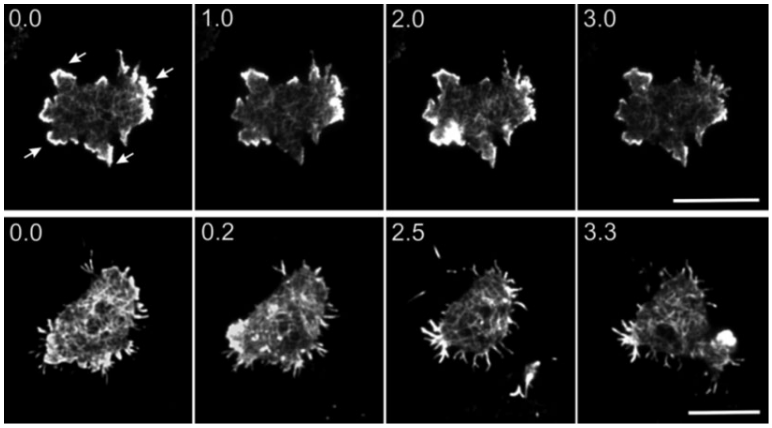

Figure 4. Actin at the cortex and the substrate-attached surface of *D. discoideum*. TIRF microscopy images show patterns visualized by GFP-LimE (a marker for actin), at the substrate-attached surface of *Dictyostelium* wild-type cells. A rapidly restructured network of single or bundled actin filaments provides a scaffold for the assembly of differentiated actin complexes: stationary foci with a lifetime of 7-10 seconds, and traveling waves. **(A)** Actin assembly observed at the cell cortex (arrows), and **(B)** the arrangement of actin assemblies close to the substratum. The numbers in the panels indicate time in seconds. Scale bars, 10 µm. Taken from **Ishikawa-Ankerhold** et al., 2019.

4.5 Characterization of immune cell migration using intravital microscopy

Intravital microscopy consists of imaging cells of a living animal through a transparent tissue or a transparent window placed in the body by surgery (Murooka & Mempel, 2012). This can allow direct observations of immune cell migration in their physiological context, and in various tissues (Weigert et al., 2010). Depending on the experiment and the invasiveness of the surgery, the animal is sacrificed at the end of the experiment. This technique requires specific labeling of the cells, which is usually performed using transgenic animals.

Some parts of the body are not trivial to access *in vivo* and require surgical skills procedure to externalize a tissue or organ to study it. *In vivo* migration experiments are often performed on mice (Abdul Hamid et al., 2020), because they are small enough to be positioned under a microscope (e.g. multiphoton microscopic system).

4.6 Multiphoton microscopy technique to study leukocyte trafficking

Multiphoton intravital microscopy (MP-IVM) is a revolutionizing high-resolution imaging method, allowing researchers to gain profound insight into inflammatory processes within tissues and organ systems *in vivo*. This is achieved by enabling real-time and long-term high-resolution deep tissue imaging, while exerting only minimal phototoxicity and stress. MP-IVM combines several microscopic advantages, including (i) efficient tissue penetration of the infrared (IR) laser beam, (ii) reduced photo-bleaching and photo-damage, (iii) imaging outside of the auto-fluorescence window, and (iv) improved fluorescence collection efficiency, as out-of-focus fluorescence is negated. Consequently, MP-IVM has become the technology of choice for high quality long-term imaging of immune responses in its different facets in native conditions. The use of MP-IVM in combination with transgenic mouse models represents a cornerstone of our research into cancer metastasis, thrombosis and immune cell migration and functions in sterile inflammation and infections.

In this work several intravital microscopy murine models were established to study leukocyte trafficking in organs as the skin, cremaster muscle, lymph nodes, brain, spleen, bone marrow, carotid artery, and novel models such as kidney, liver, fetal liver, fat tissue (visceral adipose tissue), collateral artery, stomach (gastric mucosa), gut, pancreas, yolk sac and placenta, as summarized in **Figure 5**. The immune-biological mechanism underlying inflammatory diseases, infection and tumor metastasis was investigated with a great contribution of the unique and powerful MP-IVM imaging tool combined with specific newly established models. Development of therapeutic strategies in these conditions requires a deeper understanding of complex *in vivo* multicellular processes and depends on the state-of-the-art intravital imaging in animal disease models.

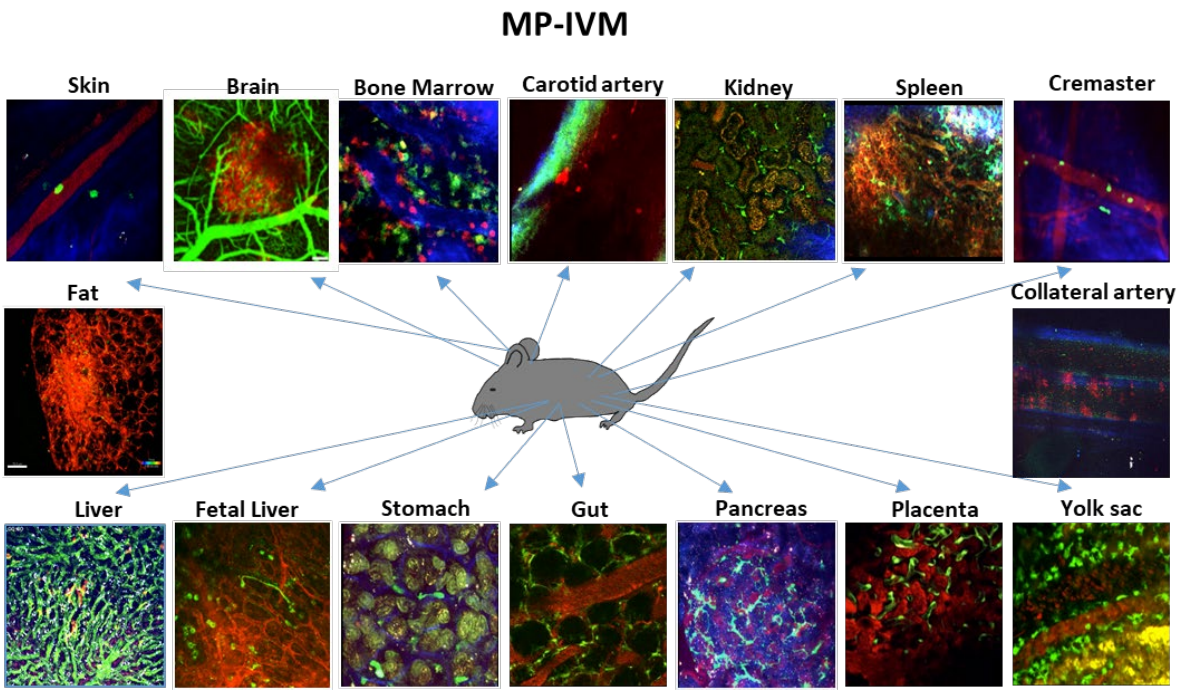

Figure 5. **Summary of the mouse tissue models established in this work used to study leukocyte trafficking by multiphoton intravital imaging (MP-IVM)**. Transgenic mice lines expressing leukocytes markers as CX3CR1-GFP and/or LysM-GFP, combined with plasma dextran dyes as Fluorescein Isothiocyanate-dextran (FITC-dextran) or Tetramethylrhodamine Isothiocyanate dextran (TRITC-dextran), and antibody labelling to visualize pathogenic microorganisms and immune cells. The blue color on the images represent the structure labelled by the second harmonic generation unique effect of the multiphoton excitation by the infra-red laser. The novel models established were bone marrow, kidney, fat, collateral artery, liver, fetal liver, stomach, gut, pancreas, placenta and yolk sac.

4.7 Establishment of novel mouse models for leukocyte trafficking studies

Inflammatory processes are associated with a broad variety of conditions such as infections, cancer metastasis, thrombosis, thrombocytopenia and ischemic diseases. However, understanding of the underlying pathophysiology has remained incomplete. Detailed elucidation of innate and adaptive immune responses *in vivo*, specifically immune cell recruitment, migration, activation and their functions within distinct niches and organ environments, is of pivotal importance for the development of novel therapeutic strategies to resolve tissue inflammation.

MP-IVM mouse models techniques were established and applied in a broad variety of scientific questions, such as the characterization of platelet migration and its interaction with *S.aureus* visualized in the infected liver (Gaertner **et al**., 2017), the interplay between the innate and adaptive immunity in organ ischemia and reperfusion injury (Funken **et al**., 2017), the immune cell recruitment during pathogen invasion in the gastric mucosa in a model of *Helicobacter pylori* infection (Schmidinger **et al**., 2022), the immune cell trafficking in tumor liver metastasis (Pfeiler **et al**., 2019) and the function of immune cells dynamics regulating the appetite and obesity (Stutte **et al**., 2022; Stutte **et al**., 2021). In addition, MP-IVM imaging was instrumental in other cooperative works, for example to visualize how platelets were forming from their megakaryocytes progenitors in the bone marrow, fetal liver (Liu, H; **Ishikawa-Ankerhold, H** et al., 2023) and the contribution of neutrophils during thrombogenesis (Petzold **et al**., 2022). The visualization of plasmacytoid dendritic cells trafficking and their interaction with megakaryocytes in the bone marrow was possible using MP-IVM (https://doi.org/10.1101/2022.05.31.494147). Our well established MP-IVM imaging also gave insides on the Chimeric Antigen Receptor T-cell (CART) immune therapy on tumor brain metastasis (Zhang **et al**., 2021) and on the role of immune cells in angiogenesis and arteriogenesis in femoral arterial ligation of smooth muscle tissue (Gotz **et al**., 2021; Kubler **et al**., 2021; Lasch **et al**., 2021). Thus, the diversity of advanced mouse models established for MP-IVM imaging (**Figure 5**) had provided significant contributions to the characterization and understanding of leukocyte trafficking and its function during inflammation and infection visualized on a high resolution cellular level.

Bellow, I am describing my contribution to some projects with the establishment of crucial novel mouse models for leukocyte trafficking studies using MP-IVM.

4.7.1 Bone Marrow Model

Plasmacytoid dendritic cells regulate megakaryocyte and platelet homeostasis in the bone marrow

In this project the mobility of plasmacytoid dendritic cells during thrombopoiesis was monitored *in vivo* by 4D MP-IVM in the bone marrow (**Figure 6**). Here, we presented for the first time that megakaryocyte (MK) homeostasis is controlled by plasmacytoid dendritic cells (pDCs) within the bone marrow (BM). pDCs are a unique subset of dendritic innate immune cells specialized in antiviral immunity. We demonstrated that pDCs constantly migrate to scan BM tissue and secrete type I interferon alpha (IFNα) upon detection of exhausted MKs to trigger megakaryopoiesis. We show that local pDC-derived IFNα synchronizes megakaryopoiesis and thrombopoiesis to maintain homeostasis of MKs and platelets in steady state and also accounts for enhanced platelet consumption in disease. Viral infection with SARS-CoV-2 can manipulate pDC-driven MK proliferation leading to inappropriate megakaryopoiesis, which has been associated with thrombotic complications during COVID-19 (Gaertner F and **Ishikawa-Ankerhold** et al., https://doi.org/10.1101/2022.05.31.494147) (**Figure 7**).

In conclusion, we identify plasmacytoid dendritic cells (pDCs) as homeostatic sensors that monitor the bone marrow for apoptotic MKs and deliver IFN-α to the MK niche triggering local on-demand proliferation and maturation of MK progenitors. This fine-tuned coordination between thrombopoiesis and megakaryopoiesis is crucial for MK and platelet homeostasis in steady state and stress. However, excessive activation of pDCs, such as by viral infections, can disturb this homeostatic circuit. Accordingly, we show that pDCs activated by SARS-CoV2 drive inappropriate megakaryopoiesis. Together, we uncover a hitherto unknown pDC-dependent homeostatic circuit that involves innate immune sensing and demand-adapted release of inflammatory mediators to maintain tissue homeostasis of the megakaryocytic lineage.

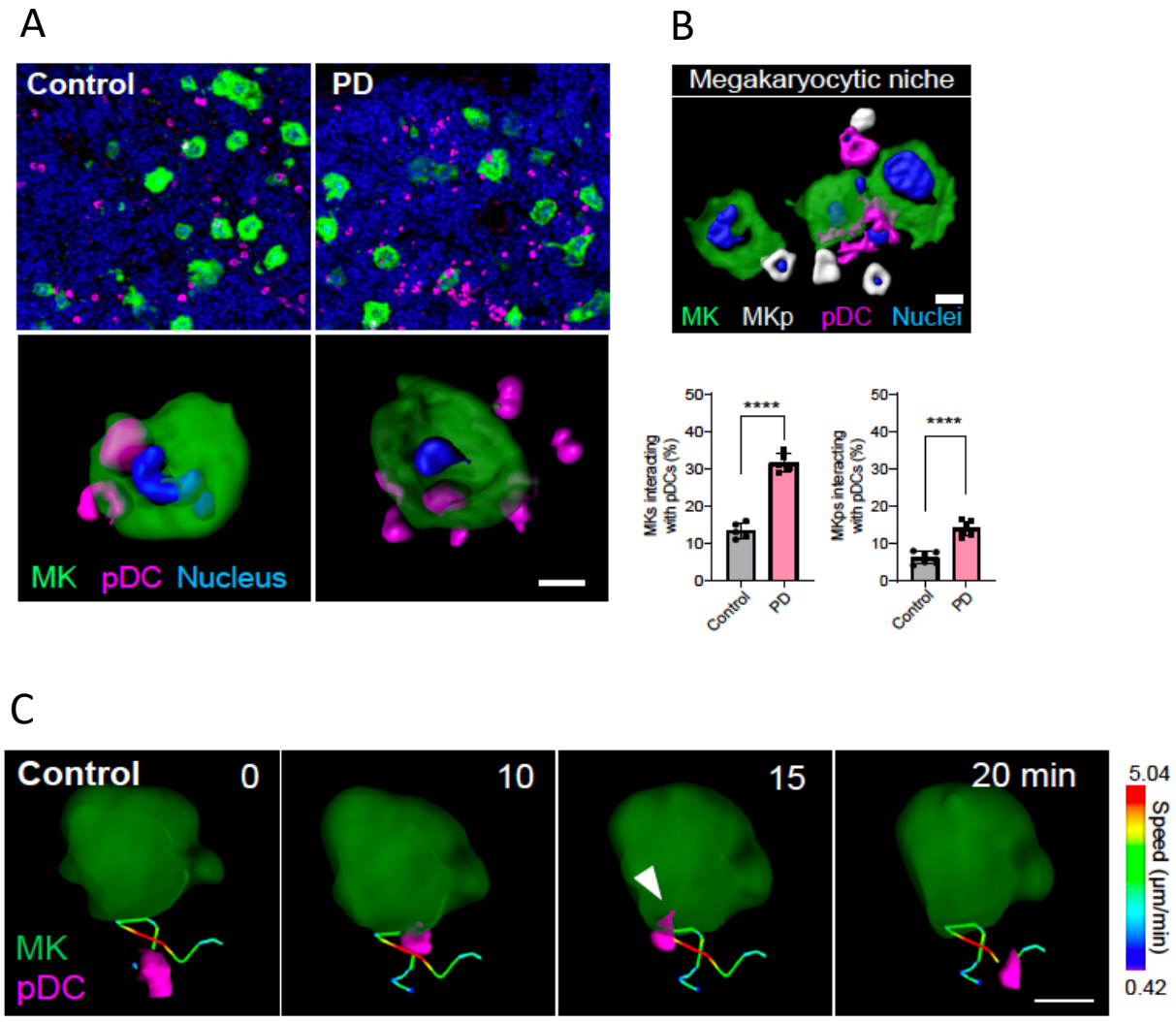

Figure 6. **Plasmacytoid cell migration in the calvarian bone marrow**. (**A**) Immunofluorescence images showing the closer interaction between plasmacytoid dendritic ceclls (pDCs) (magenta) with megakaryocytes (green) in the bone marrow. (**B**) Bone marrow megakaryocyte niche showing the proximity of pDCs with megakaryocyte progenitors (MKPs) and MKs. Histograms displaying the quantification of pDCs interacting with MKs and MKPs. (**C**) MP-IVM 4D imaging of pDC migrating and interacting with MK in the bone marrow. MP-IVM reveals migratory pattern of pDCs and long-lasting pDC-MK interactions (calvarian BM). Schematic shows experimental design MK: vWF-eGFP+ (green); pDCs: anti-siglecH-PE (magenta). pDCs protrude into MKs forming close contacts (arrowhead); color-code: pDC speed; scale bar=10 µm.

4.7.2 Adipose Tissue Model

Characterization of plasmacytoid dendritic cells trafficking, phenotype, and function in adipose tissue under high-fat diet

In this project, I have established a novel visceral adipose tissue model for MP-IVM imaging. Plasmacytoid dendritic cells (pDCs) display an increased abundance in visceral adipose tissue (VAT) of humans with obesity. In the current study, we deciphered the molecular mechanisms of their recruitment to VAT and the functional relevance of this process. We observed increased pDC numbers in murine blood, liver, spleen, and VAT in response to high-fat diet (HFD) for 3 weeks when compared with a standard diet. pDCs were migrating and enriched in fat-associated lymphoid clusters representing highly specific lymphoid regions within VAT showed by MP-IVM (**Figure 7**). HFD led to an enlargement of fat-associated lymphoid clusters with an increased density and migratory speed of pDCs as shown by multiphoton intravital microscopy. For their recruitment into VAT, pDCs employed P-selectin with E-selectin and L-selectin being only critical in response to HFD, indicating that the molecular cues underlying pDC trafficking were dependent on the nutritional state. HFD altered pDC functions by promoting their activation and type 1 interferon expression. Blocking pDC infiltration into VAT prevented weight gain and improved glucose tolerance during HFD. In summary, a HFD fundamentally alters pDC biology by promoting their trafficking, retention, and activation in VAT, which in turn seems to regulate metabolism (Stutte **et al**., 2022).

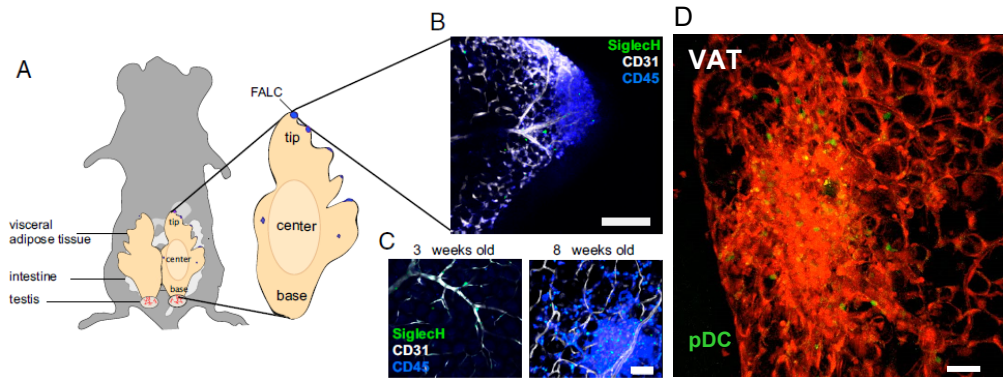

Figure 7. Distribution of pDCs within the VAT. (**A**) VAT anatomy of the mouse and localization of fat-associated lymphoid clusters (FALCs).Male VAT was separated in the tip area (facing the liver), in the center part, and in the base part, which is attached to testis and epididymis. Intravital microscopy of FALC structures within VAT from chow-treated male mice. (**B**) VAT was stained for SiglecH (pDCs, green), CD31 (endothelia cells, white), and CD45 (hematopoietic cells, blue). Scale bar, 100 mm. (**C**) Representative images of VAT of 3-wk-old (left image) and 8-wk-old male mice (right image) stained for SiglecH (green), CD31 (white), and CD45 (blue). Scale bar, 50 mm. (**D**) Multiphoton intravital imaging of the VAT from a male mouse expressing tdTomato (red), and pDCs (green). Scale bar 70 mm.

4.7.3 Liver Metastasis Model

Imaging of immune cell responses to pancreatic tumor metastasis associated with blood coagulation cascade and infection

This project aimed to understand the biological basis of thrombotic and septic diseases which are among the main causes of morbidity and mortality worldwide. The focus on elucidating the immune mechanisms underlying the activation of coagulation, and the formation of immunothrombosis in the context of systemic infections and tumor metastasis.

We have found that the activation of intravascular coagulation and the formation of microthrombi during bacterial infections is controlled by innate immune cells (such as neutrophils) recruited to the microcirculation of infected organs. Fibrin-dependent immunothrombosis helps to restrict the dissemination of systemic bacteria and to support their intravascular elimination. Changes in leukocyte trafficking and crosstalk is required to support the biological functions of coagulation but can also be the cause of its dysregulation – which is a major cause of both small and large vessel thrombosis. I have established a MP-IVM model for visualization of pancreatic tumor and leukocyte trafficking in the microvasculature of the liver to investigate their role in metastasis (**Figure 8**).

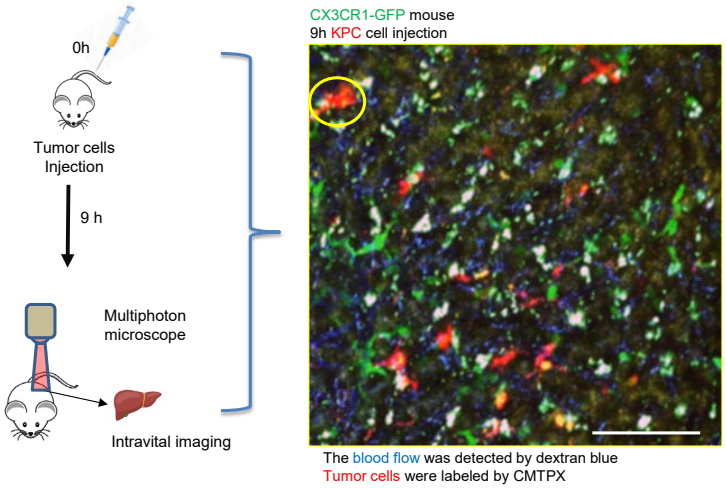

Figure 8. Multiphoton intravital imaging of the liver microvasculature. Pancreatic cells were labelled with CMTPX-red (excitation/emission 577/602 nm) and injected intravenously (i.v) into the blood stream of the CX3CR1-GFP (monocytes/macrophages are labelled in GFP) mouse. After 9h of injection, the mouse was anesthetized, and the liver tissue was exposed for MP-IVM. Dextran blue was injected for blood flow visualization. Scale bar is 50 µm.

4.7.4 Gastric Mucosa (stomach) Model

Novel intravital multiphoton imaging enables real-time study of *Helicobacter pylori* interaction with leukocytes in the mouse stomach

In this project, I have established the novel MP-IVM imaging of the gastric mucosa infected with *Helicobacter pylori* (*H. pylori*). The aim of the project was to investigate the mechanism of *H. pylori* infection in the gastric mucosa, and its modulation of the immune system. *H. pylori* infections are usually acquired in childhood and persist in life if not treated. The gastric inflammation is often asymptomatic but can also give rise to more severe gastric malignancies, such as peptic ulcer disease or gastric cancer. For a successful stomach mucosa colonization, *H. pylori* has to cope with the host immune system. The bacteria apply several immune evasion strategies, such as the prevention of Toll-like receptor recognition or skewing of effector T-cell responses. We demonstrated that *H. pylori* interferes with leukocyte migration *in vitro*, using its major virulence factor Cytotoxin-associated gene A (CagA) (Behrens **et al**., 2020). We could visualize macrophages internalizing *H. pylori in vivo* by our novel established MP-IVM of the gastric mucosa **(Figure 9)**, showing the active participation of immune cells during *H. pylori* infection.

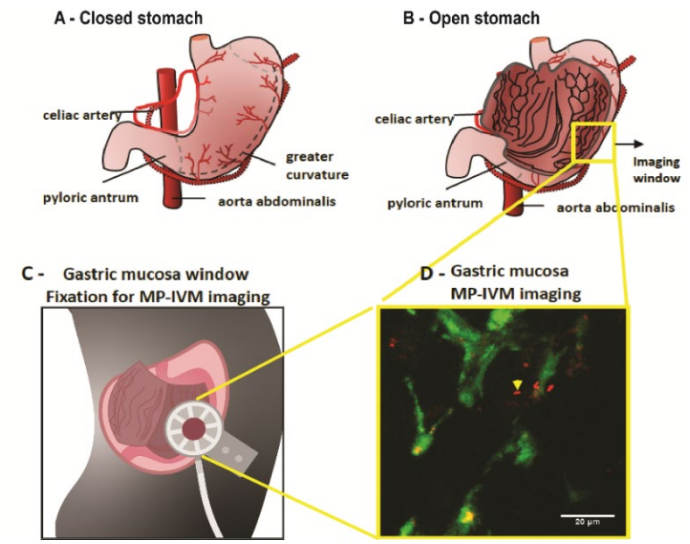

Figure 9. **Gastric mucosa preparation for multi-photon imaging**. (**A**) The mouse intact closed stomach is surgically opened (**B**) to expose the inner gastric mucosa for imaging. (**C**) The inner gastric mucosa is fixed by a suction ring, where a window of 6 mm was imaged. (**D**) MP-IVM imaging of a CX3CR1-GFP mouse gastric mucosa (showing macrophages in green), infected with RFP-*H. pylori* in red, indicated by the head arrow.

4.7.5 Femoral Collateral Artery Model

Multiphoton intravital imaging for monitoring leukocyte recruitment during arteriogenesis in a murine hindlimb model

Arteriogenesis strongly depends on leukocyte and platelet recruitment to the perivascular space of growing collateral vessels. The standard approach for analyzing collateral arteries and leukocytes in arteriogenesis is *ex vivo* (immuno-) histological methodology. However, this technique does not allow the measurement of dynamic processes such as blood flow, shear stress, cell-cell interactions, and particle velocity. In this project I have established the murine model to monitor *in vivo* processes in growing collateral arteries during arteriogenesis utilizing MP-IVM imaging (**Figure 10**). The method is a reliable tool for dynamics measurement and offers a high-contrast analysis with minimal photo-cytotoxicity, provided by multiphoton excitation microscopy. Prior to analyzing growing collateral arteries, arteriogenesis was induced in the adductor muscle of mice by unilateral ligation of the femoral artery. After the ligation, the preexisting collateral arteries started to grow due to increased shear stress. To visualize blood flow and immune cells during *in vivo* imaging, CD41-fluorescein isothiocyanate (FITC)

(platelets) and CD45-phycoerythrin (PE) (leukocytes) antibodies were injected intravenously (i.v). This method introduces intravital multiphoton imaging as an alternative or *in vivo* complementation to the commonly used static ex vivo (immuno-) histological analyses to study processes relevant for arteriogenesis. In summary, this work brings a novel and dynamic *in vivo* method to investigate immune cell trafficking, blood flow, and shear stress in a hindlimb model of arteriogenesis, which enhances evaluation possibilities notably (Lasch **et al**., 2021).

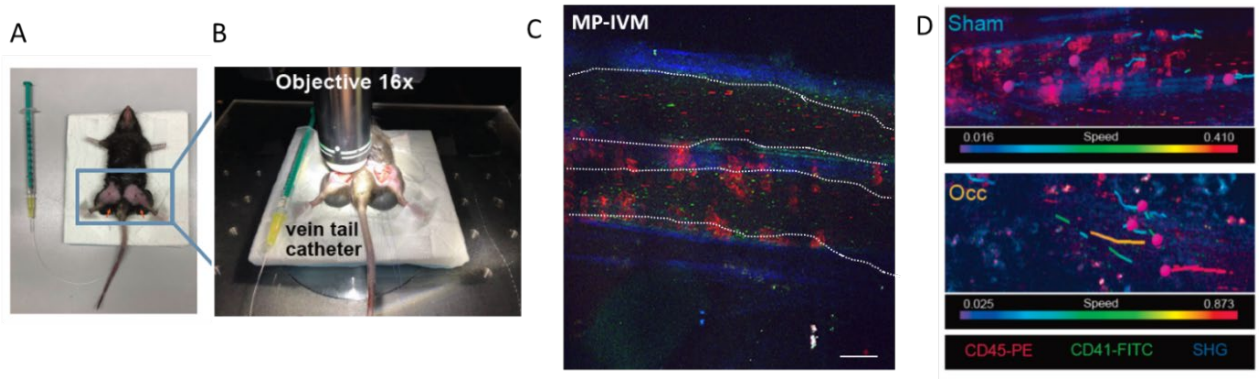

Figure 10. Multiphoton intravital imaging of a collateral artery using a femoral ligation model. (A) Mouse positioned in supine position with both hindlimbs placed on pieces of black modelling clay (prepared for imaging. (Occ) Occluded right hindlimb, and left, sham operated hindlimb were prepared for imaging. (B) Multiphoton microscope setup showing mouse placed inside the microscope chamber with the 16x objective touching the tissue covered with ultrasonic gel. (C) MP-IVM of the collateral arteries, leukocytes were labeled with injected CD45-PE antibodies (red), platelets were labelled by injected CD41-Fitc antibodies (green) and the collagen type 1 with the second harmonic generation (SHG) (blue). (D) Leukocyte speeds measured in collateral arteries of sham operated (Sham) and femoral artery ligated (Occ) hindlimbs. Representative images show the cells tracked (magenta) with the tracks color coded. The color code bar represents the cell speed with the slower cells shown by blue tracks and faster cells with red tracks. Scale bar is 50 µm.

The projects described here were beneficed with high resolution imaging MP-IVM of leukocyte trafficking *in vivo*. Thus, my contribution to these studies with the intravital imaging establishment resulted in a great dynamic visualization of immune cells trafficking in diverse contexts of inflammation, infection, and cancer progression in real time.

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The cooperative works are labelled as **et. al.,** and the first author works are labelled as **Ishikawa-Ankerhold** et. al.

6 Abreviations

ATP	Adenosine triphosphate
Cag A	Cytotoxin-associated gene A
cAMP	cyclic Adenosine mono-phosphate
CAR-T	Chimeric antigen receptor T cell
D. discoideum	*Dictyostelium discoideum*
FITC	Fluorescein isothiocyanate
FLAP	Fluorescence localization after photobleaching
FLIM	Fluorescence lifetime imaging microscopy
FLIP	Fluorescence loss in photobleaching
FPs	Fluorescent proteins
FRAP	Fluorescence recovery after photobleaching
FRET	Fluorescence resonance energy transfer
GFP	Green fluorescence protein
H. pylori	*Helicobacter pylori*
HFD	High-fat diet
IFN α	Interferon type I alpha
IVM	Intravital Microscopy
MK	Megakaryocyte
MP-IVM	Multiphoton Intravital Microscopy
PA	Photo-activatable
PA-RFP	Photo-activated red fluorescent protein
pDC	Plasmacytoid dendritic cell
pH	potential of hydrogen
SHG	Second harmonic generation
TIRF	Total Internal reflection fluorescence microscopy
TRITC	Tetramethylrodamine isothiocyanate
VAT	Visceral adipose tissue

7 List of publications / Schriftenverzeichnis

1. Originalarbeiten als Erst- oder Letztautor (Anzahl: 11, kumulativer Impact Factor 52,61):

Liu H, **Ishikawa-Ankerhold H***, Winterhalter J, Lorenz M, Vladymyrov M, Massberg S, Schulz C, and Orban M. (2023) Multiphoton In Vivo Microscopy of Embryonic Thrombopoiesis Reveals the Generation of Platelets through Budding. Cells 2023, 12(19), 2411; https://doi.org/10.3390/cells12192411

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Impact Factor: 6.00

Ishikawa-Ankerhold H, Kroll J, Heuvel DVD, Renkawitz J, Müller-Taubenberger A. (2022) Centrosome Positioning in Migrating Dictyostelium Cells. Cells. May 29;11(11):1776. doi: 10.3390/cells11111776.

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Lasch M, Vladymyrov M, van den Heuvel D, Götz P, Deindl E, **Ishikawa-Ankerhold H.** (2021) Multiphoton intravital imaging as a tool for monitoring leukocyte recruitment during arteriogenesis in vivo in a murine hindlimb model. *J Vis Exp.* 2021 Sep 30;(175).

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"You must make the choice, to take a chance,
If you want anything in life to change".