Aus dem Institut für Prophylaxe und Epidemiologie der Kreislaufkrankheiten

des Klinikums der Ludwig-Maximilians-Universität München

Direktor: Univ.-Prof. Dr. med. Christian Weber

Imaging hematopoietic cells in their native environments

Application of TPLSM to study HSCs and monocyte biology in Bone Marrow and Fat-Associated Lymphoid Clusters

Dissertation

zum Erwerb des Doktorgrades der Naturwissenschaften

an der Medizinischen Fakultät der

Ludwig-Maximilians-Universität zu München

Vorgelegt von

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aus

Varese

2019

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Tag der mündlichen Prüfung:	16.09.2020

Contents

List of abbreviations

3D	Three dimensions / -dimensional
ACKR1	Atypical chemokine receptor 1
ApoE	Apolipoprotein E
AT	Adipose tissue
BM	Bone marrow
C/EBP-B	CCAAT/enhancer binding protein beta
CAR	CXCL12 abundant reticular cells
CCR2	C-C motif chemokine receptor 2
CCR5	C-C motif chemokine receptor 5
CD	Cluster of differentiation
cDC	Classical dendritic cell
CLSM	Confocal laser scanning microscopy
CM	Classical monocyte
cMoP	Common monocyte progenitor
CMP	Common myeloid progenitor
CSFR1	Colony stimulating factor receptor 1
CX_3CR1	C-X_3-C motif chemokine receptor 1
CXCL12	C-X-C motif chemokine ligand 12
CXCR4	C-X-C motif chemokine receptor 4
DAMP	Danger associated molecular patterns
DC	Dendritic cell
Dll1	Delta like canonical Notch ligand 1
FALC	Fat-associated lymphoid cluster

FLT3	Fms related tyrosine kinase 3
G-CSF	Granulocyte colony stimulating factor
GMP	Granulocyte monocyte progenitor
HSC	Hematopoietic stem cell
HSPC	Hematopoietic stem and progenitor cell
IM	Intermediate monocyte
KIT	KIT proto-oncogene, receptor tyrosine kinase
LEPR	Leptin receptor
LFA1	Lymphocyte function-associated antigen 1
Ly6C	Lymphocyte antigen 6 complex
MDP	Monocyte and dendritic cell progenitor
MHC-II	Major histocompatibility complex II
MI	Myocardial infarction
MPP	Multipotent progenitor
NCM	Nonclassical monocyte
NEC	Nucleated erythroid cell
Nes	Nestin
NG-2	Neural/glial antigen 2
NIR	Near-infrared
NK	Natural killer
NR4A1	Nuclear receptor subfamily 4 group A member 1
PAMP	Pathogen-associated molecular patterns
PAT	Pericardial adipose tissue
PD-L1	Programmed cell death ligand 1
RNA	Ribonucleic acid

SCF	Stem cell factor
SHG	Second harmonic generation
SLAM	Signaling lymphocytic activation molecule
TLO	Tertiary lymphoid organ
TLR7	Toll-like receptor 7
TPLSM	Two-photon laser scanning microscopy
TpMo	Transitional pre-monocyte
Treg	T regulatory
TZ vessels	Transition zone vessels
UV	Ultraviolet
VLA4	Very late antigen 4

List of publications

- Publication I

Duchene J†, Novitzky-Basso I†, Thiriot A*, Casanova-Acebes M*, **Bianchini M*,[1]**, Etheridge SL*, Hub E*, Nitz K*, Artinger K, Eller K, Caamaño J, Rülicke T, Moss P, Megens RTA, von Andrian UH, Hidalgo A, Weber C, Rot A. *Atypical chemokine receptor 1 on nucleated erythroid cells regulates hematopoiesis.* **Nat Immunol**. 2017 Jul;18(7):753-761. DOI: https://doi.org/10.1038/ni.3763

- Publication II

Horckmans M, **Bianchini M[2]**, Santovito D, Megens RTA, Springael JY, Negri I, Vacca M, Di Eusanio M, Moschetta A, Weber C, Duchene J, Steffens S. *Pericardial Adipose Tissue Regulates Granulopoiesis, Fibrosis, and Cardiac Function After Myocardial Infarction.* **Circulation**. 2018 Feb 27;137(9):948-960. DOI: https://doi.org/10.1161/circulationaha.117.028833

- Publication III

Bianchini M*,[3], Duchêne J*, Santovito D, Schloss MJ, Evrard M, Winkels H, Aslani M, Mohanta SK, Horckmans M, Blanchet X, Lacy M, von Hundelshausen P, Atzler D, Habenicht A, Gerdes N, Pelisek J, Ng LG, Steffens S, Weber C†, Megens RTA†. *PD-L1 expression on nonclassical monocytes reveals their origin and immunoregulatory function*. **Sci Immunol**. 2019 Jun 21;4(36). pii:eaar3054. DOI: https://doi.org/10.1126/sciimmunol.aar3054

[1] * Joint second authors. All second authors contributed equally to this work by performing experiments and analyzing data. Mariaelvy Bianchini established and implemented TPLSM of HSC-NEC interactions in whole-mounted BM, performed spatial measurements and analyzed the resulting data.

[2] As second author, Mariaelvy Bianchini established and implemented TPLSM of lymphocytic populations of FALCs in whole PAT and of HSCs in whole-mounted BM.

[3] *Joint first authors. Mariaelvy Bianchini and Johan Duchêne share the first authorship of this work, as both authors designed the research, performed experiments, analyzed and evaluated data and wrote the manuscript.

1. Introduction

1.1. Imaging the immune system

1.1.1. Microscopic morphological and functional investigation of immune cells and tissues.

In all animal organisms, defense against pathogens and maintenance of tissue integrity are granted by a compartment of highly specialized sentinel cells that together form the immune system. These cells, termed leukocytes, circulate in the blood and eventually station at peripheral sites, such as lymph nodes, spleen, mucosae and skin, where they sense the presence of invading microbes and capture danger signals derived from damaged host tissues[1]. Following inflammatory insults, immune cells can exit these compartments and infiltrate the parenchyma of injured organs, for example the ischemic and necrotic regions of the heart after a myocardial infarction (MI)[2]. In the course of chronic inflammation, as in the case of cardiovascular disease[3] and many forms of tumors[4], B and T lymphocytes can also gather around the inflamed tissue and form organized structures, known as tertiary lymphoid organs (TLOs), where they mount a local long-standing adaptive response.

The first consistent evidences of leukocyte activation and killing of pathogens were achieved by the 1908 Nobel Laureates Elie Metchnikoff and Paul Ehrlich who, between the second half of the XIX and the beginning of the XX century, discovered the innate and adaptive branches of the immune system and described their cellular and humoral components[5]. In their experimental work, they pioneered the application of light microscopes and chemical cell-affine dyes to study the dynamic behavior of immune cells during inflammatory responses[6] and to specifically stain different classes of immune cells in frozen tissue sections[7]. Thanks to these techniques, they could identify macrophages and “microphages” (later re-named neutrophils) with phagocytic activity[8, 9], discover tissue-resident mast cells and classify different granulocyte subtypes[7], as well as describe phagocyte extravasation and recruitment to inflammatory sites[6].

Thus, elemental microscopy and histochemistry offered a unique contribution to the advent of modern immunology. Since Metchnikoff’s and Ehrlich’s work, our knowledge of different immune cell phenotypes, developmental pathways and functions under both physiological and pathological conditions has vastly expanded. However, microscopy has become only one of several tools that are nowadays used to study leukocyte plasticity and fate. In fact, most recent findings in the immunological field mainly rely on non-imaging methodologies, including multi-parametric and single-cell flow cytometry[10, 11], transcriptomics[12] and genetic fate mapping[13]. Although exceptionally powerful, these technologies lack the capacity to gather spatial information about inter-cellular relationships and interaction with the surrounding environment, which are essential aspects to

consider for a full understanding of immunological processes. Thus, microscopy still remains a pivotal means of investigation and is currently undergoing a new era of popularity, also prompted by major technological breakthrough in optics, molecular chemistry and biology and sample preparation[14]. On the one hand, methodologies and protocols for simultaneous labeling of multiple cell subsets have reached a high level of sophistication. The era of immunofluorescence started in the 1940-50s, with the development of fluorescently labeled antibodies directed against cell type-specific surface markers[15, 16]; many of these were then enlisted in the "cluster of differentiation" (CD) nomenclature protocol established in 1982 to universally immunophenotype leukocytes[17]. Nowadays, a range of various fluorescent molecules (fluorophores) with different spectral characteristics, intensity and stability can be covalently attached to both polyclonal and monoclonal antibodies, and combined into a variety of multicolor staining panels, *ad hoc* designed to readily pinpoint myeloid cells and lymphocytes inside tissues sections and whole organs. In addition, the development of numerous fluorescent dyes to target subcellular compartments in living cells[18] has enabled real-time imaging of *in vitro* cultured cells, viable explanted organs, or even *in vivo* applications. Moreover, reporter animal models (mostly mice) expressing fluorescent proteins and tags under the control of selected genes, have become increasingly sophisticated, allowing to selectively track the migration of immune cell subtypes under a variety of different stimuli *in vivo*[19, 20, 21, 22]. In parallel with the development of novel labeling strategies, the microscopy hardware has strongly evolved, enabling multicolor and multidimensional imaging with improved quality. Fluorescent widefield microscopes became used instruments in research laboratories between the 1970s and 1980s[23], when they allowed to perform bidimensional multi-color imaging of thin histological sections of hematopoietic and lymphatic organs[24, 25]. However, the quality of such images, which suffered from poor lateral resolution and out-of-focus fluorescence haze, was insufficient for the level of cellular and subcellular detail required to investigate immune responses in detail. A revolutionary improvement was the introduction of the first confocal microscope in 1955[26, 27]. Confocal laser-scanning microscopy (CLSM) consents to generate high resolution three-dimensional (3D) images, obtained by means of point-by-point scanning of highly focused laser sources on the sample. Elimination of out-of-focus fluorescence is achieved with a light-shielding filter, the *pinhole*, which is positioned at optimal distance from the focal plane of the objective lens. As a consequence, a collection of images solely consisting of in-focus information, can be stacked by shifting the focus along the z dimension. The final result is a high-quality multidimensional representation of complex sample architectures[28].

Nowadays, CLSM is still the "golden standard" for investigation of immune cell localization, activation and recruitment. However, imaging of many immunological events *in situ* can be challenging, since most tissues and organs measure several hundreds of μm in thickness when explanted for *ex vivo*

investigation and are located deeply in the body for *in vivo* imaging. Widefield and confocal microscopies do not provide the sufficient level of depth penetration and subcellular resolution, which is required for accurate visualization and analysis of such locations. Both modalities are typically limited to a maximum imaging depth of 50-80 μm inside a sample because ultraviolet (UV) and visible light is rapidly absorbed and scattered by tissues, irremediably compromising both lateral and axial resolution[28].

1.1.2. Two-photon microscopy: principles and applications in immunology.

In 1931 Maria Göppert-Mayer first theorized the phenomenon of two-photon absorption as an alternative way to bring a molecule to its excited state[29]. Based on her work, the first two-photon laser scanning microscope (TPLSM) was patented in 1990[30]. In TPLSM (also referred to as multiphoton microscopy) fluorochromes are excited within 10^{-18} s by two near-infrared (NIR) photons of ideally half the energy (wavelengths between 760 nm and 1200 nm) of the light normally required for linear single-photon excitation (**figure 1A**). Since the two-photon absorption spectra of most fluorochromes are rather broad, spanning over several hundreds of nm, two-photon microscopes are capable to excite various fluorescent molecules in the same sample simultaneously with just one wavelength. This is a noticeable advantage in terms of speed when performing multi-color imaging experiments in real time. Because long NIR wavelengths can penetrate deeper and up to a couple of mm in transparent tissues, most organs can be imaged *in situ* after minimal surgical exposure, granting high-resolution images at depths which are not accessible with conventional CLSM. In addition, the low energy carried by NIR photons consents to repeatedly scan the same volume with high frequency, yet causing only marginal photobleaching and photodamage when compared to single-photon excitation. Furthermore, because two-photon excitation is a non-linear phenomenon, the probability of a fluorochrome to be excited quadratically depends on the intensity (so the amount of photons) of the excitation pulse. Therefore two different photons need to be absorbed by the same fluorophore virtually at once, a rare event that can occur only in proximity of the focal plane of the objective lens that converges the exciting laser light (**figure 1B**). Consequently, optical sectioning of a sample is achieved without the presence of a pinhole, as for CLSM, since only one focal plane at a time is illuminated. This results in further reduction of light-induced bleaching and damage in the surrounding regions of the sample[31].

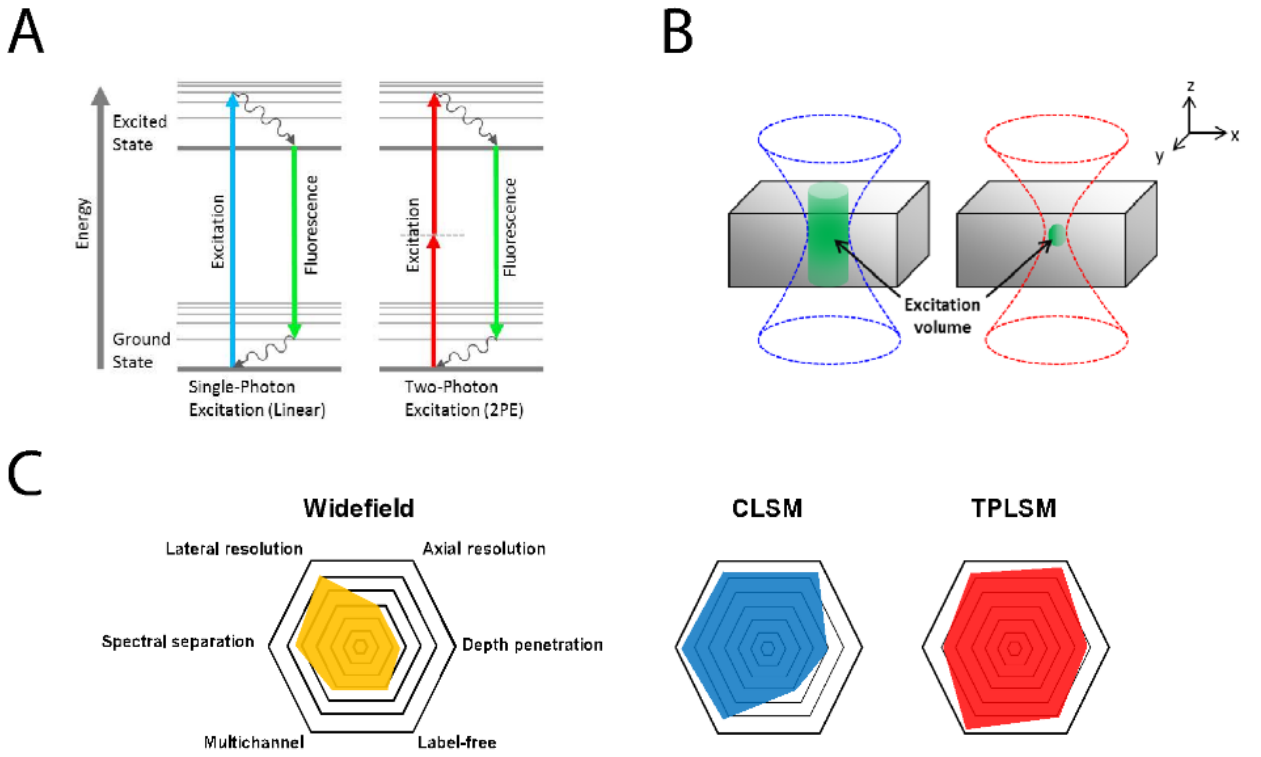

Figure 1: Principle and advantages of two-photon excitation.

(A) Jablonski diagrams comparing single-photon (as in CLSM) and two-photon excitation. In TPLSM, the energy carried by two nearly simultaneous photons of low-energy NIR light acts in an additive fashion and causes the fluorescence emission of fluorochromes. (B) Schematic representation of the excited volume (green region) in a 3D sample in CLSM (left) and TPLSM (right). In TPLSM only fluorochromes in the focal plane are reached by sufficiently high density of photons so that they can reach the excited state and emit fluorescence. Thus TPLSM allows to perform "confocal" imaging without the implementation of a *pinhole*. (C) Comparison among widefield microscopy, CLSM and TPLSM with regard to lateral and axial resolution, depth penetration, generation of label-free information, simultaneous acquisition of multiple fluorescent channels and capacity to separate overlapping emission spectra. TPLSM offers the best compromise among all characteristics; it is particularly advantageous for visualizing thick and deep-located tissues, at high resolution while collecting both label-derived and label-free fluorescence.

An additional technological advance of TPLSM imaging is that it permits the observation of several biological structures in a label-free setting. In fact, the majority of autofluorescent biological molecules also possess a two-photon excitation spectrum and can be excited in this modality[32]. In addition, non-centrosymmetric molecules, like fibrillar collagens (type I, II, III, V and XI) and elastin, that constitute the extra-cellular matrix of many mammalian tissues, have the characteristic of scattering light photons with precise geometric angles thanks to the periodicity of their aminoacidic chains. As a result, two scattered NIR photons can recombine into one with exactly half the wavelength (so with double energy), a physical phenomenon known as "second harmonic generation" (SHG)[33](**figure 2A**). By collecting the newly produced "blue" photons in a dedicated

detection channel, it is possible to visualize, among others, bone tissue, collagen capsules and the adventitial tunica of big vessels in a label-free fashion (**figure 2B**). Hence, the combination of exogenous targeted labelling combined with both autofluorescence and SHG adds significant morphological information facilitating spatial orientation and reconstruction of complex tissue architecture.

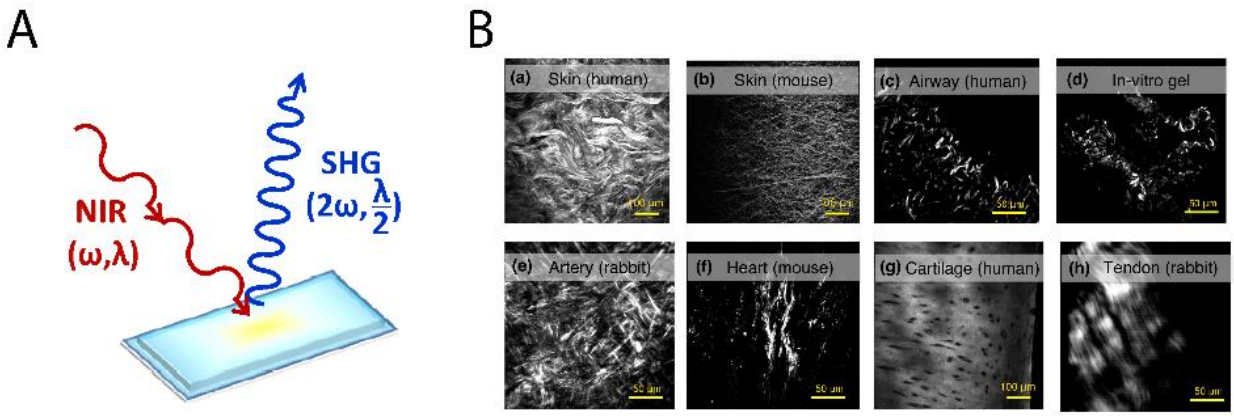

Figure 2: Label-free imaging of tissues through intrinsic SHG.

(A) Representation of the SHG principle. Two-photon excitation of non-centrosymmetric structures, like some tissue collagens, results in the scattering and recombination of the NIR-photon pair into a single photon with twice the energy (ω) and half the wavelength (λ) used to excite the sample. Detection of this SHG signal contributes to the formation of a label-free image of the generating tissue. (B) Examples of collagen bundles deposited in different types of tissues from various species and visualized via SHG. Images in panel B are reprinted from[34]; no permission required.

Altogether, TPLSM offers unique technical features that allow spatiotemporal and functional investigation of processes in both innate and adaptive immunity. Its applications have so far included measuring the migratory behavior of circulating neutrophils[19] and monocytes[35] during infection or sterile inflammation, portraying anti-tumoral and anti-metastatic activity[36, 37, 38] of different leukocyte subtypes and studying lymphocyte and dendritic cell (DC) interplay inside activated lymphoid organs[39]. In addition, TPLSM imaging is a relevant tool to image immune cell production[40], release from and homing back to[41] the bone marrow (BM) both *ex vivo* and *in vivo*. Continuous methodological improvements will further contribute to its attractiveness, making it a state-of-the-art tool for the realization of increasingly complex immunological studies.

1.2. Visualizing immune cell production

1.2.1. Hematopoiesis

A research field that greatly benefited from the technical advances of fluorescent microscopy is the study of hematopoiesis, i.e. the developmental process which leads to the production and differentiation of blood and immune cells[42]. The currently accepted model of hematopoiesis establishes that undifferentiated pluripotent hematopoietic stem cells, which reside in the yolk sac and liver during embryonic and fetal life, and in the BM after birth, give rise to lineage-restricted progenitors which, in turn, differentiate into erythrocytes, platelets and all leukocytes[43] (**figure 3**). Together with their differentiation activity, hematopoietic stem cells (HSCs) are also characterized by the ability to self-renew, for maintaining themselves throughout the whole life on an individual[44]. By contrast, HSC-derived multipotent progenitors (MPPs) and other downstream progenitors progressively show higher degree of commitment and decreased self-renewal capabilities. Such progression, which continuously replenishes the different hematopoietic compartments in the steady state, is strictly regulated by both hematopoietic stem and progenitor cell (HSPC)-intrinsic and -extrinsic factors[45]. Moreover, it becomes highly modulable during physiological aging[46], as well as in the course of inflammation, infections and malignancies[47]. In addition, it constitutes the foundation of therapeutic BM transplantation for the cure of some genetic immune-deficiencies and hematopoietic neoplasia[48]. Thus, it is of crucial importance to understand the cellular and molecular cues driving HSPC behavior.

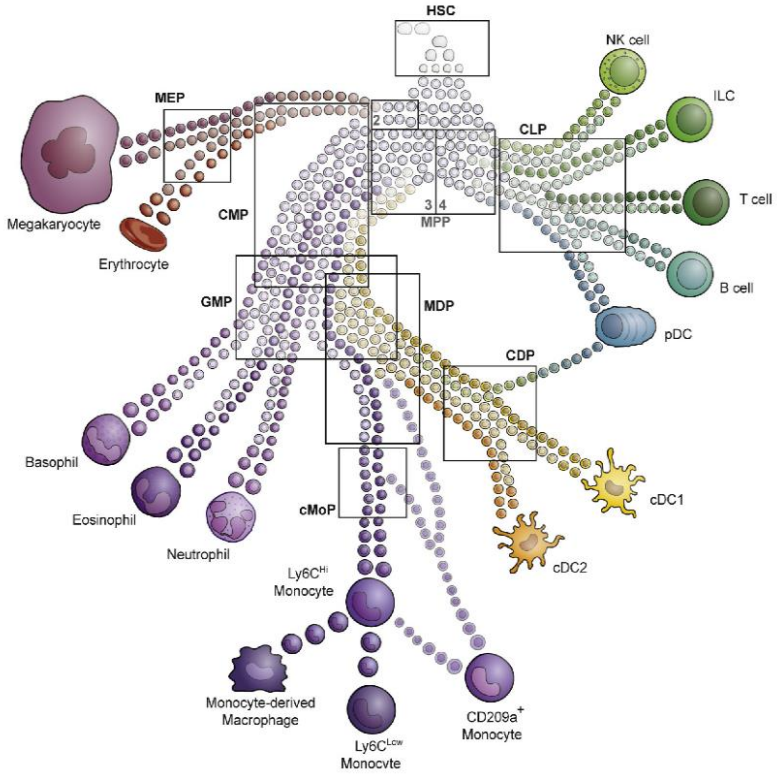

Figure 3: The hematopoietic system in the steady-state

Representation of hematopoiesis according to the most recent single-cell analysis studies, which have highlighted the presence of several pre-committed subsets within the commonly known progenitors and precursors (indicated by the squares). Few HSCs constitute the vertex of the whole system, while lineage differentiation already starts with subpopulations of MPPs, each more committed to give rise to a specific progeny. Perturbations of general hematopoietic balance progressively occur during individual's ageing or in the course of acute and chronic pathological events. Reprinted from[49] with permission from Elsevier.

1.2.2. The hematopoietic niche: insights from BM imaging studies

Thanks to recent imaging studies, it has become particularly evident that hematopoiesis is a highly spatially-regulated phenomenon. That is, the balance between HSC quiescence and activation is maintained (or altered) mainly in response to cellular and molecular cues in their immediate vicinity, a specialized microenvironment known as the "hematopoietic niche" [50]. Although the niche concept dates back to 1978 [51], when it was initially proposed, insights into its components and complexity have rapidly increased in the past decade. In fact, identification of real HSCs in the BM (hence the possibility to put them in relation to other cells and structures) in the past was mostly hampered by lack of specific markers and required complex panels of surface molecules not easily translatable to microscopy. In 2005, the SLAM family[51] of markers was proposed, that allowed to discriminate cells almost exclusively enriched in HSC activity as to be $CD48^{-}$ $CD150^{+}$ [52]. This discovery greatly facilitated successful visualization of antibody-labeled HSCs with only two colors. Moreover, due to RNA sequencing and single-cell analysis, some other genes have by now been identified which are (almost) exclusively expressed by HSCs at high level, consenting the creation of different fluorescent reporter mouse lines (**table 1**).

Mouse strain	Genetic modification	Specificity within the adult haematopoietic compartment	Analysis
Hoxb5–Tri-mCherry	Knock-in	Specific to long-term HSCs	• Modified CUBIC clearing and light- sheet microscopy • Flow cytometry • Transplantation
Ctnnal1–GFP	Knock-in	Restricted to HSCs and haematopoietic progenitors; requires KIT staining to enrich for HSCs	• Modified Murray's clearing, immunostaining and confocal and multiphoton microscopy • Flow cytometry • Transplantation
Fgd5–mCherry	Knock-in	Restricted to HSCs; low expression in haematopoietic progenitors	• Flow cytometry • Transplantation
Vwf–GFP	Transgenic	Labels platelet-biased and myeloid-biased HSCs, megakaryocyte progenitors, megakaryocytes and platelets	• Flow cytometry • Transplantation • Immunostaining and wholemount confocal microscopy
Msi2–GFP	Knock-in	Labels haematopoietic progenitors	• Confocal microscopy • Flow cytometry
Pdzk1ip1–GFP	Transgenic	Enriches for highly purified HSCs but also labels a small subpopulation of haematopoietic progenitors and mature granulocytes	• Doxycycline chase • Transplantation • Flow cytometry
Evi1–GFP	Knock-in	Labels haematopoietic progenitors	• Flow cytometry • Transplantation
Scl–tTA-H2B–GFP	Transgenic	$H2B–GFP^{high}$ label-retaining cells are enriched in quiescent long-term HSCs; also labels haematopoietic progenitors	• Doxycycline chase • Transplantation • Flow cytometry • Immunostaining and confocal microscopy
Tie2–GFP	Transgenic	Enriches for highly purified HSCs but also labels haematopoietic progenitors	• Flow cytometry • Transplantation • Immunostaining and wholemount confocal and multiphoton microscopy
Gprc5c–GFP	Transgenic	Enriches for dormant HSCs but also labels haematopoietic progenitors	• Flow cytometry • Transplantation

Hdc–GFP	Transgenic	Enriches for myeloid-biased HSCs but also labels haematopoietic progenitors and mature myeloid cells	• Flow cytometry • Transplantation • Immunostaining and confocal microscopy
Krt7–GFP	Knock-in	Specific to HSCs	Flow cytometry
Gata2–GFP	Knock-in	Enriches for HSCs and haematopoietic progenitors; SCA1 or lineage staining is required for HSC selectivity	• Flow cytometry • Transplantation • Immunostaining and microscopy
Hoxb4–YFP	Knock-in	Labels haematopoietic progenitors	• Flow cytometry • Transplantation

Ctnnal1, encodes α-catulin; CUBIC, clear, unobstructed brain imaging cocktails and computational analysis; Evi1, ecotropic virus integration site 1 homologue; Fgd5, encodes FYVE, RHOGEF and PH domain- containing 5; Gata2, encodes GATA-binding 2; Gprc5c, encodes G-protein coupled receptor family C group 5 member C; H2B, histone H2B; Hdc, encodes histidine decarboxylase; HSCs, haematopoietic stem cells; HSPCs, haematopoietic stem and progenitor cells; Hoxb5 encodes homeobox b5;Krt7, encodes keratin 7; Msi2, encodes Musashi 2; Pdzk1ip1, encodes PDZK1-interacting 1; SCA1, stem cell antigen 1; Scl, stem cell leukaemia; Tie2, encodes tyrosine kinase with immunoglobulin and EGF homology domains 2; tTA,tetracycline-controlled transactivator protein; vWF, encodes von Willebrand factor.

Table 1: Reporter mice for HSC/HSPC identification

List of reporter mice developed for specific identification of HSCs and HSPCs. Note that the majority of models have already been utilized for optical microscopic applications. Reprinted by permission from Springer Nature[53].

The combination of these newly developed labeling strategies with advanced *in situ* deep fluorescence microscopy (CLSM and TPLSM), in some cases also benefiting from more or less sophisticated sample-clearing methods[54, 55], has finally allowed to gather important, although still preliminary, information regarding HSC interaction with multiple niche components. In this regard, a pivotal role is played by BM stromal cells, described with different names and markers (for instance CXCL12 abundant reticular (CAR) cells[56], also defined as leptin receptor $(LEPR)^+$ cells[57]). They are the major source of BM-derived CXCL12 and stem cell factor (SCF), the two most important regulators of HSPC maintenance and retention, by acting on HSPC-expressed receptors CXCR4[58] and KIT[59], respectively. Several recent studies have shown that HSCs establish close contacts also with the endothelial cells of BM blood vessels, sinusoids and arterioles. Indeed, the great majority of HSCs is found in perisinusoidal regions, in a quiescent and hypoxic state[52, 54, 60]. However, a minor fraction (about 10%) of HSCs are also localized in proximity of arterioles and some other studies have pointed out the role of peri-arteriolar nestin $(Nes)^{+}$[61] and neural/glial antigen 2 $(NG\text{-}2)^{+}$[62] cells in the maintenance of the niche. Even if the respective contribution of sinusoids and arterioles and their perivascular cells is still a matter of debate, it is nonetheless clear that the hematopoietic niche is primarily a vascular niche. In support of this, the direct role of endothelial cells (both arteriolar and sinusoidal) on HSCs has also been investigated and mechanistic evidences of their involvement in HSCs biology has been proved[63], with special contribution of the endothelial Notch-derived signals[64]. Nevertheless, several evidences derived from additional imaging experimental models, have also pinpointed the importance of other cells of both hematopoietic and non-hematopoietic origins, including megakaryocytes[65], T regulatorary (Treg) lymphocytes[66], adipocytes[67], osteoblasts[68] and sympathetic neurons[69] whose fibers run alongside BM arterioles (**figure 4**). At present the exact role of each of these actors still remains controversial, as they have been shown to affect HSC biology

in sometimes contrasting ways, depending on the model and experimental settings, in some cases promoting HSC expansion and, in some others, leading to their exhaustion[54, 57, 70]. Moreover, important regulators of either HSCs or the aforementioned niche players also include long-distance signals such as secreted granulocyte-colony stimulating factor (G-CSF), a potent inducer of HSC release from the BM and myeloid cell production[71, 72].

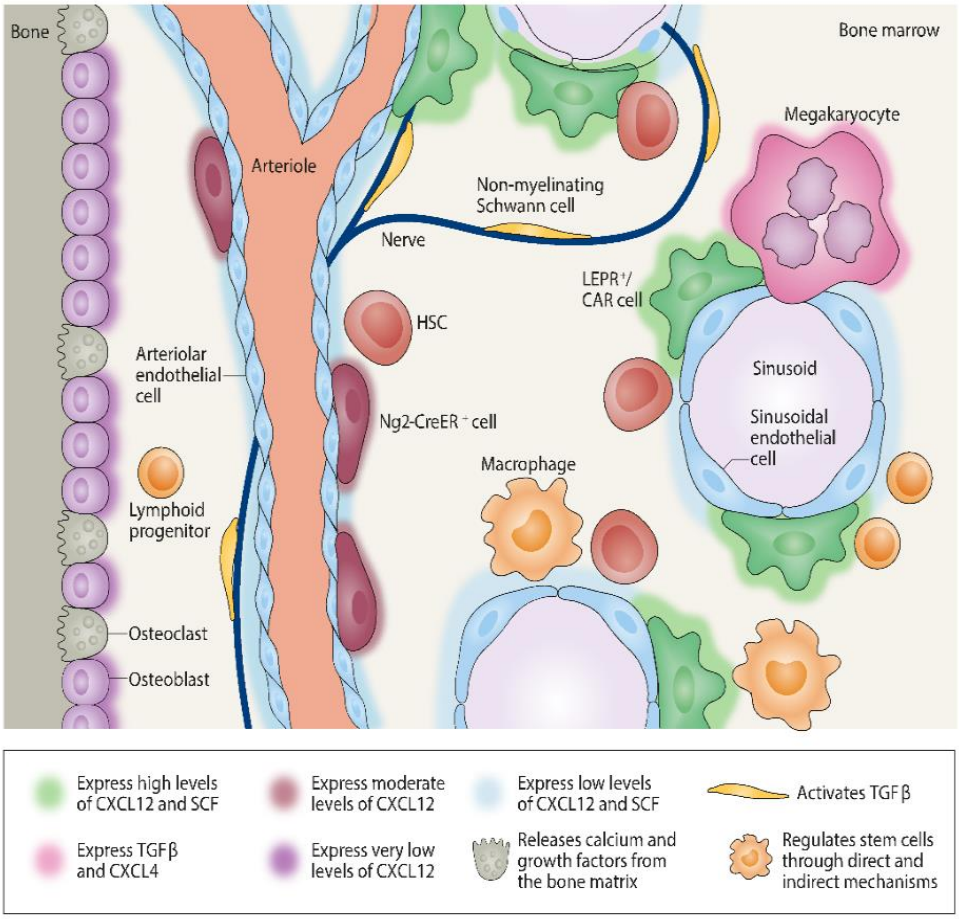

Figure 4: Identified components of the hematopoietic niche

Schematic depiction of the main niche cell types. To some of them, contrasting roles and regulatory functions on the activity of HSCs have been attributed in different studies and models. With the exception of osteo-lineage cells, for which a role in the maintenance of HSC viability and self-renewal had initially been proposed (now restricted solely to maintenance of the lymphoid lineage[73]), all other niche cells locate in proximity of central BM sinusoids and arterioles. Hematopoiesis is thus believed to be a vasculature-associated process. Reprinted by permission from Springer Nature[50].

It has been recently shown that the major contribution to the various hematopoietic lineages in the steady state is derived from the active proliferation of more differentiated progenitors[74, 75], while HSCs mostly rest in an inactive state and get activated only after major BM insults[76, 77, 78].

Unfortunately, to date little effort has been made to image hematopoietic progenitor cells downstream of HSCs[79], although recent work has characterized the *in situ* step-wise production of subpopulations of leukocytes, namely neutrophils, under both healthy and inflammatory conditions[80]. However many questions regarding physiological and pathological production of other specific hematopoietic subsets still await to be answered.

1.3. Investigating monocyte development and activity

1.3.1. Developmental and phenotypical aspects of monocytes

In healthy individuals, monocytes arise from HSCs in the fetal liver and in the BM during embryonic and adult hematopoiesis, respectively. Production in the spleen has also been observed in mice in the course of inflammatory responses[81]. In BM, the earliest CD115$^+$ monocyte-committed progenitor described is the monocyte and dendritic cell progenitor (MDP)[82, 83], which derives from the common myeloid progenitor (CMP) and/or the granulocyte monocyte progenitor (GMP)[84] (although their existence as a homogeneous entity has been recently questioned in both mice[85] and humans[86]). MDPs are characterized by high expression of KIT/CD117 and FLT3/CD135, and low expression of Ly6C. *In vivo*, these cells can generate both cDCs and monocytes. In particular, they give rise to a CD115 (CSFR1)$^+$ monocyte-restricted progenitor called common monocyte progenitor (cMoP)[87], which differs from MDPs for its lack of FLT3 expression while being Ly6C$^+$. cMoPs have long been considered direct precursors of mature monocytes. However, a BM-resident population of immature monocytes has been recently described and named “transitional pre-monocytes” (TpMos)[88]. These cells permanently reside in the BM due to high expression of the chemokine receptor CXCR4 (which characterizes all BM-resident hematopoietic cells) and constitute a readily-available source of monocytes which proliferate and are mobilized upon downregulation of CXCR4 in response to inflammatory stimuli. In summary, some myeloid-committed early progenitors sequentially differentiate into MDPs, cMoPs and TpMos prior to generating mature monocytes (**figure 3**).

In both mice[89] and humans[90] circulating and BM monocytes comprise two main distinct subsets, known as classical and nonclassical monocytes (CMs and NCMs). In flow cytometry analysis, CMs and NCMs in mice are distinguished on the basis of the membrane expression of Ly6C, with CMs being Ly6C^{high} and NCMs being Ly6C^{low}. Monocytes characterized by intermediate expression of Ly6C are considered to be a transitional developmental stage between CMs and NCMs and are called intermediate monocytes (IMs)[91]. In addition, CMs harbor high levels of the chemokine receptor CCR2, and moderate amounts of the chemokine receptor CX_3CR1. Conversely, NCMs are CCR2-negative, while expressing the highest levels of CX_3CR1. In human blood, CMs and NCMs maintain the same dichotomy as observed in mice with regard to CCR2 and CX_3CR1 expression[92], but are

otherwise distinguishable on the basis of the differential levels of the Fc-receptors CD14 and CD16. In particular, human CMs are $CD14^{high}$ $CD16^{-}$ $CCR2^{high}$ CX_3CR1^{+}, whereas human NCMs are $CD14^{low}$ $CD16^{+}$ $CCR2^{low}$ CX_3CR1^{high}. Furthermore, transitional IMs are characterized as $CD14^{+}$ $CD16^{+}$. Hence, mouse CMs and NCMs share several phenotypical, as well as functional, aspects with human CMs and NCMs so that both subsets can be generally related between the two species. However, gene expression profiling of human and mouse monocytes also indicated that a number of differences exist and that some genetic programs of a particular subset are species-specific[92]. A major difference between the two species is the respective subset ratio in the blood of healthy mice or humans, respectively. While CMs and NCMs are equally distributed in the mouse circulation, about 80% of human monocytes are CMs, with the remaining 20% being further shared between IMs and NCMs, possibly reflecting species-specific variations in the control of monocyte production rate, life-span and clearance. Indeed, based on pulse-labeling and fate-mapping experiments, it has been calculated that human circulating NCMs survive for approximately 7 days[93], whereas their half-life is only 2 days in mice, although it can be extended depending on the environmental conditions[94].

In the experimental setting of *in vitro* colony forming assays, MDPs have been shown to produce both circulating CMs and NCMs, albeit with different kinetics. As an *in vivo* confirmation, adoptively transplanted MDPs and cMoPs gave rise to CMs within 1 to 4 days after transfer, and to NCMs between 3 and 6 days, in PB, BM and spleen[87]. These results suggested that either MDPs (and MDP-derived cMoPs) were in fact a heterogeneous mixture of different CM- and NCM-biased precursors, or that NCMs constituted a more terminally differentiated stage of monocyte development, following CMs. Fate-mapping tracing and single-cell transcriptomic analysis of monocyte subsets successively revealed that indeed CMs can convert into NCMs[94], while transiently giving rise also to IMs, and that this process is strongly dependent on the expression and activity of the transcription factors C/EBP-B and NR4A1, since both *Cebpb*- and *Nr4a1*-deficient mice lack NCMs from their circulation[91, 95, 96]. Although these studies have concluded that monocyte conversion is a blood-borne phenomenon, other investigations provided convincing evidence that CMs can turn into NCMs also in the BM and spleen[97, 98]. Specifically in the spleen, NCM production has been linked to a Notch-2-dependent mechanism triggered by Dll1 (a Notch ligand) expression on splenic sinusoids[95]. However, the mechanism regulating monocyte conversion in the BM has not been investigated so far.

1.3.2. Monocyte functions in health and disease

For decades circulating monocytes have been regarded exclusively as a transitional developmental state between immature BM precursors and activated tissue macrophages. However, the discovery

of multiple monocyte subsets together with the advent of more sophisticated investigative tools contributed to substantially revise the role of monocytes under both healthy and inflammatory conditions, uncovering new and complex aspects of monocyte behavior as well as re-assigning to monocytes functional characteristics, which were previously believed to exclusively belong to macrophages and cDCs[49] (**figure 5**).

CMs are also commonly known as "inflammatory" monocytes since they used to, and partially still do, represent a paradigm for flow-dependent circulating cells which roll on and adhere to activated endothelial surfaces at sites of inflammation in response to soluble cytokines secreted by injured parenchymal cells, endothelial cells, other leukocytes and platelets; thereafter, they transmigrate into inflamed tissues following chemokine gradients that mainly involve their surface receptors CCR2, CCR5 and CX_3CR1[99, 100]. Once entering the inflammatory milieu, CMs can undergo various destinies depending on the specific environmental conditions of the infiltrated tissue/organ, the microbial status and the secretion of pro- versus anti-inflammatory mediators from by-standing immune and stromal cells. While it is still true that CMs can develop into *bona fide* macrophages and cDCs by fully activating distinctive genetic programs, it is now becoming clear that they can also maintain a monocytic status and only acquire some functional characteristics and markers (up-regulation of Cd11c and MHC-II) that make them act and phenotypically resemble tissue phagocytes. In either case, CMs exhibit a wide range of plasticity, performing numerous pro-inflammatory tasks which span from pathogen killing and cytotoxicity, antigen presentation, T cell priming to anti-inflammatory and reparative pro-fibrotic and pro-angiogenic functions[49].

Alternatively, steady-state CMs can also turn into NCMs, which have been first described over a decade ago as blood-resident leukocytes that crawl on the luminal surface of microcirculatory blood vessels in a flow-independent fashion in search of endothelial damage cues[101, 102, 103]. This ability to monitor the healthy status of the microvasculature has gained them the definition of "patrolling" or "sentinel" monocytes. From a molecular point of view, NCMs peculiar motility relies on the integrins LFA1 and VLA4, but not on CX_3CR1. Moreover, NCMs can sense specific pathogen-/danger-associated molecular patterns (PAMPs and DAMPs) that activate TLR7, and respond by secreting neutrophil-attracting mediators to promote neutrophil recruitment to the site of injury, where they also contribute to the disposal of cell debris after pathogen clearance. More recently, NCMs have been observed in the lumen of big early atherosclerotic arteries of ApoE-deficient mice, where their patrolling behavior was enhanced by diet-induced hypercholesterolemia[104]. As a consequence, depletion of NCMs increased atherosclerotic severity and plaque burden[105]. A role for NCMs in atherosclerosis had been previously proposed in more advanced stages of plaque formation, and involved CCR5-dependent recruitment of NCM inside the lesions[99]. Nevertheless, the capacity of NCMs to extravasate and infiltrate tissues remains controversial although demonstrated in some

contexts, e.g. in the injured myocardium during the latest inflammatory stages of MI[106]. Finally, NCMs have also been reported to be part of the pro-tumoral milieu in the lung metastatic niche via inhibition of the cytotoxic activity of natural killer (NK) cells[106]. Thus, while several mechanisms of action have been proposed for NCMs in both health and disease, many aspects of their biology still remain enigmatic, largely due to the lack of an unambiguous methodology to specifically visualize NCMs in their physiological environment.

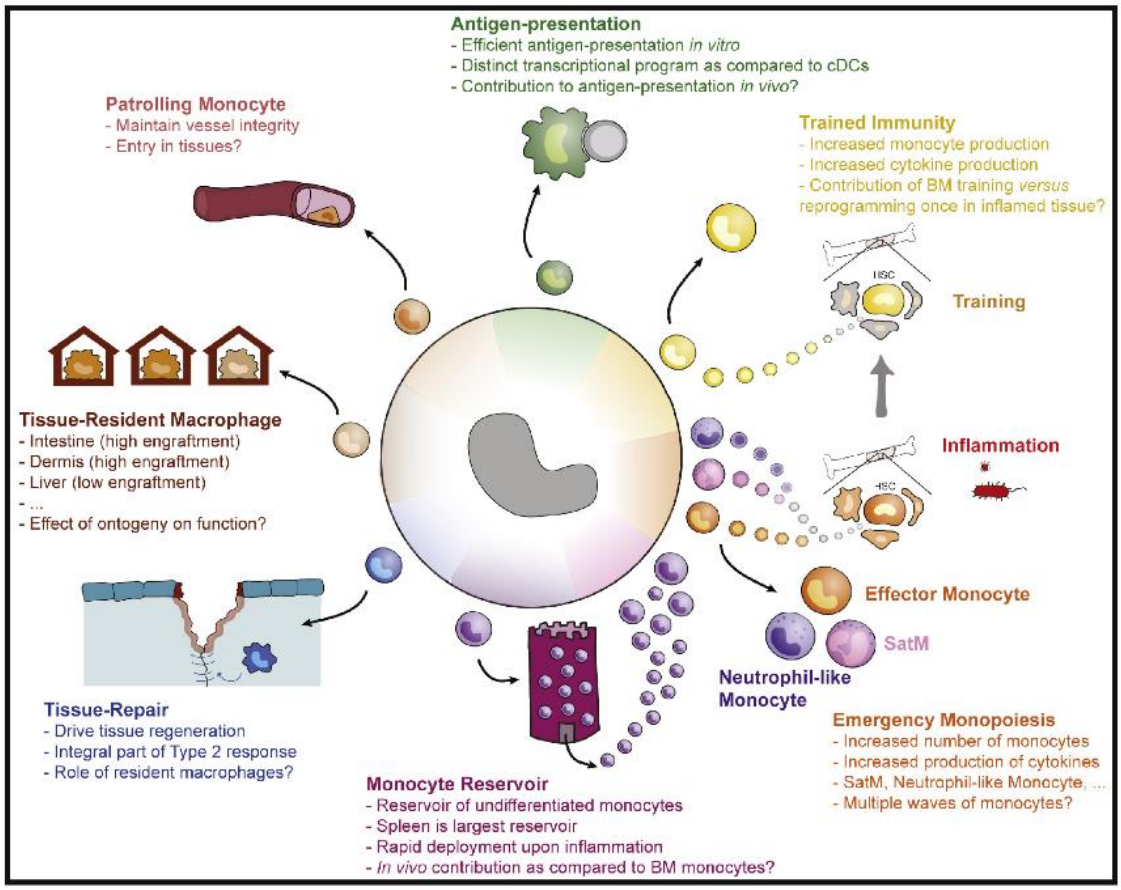

Figure 5: Diversity of monocyte functions

Schematic representation of monocyte roles and fate in healthy and diseased states. CMs can either function as precursors of tissue macrophages and DCs or maintain an essentially distinct genetic program, while exerting tissue-repair and antigen-presenting activities. Alternatively, they can give rise to new subtypes of effector monocytes with emergency functions or convert in vessel-patrolling NCMs. Reprinted from[49] with permission from Elsevier.

1.4. Aim of the thesis.

The current thesis presents how *in situ* TPLSM imaging of hematopoietic cells in their native environments can be implemented to unveil novel aspects of their development, interactions and function in steady state and inflammation. In particular, the three studies included here aimed to:

- develop a strategy to label and visualize HSCs in whole-mounted BM in the steady state, in mice with different genetic background in order to analyze their mutual spatial relationship with other BM-resident cells, namely nucleated erythroid cells (NECs) (study 1);
- visualize the effects of myocardial infarction (MI) on HSCs in the BM and lymphoid populations in fat-associated lymphoid clusters (FALCs) within the pericardial fat (study 2);
- identify a novel marker to selectivity label and track NCMs in blood and tissues, uncovering the spatiotemporal features of physiological monocyte conversion in the BM and the role of extra-vascular NCMs in MI-activated FALCs (study 3).

2. Publications

2.1. Publication I

Duchene J†, Novitzky-Basso I†, Thiriot A*, Casanova-Acebes M*, **Bianchini M***[4] , Etheridge SL*, Hub E*, Nitz K*, Artinger K, Eller K, Caamaño J, Rülicke T, Moss P, Megens RTA, von Andrian UH, Hidalgo A, Weber C, Rot A. *Atypical chemokine receptor 1 on nucleated erythroid cells regulates hematopoiesis.* **Nat Immunol**. 2017 Jul;18(7):753-761. DOI: https://doi.org/10.1038/ni.3763

2.1.1. Regulation of the HSC niche by erythroid cells in the BM.

Background:

Nucleated erythroid cells (NECs) are the precursors of circulating erythrocytes and constitute the majority of all hematopoietic cells in the BM. However, their contribution to BM homeostasis and in particular to hematopoiesis is unknown. The hematopoietic niche is controlled by several molecular cues including chemokines and their cognate receptors, of which the best described are CXCL12 and CXCR4. Cells of the erythroid lineage, along with some blood vessels in the BM, are characterized by the expression of ACKR1, an atypical chemokine receptor, which acts as a molecular sink and transcellular transporter for more than twenty chemokines. Interestingly, the rs2814778(G) polymorphism in the *Ackr1* gene leads to the absence of this receptor specifically from erythroid cells in individuals of African ancestry, as well as being strongly associated with benign neutropenia, although the molecular mechanisms of this phenotype are not yet understood.

Aim:

Investigating the role of ACKR1 on NECs as a possible modulator of hematopoiesis.

Key findings:

- HSCs and NECs can be visualized in whole-mounted BM as $CD150^+$ Lin^- $CD48^-$ $CD41^-$ and $CD71^+$ cells, respectively, based on the expression of these markers on the cell surface.
- In *Ackr1*$^{-/-}$ mice, HSCs interact less with NECs and are located at further distances.
- NECs regulate hematopoiesis via physical interactions with HSCs depending on ACKR1.

[4] Contribution as co-second author:
- Implementation of a labeling strategy to visualize HSCs and NECs with TPLSM in whole-mounted BM specimens (Supplementary fig. 7 A-B).
- Measurement of minimal distances between HSCs and NECs in 3D (Supplementary fig. 7C).
- Quantification of HSC-NEC interactions and comparison between *Ackr1*$^{+/+}$ and *Ackr1*$^{-/-}$ mice (Fig. 6 A-C).

2.2. Publication II

Horckmans M, **Bianchini M**[5], Santovito D, Megens RTA, Springael JY, Negri I, Vacca M, Di Eusanio M, Moschetta A, Weber C, Duchene J, Steffens S. *Pericardial Adipose Tissue Regulates Granulopoiesis, Fibrosis, and Cardiac Function After Myocardial Infarction.* **Circulation**. 2018 Feb 27;137(9):948-960. DOI: https://doi.org/10.1161/circulationaha.117.028833

2.2.1. Role of pericardial FALCs as regulators of post-MI hematopoiesis and immune response.

Background:

Cardiac ischemia and infarction result in the massive necrosis of cardiomyocytes with consequential impairment of cardiac function and systemic repercussions on the homeostasis of the whole organism. Locally, the release of pro-inflammatory mediators from the injured tissue promotes the onset of an acute inflammatory response, which eventually initiates post-MI cardiac repair and the formation of a fibrotic scar replacing the damaged muscle. Such response involves a massive recruitment of inflammatory innate cells during the initial phases of the disease and of B and T lymphocytes at later stages. However, recruitment of adaptive immune cells from the circulation is also observed within hours after the onset of MI. The epicardial AT in humans and the PAT in mice, which are located in proximity of the infarcted area, contain numerous FALCs, arising the hypothesis of a potential involvement of FALCs in the immune response to MI.

Aim:

Evaluating how MI affects cell dynamics in FALCs and hematopoiesis in the BM.

Key findings:

- B and T cells are visualized in whole-mounted PAT by surface expression of the markers B220 and CD3, respectively, and are exclusively clustered in FALCs.
- Both B and T cells substantially expand in FALCs after MI and are more numerous in knockout mice with increased basal numbers of peripheral B cells.
- Pericardial FALC B cell activation is linked to enhanced HSC expansion in the BM, both in mice with basally elevated B cell counts as well as in response to MI.

[5] Contribution as second co-author:

- Implementation of B and T cell imaging with TPLSM in whole-mounted PAT in a mouse model of MI (Fig. 2D and 4A).
- Comparative visualization of FALCs between mice with physiological and increased basal B cell burden before and after MI (Fig. 2D and 4A).
- Comparative TPLSM imaging of HSCs in whole-mounted BM specimens between mice with physiological and increased basal B cell burden after MI (Fig. 5D and Supplementary fig. 6B).

2.3. Publication III

Bianchini M*[6] , Duchêne J*, Santovito D, Schloss MJ, Evrard M, Winkels H, Aslani M, Mohanta SK, Horckmans M, Blanchet X, Lacy M, von Hundelshausen P, Atzler D, Habenicht A, Gerdes N, Pelisek J, Ng LG, Steffens S, Weber C†, Megens RTA†. *PD-L1 expression on nonclassical monocytes reveals their origin and immunoregulatory function.* **Sci Immunol**. 2019 Jun 21;4(36). pii:eaar3054. DOI: https://doi.org/10.1126/sciimmunol.aar3054

2.3.1. A NCM marker for imaging studies of monocyte conversion and NCM function.

Background:

NCMs are a subset of leukocytes residing in the peripheral circulation, where they crawl on the luminal surface of blood vessels to sense early endothelial damage. While reporter *$Cx_3cr1^{+/gfp}$* mice have been instrumental to initially characterize the patrolling behavior of NCMs, this chemokine receptor is expressed by the whole monocyte-macrophage compartment, thus requiring the application of a broader panel of markers to distinguish individual cell populations as commonly performed in flow cytometry experiments. However, optical fluorescence microscopy relies on a more limited number of detection channels, making it particularly challenging to transfer the same panels utilized in flow cytometry to imaging technologies. As such, there is a strong need to identify a NCM-restricted marker for discrimination of these cells in complex tissues, when simultaneous visualization of multiple cell types and structures is demanded.

Aim:

Identification, validation and application of a novel positive surface marker for microscopic studies of NCM monocyte biology.

Key findings:

- *Programmed cell death 1 ligand 1* (PD-L1) marks NCMs *ex vivo* and *in vivo* in blood and BM both under steady-state and inflammatory conditions (MI model).
- $PD\text{-}L1^-$ CM-to- $PD\text{-}L1^+$ NCM conversion in the BM is associated to a specific vascular niche and altered during ageing.
- After MI, NCMs accumulate in pericardial FALCs where they negatively regulate T cell survival via PD-L1.

[6] Contribution as co-first author:
- Study design.
- Flow cytometry validation of PD-L1 expression in blood and BM.
- Microscopic imaging of NCMs and their environment in blood, BM and PAT in different mouse models.
- Data quantification, analysis and interpretation.
- Manuscript writing.

3. Summary

TPLSM is a powerful imaging tool to investigate the spatial and temporal aspects of immune cell development, organization and functions under a variety of physiological and pathological conditions. By generating multicolor images of virtually unaltered tissues with subcellular resolution at considerable imaging depths, it provides both architectural and mechanistic insights into immunological processes *in vivo* and *ex vivo*, thus significantly contributing to the expansion of our knowledge of the immune system. This thesis includes three studies in which TPLSM was applied to unveil novel features of hematopoiesis and monopoiesis in the BM, as well as to investigate the dynamics of activated pericardial FALCs following MI.

The first study addressed the role of NECs as regulators of HSC differentiation in the BM. NECs are the most abundant cells of hematopoietic origin inside the BM and, together with sinusoidal endothelial cells, express high levels of the ACKR1, an atypical chemokine receptor which binds several inflammatory chemokines without inducing cell migration. The human rs2814778(G) polymorphism in the promoter of the *Ackr1* gene prevents gene expression in cells of the erythroid lineage. This genetic variant is also associated with benign neutropenia in individuals of African ancestry, although the molecular basis of such phenotype has so far been unknown. Therefore, the aim of this study was to investigate how the genetic deletion of *Ackr1* on NECs affects neutrophil production and hematopoiesis in general. *Ackr1*$^{-/-}$ mice displayed different frequencies of HSPCs, which also expressed higher levels of neutrophil-associated effector molecules. These changes were specifically ascribed to erythroid ACKR1 in the BM, but not to the vascular compartment.TPLSM of whole-mounted femoral BM to visualize HSCs and NECs in an unaltered 3D environment, revealed that ACKR1 promotes direct physical contacts between these two cell populations, thereby highlighting a previously unidentified contribution of NECs to the hematopoietic niche. The alternative pattern of hematopoiesis that developped in absence of ACKR1 resulted in increased expression of effector molecules on mature neutrophils, which are more prone to leave the circulation and accumulate in spleen, thus causing neutropenia.

The second study investigated the function of pericardial FALCs as coordinators of post-MI hematopoiesis and immune response. FALCs are tertiary lymphoid structures present in the adipose tissues and particularly abundant in the PAT, where they sense danger signals derived from the infarcted tissue. TPLSM of whole mounted PAT in mice 3 days after induction of MI showed local increment of both B and T cells specifically inside FALCs, in response to pro-inflammatory cytokines. Such expansion was due to local proliferation of GM-CSF^{+} B cells and recruitment of T cells through CCR7^{+} DCs. Effects on both DCs and T cell numbers and activation were enhanced in *Cb2*$^{-/-}$ mice

(which present higher basal levels of circulating and peripheral B cells) and abrogated after depletion of B cells or blocking of GM-CSF, corroborating the substantial role of B cells in triggering a local adaptive immune response in the PAT post-MI. The activation of lymphoid cells within FALCs also caused augmented production of G-CSF, a regulator of the hematopoietic niche. Emergency hematopoiesis and granulopoiesis, also assessed with TPLSM of whole mounted femurs, caused a second wave of neutrophils infiltrating the infarcted heart. Finally, enhanced FALC activation as seen in *Cb2*$^{-/-}$ mice resulted in aggravated fibrosis 7 days post-MI and impaired heart function.

The third study aimed at discovering and applying a novel marker to specifically visualize and track NCMs in circulation and tissue via microscopy. Herein, the immune check-point PD-L1 was identified and validated as a membrane marker uniquely expressed by circulating NCMs both *ex vivo* and *in vivo*, with higher specificity compared to the commonly used CX_3CR1. PD-L1 also marked NCMs and IMs in the BM. Thus a PD-L1-based multicolor labeling strategy was implemented to quantify monocyte subsets and vascular compartments in whole mounted femurs with TPLSM. NCMs and IMs were especially enriched in the epiphysis where their numbers correlated with the extension of transition zone (TZ) vessels. Moreover, BM monocytes were distributed within specific vascular microdomains, with CMs being in direct contact and more proximal to TZ vessels, while IMs and NCMs were increasingly more distant. Time-course analysis of monocyte conversion *in situ* demonstrated that this spatial distribution reflects progressive differentiation of CMs into NCMs, potentially triggered by engagement of CMs by TZ vessels. In aged mice, defective conversion was indeed associated to loss of TZ vessels. NCMs retained the exclusive expression of PD-L1 among blood leukocytes after MI and were found in the extravascular space of pericardial FALCs, as well as in human TLOs of atherosclerotic patients. NCMs significantly increased in FALCs 3 days after MI, where they actively interacted with T cells and induce their later apoptosis through PD-L1.

Hence, the studies presented in this thesis applied TPLSM to uncover novel regulators of hematopoiesis, both locally during the steady-state (NECs) and remotely during MI-driven inflammation (pericardial FALCs). Moreover, due to the application of PD-L1 as a specific NCM marker, the development of NCMs in BM could be assigned to a specific vascular niche. Finally, TPLSM established a previously unknown PD-L1-dependent immunomodulatory function of NCMs inside FALCs after MI.

4. Zusammenfassung

Die Zwei-Photonen-Laser-Scanning-Mikroskopie („Two-photon laser scanning microscopy“, TPLSM) ist eine leistungsstarke Technologie, um Immunzellen in ihrer Reifung, Organisation und Funktion unter physiologischen oder auch pathophysiologischen Bedingungen zu untersuchen, unter Berücksichtigung von räumlichen und zeitlichen Aspekten. Es lassen sich hiermit, mehrfarbige Fluoreszenzbilder von nahezu unmodifizierten Präparaten mit einer beachtlichen subzellulären Auflösung aufnehmen. Mittels dieser neuartigen *in vivo* oder auch *ex vivo* Analyse lassen sich neue mechanistische Erkenntnisse über immunologische Prozesse ableiten, was signifikant zu unserem Verständnis über das Immunsystem beiträgt. Die vorliegende Dissertation beinhaltet drei Studien, in denen mithilfe der TPLSM neue Charakteristika der Hämatopoese und Monopoese im Knochenmark untersucht wurden. Zudem wurde die Dynamik der Aktivierung von Lymphozyten-Clustern im perikardialen Fettgewebe („fat-associated lymphoid clusters“, FALCs) nach Herzinfarkt mittels TPLSM untersucht.

In der ersten Studie wurde die Rolle der kernhaltigen Erythrozytenvorläufer („nucleated erythroid cells“, NECs) als Regulatoren der hämatopoetischen Stammzell (HSC)-Reifung erforscht. Im Knochenmark sind NECs der am häufigsten vorhandene hämatopoetische Zelltyp. Gemeinsam mit sinusoidalen Endothelzellen weisen NECs eine hohe Expression des atypischen Chemokinrezeptors ACKR1 auf. ACKR1 bindet zwar verschiedene inflammatorische Chemokine, löst aber keine Zellmigration aus. Der humane Polymorphismus rs2814778(G) im Promotor des *Ackr1* Gens ist mit einer fehlenden Expression des Chemokin-Rezeptors in Zellen erythroiden Ursprungs verbunden. Zudem ist diese genetische Variante mit einer benignen Neutrophilie assoziiert und tritt gehäuft in Menschen mit afrikanischer Abstammung auf. Die molekularen Grundlagen der Neutrophilie sind bislang jedoch unbekannt. Im Rahmen dieser Studie sollte daher untersucht werden, wie die genetische Defizienz von *Ackr1* in NECs die Neutrophilen-Produktion und die Hämatopoese im Allgemeinen beeinflusst. Die vorliegende Arbeit zeigt, dass *Ackr1*-defiziente Mäuse eine veränderte Anzahl an hämatopoetischen Vorläufern aufweisen, die zudem durch einen höheren Anteil von neutrophilen Effektor-Molekülen charakterisiert sind. Diese Veränderungen sind dem Erythrozyten-ACKR1 zuzuschreiben, während der vaskuläre ACKR1 dabei keine Rolle spielt. Mittels 3-dimensionaler TPLSM Analyse des femoralen Knochenmarks in seiner Gesamtstruktur konnte zudem gezeigt werden, dass ACKR1 direkte Zell-Zell-Kontakte zwischen NECs und HSCs vermittelt. Somit wurde eine neue Funktion der NECs innerhalb der hämatopoetischen Knochenmarksnische aufgedeckt. Die veränderte Hämatopoese in Abwesenheit des ACKR1 resultiert weiterhin in einer vermehrten Bildung von reifen Neutrophilen, welche mit mehr

Effektor-Molekülen ausgestattet sind, vermehrt in die Zirkulation mobilisiert werden und sich in der Milz ansammeln, was eine Neutrophilie zur Folge hat.

Die zweite Studie beschäftigte sich mit der Funktion perikardialer FALCs als Koordinatorender Hämatopoese und der Immunantwort nach Herzinfarkt. FALCs sind tertiäre lymphoide Strukturen, die im adipösen Gewebe lokalisiert sind, insbesondere im perikardialen Fett. Hier nehmen sie Gefahr-Signale wahr, die vom infarzierten Areal ausgesandt werden werden. Die TPLSM des perikardialen Fettgewebes in seiner Gesamtstruktur 3 Tage nach Induktion des Herzinfarkts ergab eine Expansion von B- und T-Zellen innerhalb der FALCs, als Antwort auf erhöhte Spiegel proinflammatorischer Zytokine. Es zeigte sich, dass hierbei insbesondere GM-CSF-sezernierende B Zellen sowie die CCR7-abhängige Migration von Dendritischen Zellen in die perikardialen FALCs eine entscheidende Rolle spielen. Die Effekte auf T Zellen und Dendritische Zellen treten deutlich verstärkt in *Cb2*-defizienten Mäusen mit erhöhten zirkulierenden und peripheren B-Zellzahlen auf und lassen sich umgekehrt durch B-Zell-Depletion oder GM-CSF-Blockade hemmen. Diese funktionellen Experimente untermauern die essentielle Rolle der B-Zellen als Antreiber der lokalen adaptiven Immunantwort im perikardialen Fettgewebe nach Myokardinfarkt. Weiterhin führt die Aktivierung der perikardialen FALCs zu erhöhter G-CSF-Produktion und somit der Notfall-Granulopoese im Knochenmark. Dies wurde anhand von TPSLM des femoralen Knochenmarks in seiner Gesamtstruktur dokumentiert. Letztendlich bewirkt die vermehrte Aktivierung des perikardialen Fettgewebes in den *Cb2*-defizienten Mäusen eine vermehrte Fibrose und schlechtere Herzfunktion 7 Tage nach Infarkt.

Ziel der dritten Studie war es, einen neuen Marker für nicht-klassische Monozyten („non-classical monocytes“, NCM) zu identifizieren, der eine *in vivo* Zellmarkierung zur Visualisierung und Verfolgung der Zellen in Zirkulation und Gewebe mittels Mikroskopie ermöglicht. Als spezifischer Zelloberflächenmarker für NCM wurde der Immuncheckpoint PD-L1 gefunden und sowohl *in vivo* als auch *ex vivo* validiert. Dabei zeigte sich eine deutlich bessere Spezifität im Vergleich zum herkömmlich verwendeten Marker CX_3CR1. PD-L1 markiert zudem NCM und intermediäre Monozyten (IM) im Knochenmark. Infolgedessen wurde eine mehrfarbige Markierungsstrategie für die TPLSM entwickelt, um Monozyten-Subpopulationen im Bezug zu den vaskulären Kompartimenten in Gesamtgewebestrukturen des femoralen Knochenmarks zu quantifizieren. Es zeigte sich, das NCM und IM vorwiegend im Bereich der Epiphyse anzufinden sind, die reich an Transitionszonen (TZ)-Gefäßen ist. Darüber hinaus sind Knochenmarks-Monozyten im Bereich spezifischer Gefäß-Mikrodomänen angehäuft. Dabei sind klassische Monozyten („classical monocytes“, CM) in direktem Kontakt und eher proximal zu TZ-Gefäßen lokalisiert, wohingegen IM und NCM zunehmend weiter entfernt positioniert sind. Die *in situ* Untersuchung der Monozyten-Konversion im Zeitverlauf unterstützt die Beobachtung, dass die räumliche Verteilung auf eine

progressive Differenzierung von CM zu NCM zurückzuführen ist. Dabei spielt die Interaktion der CM mit den TZ Gefäßen möglicherweise eine direkte Rolle. In gealterten Mäusen geht der Verlust der Monozyten-Konversion mit einem Verschwinden der TZ Gefäße einher. Die spezifische Expression des Markers PD-L1 bleibt auch nach einem Herzinfarkt stabil und selektiv auf den NCM exprimiert, sowohl im Blut als auch im perikardialen Fettgewebe. Auch in humanen tertiären lymphoiden Organen in Aortenpräparaten von Patienten mit Atherosklerose waren PD-L1-exprimierende Monozyten nachweisbar. Im Hinblick auf eine mögliche funktionelle Rolle im Kontext eines Herzinfarkts zeigte sich, dass NCMs innerhalb von 3 Tagen massiv in perikardiale FALCs von Infarkt-Mäusen einwandern und dort mit T Zellen interagieren. Diese funktionelle PD-L1-vermittelte Interaktion zwischen NCM und T Zellen führt zur Apoptose-Induktion in den T Zellen.

Zusammenfassend zeigen diese Studien, dass mithilfe der TPLSM neue Regulatoren der Hämatopoese identifiziert werden konnten, sowohl homöostatisch (was die Rolle der NECs betrifft) als auch unter Herzinfarkt-vermittelter Entzündung (bezüglich perikardialer FALCs). Darüber hinaus ermöglichte die Etablierung des NCM Markers PD-L1, dass die NCM Konversion im Knochenmark einer spezifischen Gefäßnische zugeordnet werden konnte. Zudem verhalf die TPLSM letztendlich zur Aufdeckung einer bislang unbekannten PD-L1-vermittelten immunomodulatorischen Funktion der NCM innerhalb perikardialer FALCs im Zusammenhang mit einem Herzinfarkt.

5. Perspectives

Understanding how immune cell production is regulated under steady-state conditions and how this gets modified during different physiological and pathological states (for example genetic variation, aging and inflammation) is of critical importance to further progress our knowledge of immunology. Currently known modalities of alternative hematopoiesis, which include emergency[47], trained[107] and clonal[108] hematopoiesis, result into the production of immune cell subtypes with different phenotypical and behavioral characteristics from what observed under steady-state conditions. Detailed comprehension of how HSPCs and their progenies are modulated requires thorough characterization of the spatial components of early and later hematopoietic niches. Nowadays this is possible thanks to advanced optical imaging methodologies among which TPLSM stands out for informativity and resolution power at considerable imaging depth in a sample.

In this thesis, evidence is provided that proliferation and differentiation of HSCs in the BM is modulated by surrounding NECs, and in particular by the ACKR1 expressed on NEC surface. Absence of ACKR1 from this specific cellular compartment, as it occurs in individuals of African ancestry, leads to an alternative priming of HSCs, which results into by-passed or accelerated differentiation of downstream MPPs, overall myeloid instruction of HSPCs and, ultimately, the production of neutrophils with increased effector molecular signatures and extravasation capability. Although *in situ* imaging showed that ACKR1 presence on NECs is unequivocally required for HSC scaffolding inside NEC clusters, how exactly cell-cell contacts are mediated by ACKR1 still needs to be elucidated. In fact, ACKR1 could act *in trans* as a molecular anchor for chemokines involved in HSC regulation or through hypothetical oligomerization with other chemokine receptors such as CXCR4, whose cognate ligand CXCL12 is the main responsible of HSPC retention in the BM, but does not directly bind to ACKR1. Alternatively, ACKR1 might engage a non-chemokine partner on HSCs, such as CD82[109], and directly signal to HSCs. For this, application of advanced imaging modalities, such as super-resolution microscopy, could offer valuable insights into the exact positioning of ACKR1 and its counterpart(s) within HSC-NEC units. Of notice, NECs form by far the most abundant cellular population in the BM, approximately 30-50% of non-stromal cells, while HSCs are estimated to be only around 0.007%[52]. Considering that all NECs express ACKR1, albeit at different levels depending on the maturation stage, and that they are rather homogeneously distributed throughout bone cavities, it is tempting to suppose that other structural and cellular entities contribute to define the exact localization of NECs specifically involved with HSCs. ACKR1 on NECs may act as a direct or indirect molecular platform for other niche players which in turn influence HSC behavior. Thus, visualization of HSC-NEC niches in combination with other known

niche components, blood vessels and mesenchymal cells among others, may help to better characterize these aspects.

Aside from being regulated by local cues, BM hematopoietic niches can also respond to danger signals originating from distant organs, consequently boosting the development of preferential forms of immune cell subtypes, that can also sensibly differ from their steady-state forms, in a process overall known as emergency hematopoiesis. Here it is reported that increased systemic G-CSF levels in response to MI enhance granulopoiesis, in a mechanism driven by lymphocyte and DC activation in pericardial FALCs. Complete removal of PAT, as well as inhibition of various FALC components, both demonstrated the centrality of these tertiary lymphatic structures in regulating the activation of neutrophil-biased HSPCs. In this regard, microscopy was pivotal to spatially define the distinctive expansion of lymphocytes in response to MI in confined, organized cellular units inside the adipose tissue, where communication between B, T cells and DCs can be efficiently modulated. However, the precise mechanisms by which the infarcted heart drives B cell proliferation and proliferation/attraction of DCs and T cells remains to be fully elucidated. To this extent, it would be interesting to define the involvement of the vascular blood and lymphatic networks connecting the heart and the pericardium to the juxtaposed adipose tissue, by evaluating their post-MI morphological remodeling. Likewise, since the sympathetic nervous system plays a fundamental role in modulating and synchronizing many aspects of immune cell development and function[110], local changes in sympathetic innervation might also be involved in FALC activation and should be investigated further.

In addition, other cell types residing in visceral fat deposits (e.g. macrophages, innate lymphoid cells and adipocytes themselves) should be examined as well and their relationship with FALC-residing lymphocytes and DCs explored.

In the third study, it is demonstrated that PD-L1^{+} NCMs readily accumulate specifically in post-MI FALCs, where they restrict the expansion of T cells over time by inducing their apoptosis. It is still not clear if, in the context of MI, T cell immunomodulation by NCMs also has an impact on FALC-controlled granulopoiesis, neutrophil recruitment to the heart and tissue repair. However, these findings open the way to interesting new concepts about the role played by NCMs in inflammatory diseases, on top of the vascular patrolling and neutrophil-attraction activities so far attributed to them. The additional identification of PD-L1^{+} NCMs in human TLOs under chronic inflammatory conditions further endorses the hypothesis that immunomodulation by NCMs regulating adaptive immune responses inside local TLOs, could play a broader role in chronic pathologies, not limited to cardiovascular disease. In this context, it is particularly interesting that immunomodulatory therapies targeting PD-L1 are already in clinical use, as in the case of several types of tumors[111].

NCMs gathering in FALCs after MI were likely recruited from the circulatory pool, as suggested by the presence of some of these cells attached to the outer wall of FALC-perfusing vessels. This would also be in line with the notion that NCMs infiltrate the injured heart independently of previous recruitment of CMs[106], which do not convert locally, although *in vivo* imaging studies using PD-L1 as a novel and highly specific tracking tool would be required to fully demonstrate NCM extravasation and migration inside the PAT parenchyma. In such a scenario, it would be important to clarify whether "immunomodulatory" PD-L1$^+$ NCMs totally overlap with their steady-state patrolling counterparts or are *de novo* generated cells, recruited from the BM during emergency hematopoiesis. In the steady-state, NCMs originate upon conversion of CMs in the peripheral blood and other hematopoietic organs. In this work, application of PD-L1 as an IM- and NCM-marker consented to investigate monocyte conversion *in situ* in the BM, revealing it to be a strictly temporally and spatially regulated phenomenon. In particular, the physical engagement of CMs by endosteal TZ vessels was proposed as a necessary step for subsequent production of IMs and NCMs. Indeed, aged mice that harbored altered TZ vessels were characterized by a dramatic reduction of IMs and NCMs, both in BM and blood. However, further TPLSM or nanoscopic characterization of CM-TZ vessel cross-talk is required in order to identify the exact molecular determinants responsible for CM priming and how this event is possibly modulated under non-physiological conditions. In this regard, over-production of NCMs has been observed in the course of some inflammatory pathologies[112, 113, 114], hinting at an altered conversion process. Moreover, alternative pathways of monopoiesis have also been described during infection[84] and fibrosis[115], involving the activation of non-canonical monocyte precursors, suggesting that biological stress could induce other ontogenetic routes of NCM production either in addition or in replacement of conversion. TPLSM imaging of monocyte subsets in different mouse models of inflammatory disease will help to further elucidate these aspects. The novel specific NCM-marker PD-L1 will be central for such studies.

Taken together, the studies presented in this thesis exemplify three applications of TPLSM for the visualization and analysis of immune cell production and interaction in specific physiological and pathological contexts, unraveling previously unknown spatial and mechanistic aspects, which will constitute the basis for future research on these subjects.

6. References

1. Parkin J, Cohen B. An overview of the immune system. *Lancet (London, England)* 2001, **357**(9270)**:** 1777-1789.

2. Swirski FK, Nahrendorf M. Cardioimmunology: the immune system in cardiac homeostasis and disease. *Nature reviews Immunology* 2018, **18**(12)**:** 733-744.

3. Mohanta SK, Yin C, Peng L, Srikakulapu P, Bontha V, Hu D*, et al.* Artery tertiary lymphoid organs contribute to innate and adaptive immune responses in advanced mouse atherosclerosis. *Circulation research* 2014, **114**(11)**:** 1772-1787.

4. Sautes-Fridman C, Petitprez F, Calderaro J, Fridman WH. Tertiary lymphoid structures in the era of cancer immunotherapy. *Nature reviews Cancer* 2019, **19**(6)**:** 307-325.

5. Kaufmann SH. Immunology's foundation: the 100-year anniversary of the Nobel Prize to Paul Ehrlich and Elie Metchnikoff. *Nature immunology* 2008, **9**(7)**:** 705-712.

6. Metschnikoff E. Untersuchungen uber die intracellulare Verdauung bei wirbellosen Thieren. *Arb Zool Inst Univ Wien u Zool Stat Triest* 1884, **5:** 141.

7. Ehrlich PR, Himmelweit F. *The collected papers of Paul Ehrlich*, vol. 1. Pergamon, 1956.

8. Metchnikoff E. Untersuchungen über die mesodermalen Phagocyten einiger Wirbeltiere. *Biol Zentralbl* 1883, **3:** 560-565.

9. Metschnikoff E. Ueber den Kampf der Zellen gegen Erysipel-kokken. *Archiv für pathologische Anatomie und Physiologie und für klinische Medicin* 1887, **107**(2)**:** 209-249.

10. McKinnon KM. Flow Cytometry: An Overview. *Current protocols in immunology* 2018, **120:** 5.1.1-5.1.11.

11. Spitzer MH, Nolan GP. Mass Cytometry: Single Cells, Many Features. *Cell* 2016, **165**(4)**:** 780-791.

12. Papalexi E, Satija R. Single-cell RNA sequencing to explore immune cell heterogeneity. *Nature reviews Immunology* 2018, **18**(1)**:** 35-45.

13. Hofer T, Busch K, Klapproth K, Rodewald HR. Fate Mapping and Quantitation of Hematopoiesis In Vivo. *Annual review of immunology* 2016, **34:** 449-478.

14. Bajenoff M, Germain RN. Seeing is believing: a focus on the contribution of microscopic imaging to our understanding of immune system function. *European journal of immunology* 2007, **37 Suppl 1:** S18-33.

15. Coons AH. The demonstration of pneumococcal antigen in tissues by the use of fluorescent antibody. *J Immunol* 1942, **45:** 159.

16. Hiramoto R, Engel K, Pressman D. Tetramethylrhodamine as immunohistochemical fluorescent label in the study of chronic thyroiditis. *Proceedings of the Society for Experimental Biology and Medicine* 1958, **97**(3)**:** 611-614.

17. Bernard A, Boumsell L. The clusters of differentiation (CD) defined by the first international workshop on human leucocyte differentiation antigens. *Human immunology* 1984, **11**(1)**:** 1-10.

18. Zhang J, Campbell RE, Ting AY, Tsien RY. Creating new fluorescent probes for cell biology. *Nature reviews Molecular cell biology* 2002, **3**(12)**:** 906-918.

19. Li JL, Goh CC, Keeble JL, Qin JS, Roediger B, Jain R*, et al.* Intravital multiphoton imaging of immune responses in the mouse ear skin. *Nature protocols* 2012, **7**(2)**:** 221-234.

20. Coombes JL, Robey EA. Dynamic imaging of host–pathogen interactions in vivo. *Nature Reviews Immunology* 2010, **10**(5)**:** 353-364.

21. Geissmann F, Cameron TO, Sidobre S, Manlongat N, Kronenberg M, Briskin MJ*, et al.* Intravascular immune surveillance by CXCR6+ NKT cells patrolling liver sinusoids. *PLoS biology* 2005, **3**(4)**:** e113.

22. Shakhar G, Lindquist RL, Skokos D, Dudziak D, Huang JH, Nussenzweig MC*, et al.* Stable T cell-dendritic cell interactions precede the development of both tolerance and immunity in vivo. *Nature immunology* 2005, **6**(7)**:** 707-714.

23. Kasten F. The origin of modern fluorescence microscopy. chap 1. Academic Press, San Diego; 1989.

24. Raff MC. Two distinct populations of peripheral lymphocytes in mice distinguishable by immunofluorescence. *Immunology* 1970, **19**(4)**:** 637-650.

25. Morrison WI, Murray M, Hinson CA. The response of the murine lymphoid system to a chronic infection with Trypanosoma congolense. II. The lymph nodes, thymus and liver. *The Journal of pathology* 1982, **138**(3)**:** 273-288.

26. Minsky M. Memoir on inventing the confocal scanning microscope. *Scanning* 1988, **10**(4)**:** 128-138.

27. Brakenhoff GJ, van der Voort HT, van Spronsen EA, Linnemans WA, Nanninga N. Three-dimensional chromatin distribution in neuroblastoma nuclei shown by confocal scanning laser microscopy. *Nature* 1985, **317**(6039)**:** 748-749.

28. Bayguinov PO, Oakley DM, Shih CC, Geanon DJ, Joens MS, Fitzpatrick JAJ. Modern Laser Scanning Confocal Microscopy. *Current protocols in cytometry* 2018, **85**(1)**:** e39.

29. Göppert-Mayer M. Elementary processes with two quantum transitions. *Annalen der Physik* 2009, **18**(7-8)**:** 466-479.

30. Denk W, Strickler JH, Webb WW. Two-photon laser scanning fluorescence microscopy. *Science* 1990, **248**(4951)**:** 73-76.

31. Wu Z, Rademakers T, Kiessling F, Vogt M, Westein E, Weber C*, et al.* Multi-photon microscopy in cardiovascular research. *Methods (San Diego, Calif)* 2017, **130:** 79-89.

32. Shirshin EA, Yakimov BP, Darvin ME, Omelyanenko NP, Rodionov SA, Gurfinkel YI*, et al.* Label-Free Multiphoton Microscopy: The Origin of Fluorophores and Capabilities for Analyzing Biochemical Processes. *Biochemistry (Moscow)* 2019, **84**(1)**:** 69-88.

33. Campagnola P. Second harmonic generation imaging microscopy: applications to diseases diagnostics. ACS Publications; 2011.

34. Mostaço-Guidolin L, Rosin NL, Hackett T-L. Imaging Collagen in Scar Tissue: Developments in Second Harmonic Generation Microscopy for Biomedical Applications. *International Journal of Molecular Sciences* 2017, **18**(8)**:** 1772.

35. Evrard M, Chong SZ, Devi S, Chew WK, Lee B, Poidinger M*, et al.* Visualization of bone marrow monocyte mobilization using Cx3cr1gfp/+Flt3L-/- reporter mouse by multiphoton intravital microscopy. *Journal of leukocyte biology* 2015, **97**(3)**:** 611-619.

36. Laviron M, Combadiere C, Boissonnas A. Tracking Monocytes and Macrophages in Tumors With Live Imaging. *Frontiers in immunology* 2019, **10:** 1201.

37. Sody S, Uddin M, Gruneboom A, Gorgens A, Giebel B, Gunzer M*, et al.* Distinct Spatio-Temporal Dynamics of Tumor-Associated Neutrophils in Small Tumor Lesions. *Frontiers in immunology* 2019, **10:** 1419.

38. Ng LG, Mrass P, Kinjyo I, Reiner SL, Weninger W. Two-photon imaging of effector T-cell behavior: lessons from a tumor model. *Immunological reviews* 2008, **221:** 147-162.

39. van Panhuys N. Studying Dendritic Cell-T Cell Interactions Under In Vivo Conditions. *Methods in molecular biology (Clifton, NJ)* 2017, **1584:** 569-583.

40. Malide D, Metais JY, Dunbar CE. In vivo clonal tracking of hematopoietic stem and progenitor cells marked by five fluorescent proteins using confocal and multiphoton microscopy. *Journal of visualized experiments : JoVE* 2014(90)**:** e51669.

41. Devi S, Wang Y, Chew WK, Lima R, N AG, Mattar CN*, et al.* Neutrophil mobilization via plerixafor-mediated CXCR4 inhibition arises from lung demargination and blockade of neutrophil homing to the bone marrow. *The Journal of experimental medicine* 2013, **210**(11)**:** 2321-2336.

42. Jagannathan-Bogdan M, Zon LI. Hematopoiesis. *Development* 2013, **140**(12)**:** 2463-2467.

43. Laurenti E, Gottgens B. From haematopoietic stem cells to complex differentiation landscapes. *Nature* 2018, **553**(7689)**:** 418-426.

44. Eaves CJ. Hematopoietic stem cells: concepts, definitions, and the new reality. *Blood* 2015, **125**(17)**:** 2605-2613.

45. Wei Q, Frenette PS. Niches for Hematopoietic Stem Cells and Their Progeny. *Immunity* 2018, **48**(4)**:** 632-648.

46. de Haan G, Lazare SS. Aging of hematopoietic stem cells. *Blood* 2018, **131**(5)**:** 479-487.

47. Boettcher S, Manz MG. Regulation of Inflammation- and Infection-Driven Hematopoiesis. *Trends in immunology* 2017, **38**(5)**:** 345-357.

48. Armitage JO. Bone marrow transplantation. *The New England journal of medicine* 1994, **330**(12)**:** 827-838.

49. Guilliams M, Mildner A, Yona S. Developmental and Functional Heterogeneity of Monocytes. *Immunity* 2018, **49**(4)**:** 595-613.

50. Crane GM, Jeffery E, Morrison SJ. Adult haematopoietic stem cell niches. *Nature reviews Immunology* 2017, **17**(9)**:** 573-590.

51. Schofield R. The relationship between the spleen colony-forming cell and the haemopoietic stem cell. *Blood cells* 1978, **4**(1-2)**:** 7-25.

52. Kiel MJ, Yilmaz OH, Iwashita T, Yilmaz OH, Terhorst C, Morrison SJ. SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell* 2005, **121**(7)**:** 1109-1121.

53. Pinho S, Frenette PS. Haematopoietic stem cell activity and interactions with the niche. *Nature reviews Molecular cell biology* 2019, **20**(5)**:** 303-320.

54. Acar M, Kocherlakota KS, Murphy MM, Peyer JG, Oguro H, Inra CN*, et al.* Deep imaging of bone marrow shows non-dividing stem cells are mainly perisinusoidal. *Nature* 2015, **526**(7571)**:** 126-130.

55. Gomariz A, Helbling PM, Isringhausen S, Suessbier U, Becker A, Boss A*, et al.* Quantitative spatial analysis of haematopoiesis-regulating stromal cells in the bone marrow microenvironment by 3D microscopy. *Nature communications* 2018, **9**(1)**:** 2532.

56. Sugiyama T, Kohara H, Noda M, Nagasawa T. Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. *Immunity* 2006, **25**(6)**:** 977-988.

57. Ding L, Saunders TL, Enikolopov G, Morrison SJ. Endothelial and perivascular cells maintain haematopoietic stem cells. *Nature* 2012, **481**(7382)**:** 457-462.

58. Zou YR, Kottmann AH, Kuroda M, Taniuchi I, Littman DR. Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. *Nature* 1998, **393**(6685)**:** 595-599.

59. Williams DE, Eisenman J, Baird A, Rauch C, Van Ness K, March CJ*, et al.* Identification of a ligand for the c-kit proto-oncogene. *Cell* 1990, **63**(1)**:** 167-174.

60. Nombela-Arrieta C, Pivarnik G, Winkel B, Canty KJ, Harley B, Mahoney JE*, et al.* Quantitative imaging of haematopoietic stem and progenitor cell localization and hypoxic status in the bone marrow microenvironment. *Nature cell biology* 2013, **15**(5)**:** 533-543.

61. Mendez-Ferrer S, Michurina TV, Ferraro F, Mazloom AR, Macarthur BD, Lira SA*, et al.* Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature* 2010, **466**(7308)**:** 829-834.

62. Kunisaki Y, Bruns I, Scheiermann C, Ahmed J, Pinho S, Zhang D*, et al.* Arteriolar niches maintain haematopoietic stem cell quiescence. *Nature* 2013, **502**(7473)**:** 637-643.

63. Hooper AT, Butler JM, Nolan DJ, Kranz A, Iida K, Kobayashi M*, et al.* Engraftment and reconstitution of hematopoiesis is dependent on VEGFR2-mediated regeneration of sinusoidal endothelial cells. *Cell stem cell* 2009, **4**(3)**:** 263-274.

64. Kusumbe AP, Ramasamy SK, Itkin T, Mae MA, Langen UH, Betsholtz C*, et al.* Age-dependent modulation of vascular niches for haematopoietic stem cells. *Nature* 2016, **532**(7599)**:** 380-384.

65. Bruns I, Lucas D, Pinho S, Ahmed J, Lambert MP, Kunisaki Y*, et al.* Megakaryocytes regulate hematopoietic stem cell quiescence through CXCL4 secretion. *Nature medicine* 2014, **20**(11)**:** 1315-1320.

66. Fujisaki J, Wu J, Carlson AL, Silberstein L, Putheti P, Larocca R*, et al.* In vivo imaging of Treg cells providing immune privilege to the haematopoietic stem-cell niche. *Nature* 2011, **474**(7350)**:** 216-219.

67. Naveiras O, Nardi V, Wenzel PL, Hauschka PV, Fahey F, Daley GQ. Bone-marrow adipocytes as negative regulators of the haematopoietic microenvironment. *Nature* 2009, **460**(7252)**:** 259-263.

68. Visnjic D, Kalajzic Z, Rowe DW, Katavic V, Lorenzo J, Aguila HL. Hematopoiesis is severely altered in mice with an induced osteoblast deficiency. *Blood* 2004, **103**(9)**:** 3258-3264.

69. Lucas D, Scheiermann C, Chow A, Kunisaki Y, Bruns I, Barrick C*, et al.* Chemotherapy-induced bone marrow nerve injury impairs hematopoietic regeneration. *Nature medicine* 2013, **19**(6)**:** 695-703.

70. Isern J, Garcia-Garcia A, Martin AM, Arranz L, Martin-Perez D, Torroja C*, et al.* The neural crest is a source of mesenchymal stem cells with specialized hematopoietic stem cell niche function. *eLife* 2014, **3:** e03696.

71. Burberry A, Zeng MY, Ding L, Wicks I, Inohara N, Morrison SJ*, et al.* Infection mobilizes hematopoietic stem cells through cooperative NOD-like receptor and Toll-like receptor signaling. *Cell host & microbe* 2014, **15**(6)**:** 779-791.

72. Johns JL, Borjesson DL. Downregulation of CXCL12 signaling and altered hematopoietic stem and progenitor cell trafficking in a murine model of acute Anaplasma phagocytophilum infection. *Innate immunity* 2012, **18**(3)**:** 418-428.

73. Zhu J, Garrett R, Jung Y, Zhang Y, Kim N, Wang J*, et al.* Osteoblasts support B-lymphocyte commitment and differentiation from hematopoietic stem cells. *Blood* 2007, **109**(9)**:** 3706-3712.

74. Busch K, Klapproth K, Barile M, Flossdorf M, Holland-Letz T, Schlenner SM*, et al.* Fundamental properties of unperturbed haematopoiesis from stem cells in vivo. *Nature* 2015, **518**(7540)**:** 542-546.

75. Sun J, Ramos A, Chapman B, Johnnidis JB, Le L, Ho YJ*, et al.* Clonal dynamics of native haematopoiesis. *Nature* 2014, **514**(7522)**:** 322-327.

76. Wilson A, Laurenti E, Oser G, van der Wath RC, Blanco-Bose W, Jaworski M*, et al.* Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. *Cell* 2008, **135**(6)**:** 1118-1129.

77. Baldridge MT, King KY, Boles NC, Weksberg DC, Goodell MA. Quiescent haematopoietic stem cells are activated by IFN-gamma in response to chronic infection. *Nature* 2010, **465**(7299)**:** 793-797.

78. Walter D, Lier A, Geiselhart A, Thalheimer FB, Huntscha S, Sobotta MC*, et al.* Exit from dormancy provokes DNA-damage-induced attrition in haematopoietic stem cells. *Nature* 2015, **520**(7548)**:** 549-552.

79. Cordeiro Gomes A, Hara T, Lim VY, Herndler-Brandstetter D, Nevius E, Sugiyama T*, et al.* Hematopoietic Stem Cell Niches Produce Lineage-Instructive Signals to Control Multipotent Progenitor Differentiation. *Immunity* 2016, **45**(6)**:** 1219-1231.

80. Evrard M, Kwok IWH, Chong SZ, Teng KWW, Becht E, Chen J*, et al.* Developmental Analysis of Bone Marrow Neutrophils Reveals Populations Specialized in Expansion, Trafficking, and Effector Functions. *Immunity* 2018, **48**(2)**:** 364-379.e368.

81. Robbins CS, Chudnovskiy A, Rauch PJ, Figueiredo JL, Iwamoto Y, Gorbatov R*, et al.* Extramedullary hematopoiesis generates Ly-6C(high) monocytes that infiltrate atherosclerotic lesions. *Circulation* 2012, **125**(2)**:** 364-374.

82. Fogg DK, Sibon C, Miled C, Jung S, Aucouturier P, Littman DR*, et al.* A clonogenic bone marrow progenitor specific for macrophages and dendritic cells. *Science* 2006, **311**(5757)**:** 83-87.

83. Lee J, Breton G, Oliveira TY, Zhou YJ, Aljoufi A, Puhr S*, et al.* Restricted dendritic cell and monocyte progenitors in human cord blood and bone marrow. *The Journal of experimental medicine* 2015, **212**(3)**:** 385-399.

84. Yanez A, Coetzee SG, Olsson A, Muench DE, Berman BP, Hazelett DJ*, et al.* Granulocyte-Monocyte Progenitors and Monocyte-Dendritic Cell Progenitors Independently Produce Functionally Distinct Monocytes. *Immunity* 2017, **47**(5)**:** 890-902.e894.

85. Sathe P, Metcalf D, Vremec D, Naik SH, Langdon WY, Huntington ND*, et al.* Lymphoid tissue and plasmacytoid dendritic cells and macrophages do not share a common macrophage-dendritic cell-restricted progenitor. *Immunity* 2014, **41**(1)**:** 104-115.

86. Kawamura S, Onai N, Miya F, Sato T, Tsunoda T, Kurabayashi K*, et al.* Identification of a Human Clonogenic Progenitor with Strict Monocyte Differentiation Potential: A Counterpart of Mouse cMoPs. *Immunity* 2017, **46**(5)**:** 835-848.e834.

87. Hettinger J, Richards DM, Hansson J, Barra MM, Joschko AC, Krijgsveld J*, et al.* Origin of monocytes and macrophages in a committed progenitor. *Nature immunology* 2013, **14**(8)**:** 821-830.

88. Chong SZ, Evrard M, Devi S, Chen J, Lim JY, See P*, et al.* CXCR4 identifies transitional bone marrow premonocytes that replenish the mature monocyte pool for peripheral responses. *The Journal of experimental medicine* 2016, **213**(11)**:** 2293-2314.

89. Geissmann F, Jung S, Littman DR. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity* 2003, **19**(1)**:** 71-82.

90. Passlick B, Flieger D, Ziegler-Heitbrock HW. Identification and characterization of a novel monocyte subpopulation in human peripheral blood. *Blood* 1989, **74**(7)**:** 2527-2534.

91. Mildner A, Schonheit J, Giladi A, David E, Lara-Astiaso D, Lorenzo-Vivas E*, et al.* Genomic Characterization of Murine Monocytes Reveals C/EBPbeta Transcription Factor Dependence of Ly6C(-) Cells. *Immunity* 2017, **46**(5)**:** 849-862.e847.

92. Ingersoll MA, Spanbroek R, Lottaz C, Gautier EL, Frankenberger M, Hoffmann R*, et al.* Comparison of gene expression profiles between human and mouse monocyte subsets. *Blood* 2010, **115**(3)**:** e10-19.

93. Patel AA, Zhang Y, Fullerton JN, Boelen L, Rongvaux A, Maini AA*, et al.* The fate and lifespan of human monocyte subsets in steady state and systemic inflammation. *The Journal of experimental medicine* 2017, **214**(7)**:** 1913-1923.

94. Yona S, Kim KW, Wolf Y, Mildner A, Varol D, Breker M*, et al.* Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. *Immunity* 2013, **38**(1)**:** 79-91.

95. Hanna RN, Carlin LM, Hubbeling HG, Nackiewicz D, Green AM, Punt JA*, et al.* The transcription factor NR4A1 (Nur77) controls bone marrow differentiation and the survival of Ly6C- monocytes. *Nature immunology* 2011, **12**(8)**:** 778-785.

96. Thomas GD, Hanna RN, Vasudevan NT, Hamers AA, Romanoski CE, McArdle S*, et al.* Deleting an Nr4a1 Super-Enhancer Subdomain Ablates Ly6C(low) Monocytes while Preserving Macrophage Gene Function. *Immunity* 2016, **45**(5)**:** 975-987.

97. Lessard AJ, LeBel M, Egarnes B, Prefontaine P, Theriault P, Droit A*, et al.* Triggering of NOD2 Receptor Converts Inflammatory Ly6C(high) into Ly6C(low) Monocytes with Patrolling Properties. *Cell reports* 2017, **20**(8)**:** 1830-1843.

98. Selimoglu-Buet D, Riviere J, Ghamlouch H, Bencheikh L, Lacout C, Morabito M*, et al.* A miR-150/TET3 pathway regulates the generation of mouse and human non-classical monocyte subset. *Nature communications* 2018, **9**(1)**:** 5455.

99. Tacke F, Alvarez D, Kaplan TJ, Jakubzick C, Spanbroek R, Llodra J*, et al.* Monocyte subsets differentially employ CCR2, CCR5, and CX3CR1 to accumulate within atherosclerotic plaques. *The Journal of clinical investigation* 2007, **117**(1)**:** 185-194.

100. Fife BT, Huffnagle GB, Kuziel WA, Karpus WJ. CC chemokine receptor 2 is critical for induction of experimental autoimmune encephalomyelitis. *The Journal of experimental medicine* 2000, **192**(6)**:** 899-905.

101. Auffray C, Fogg D, Garfa M, Elain G, Join-Lambert O, Kayal S*, et al.* Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. *Science* 2007, **317**(5838)**:** 666-670.

102. Carlin LM, Stamatiades EG, Auffray C, Hanna RN, Glover L, Vizcay-Barrena G*, et al.* Nr4a1-dependent Ly6C(low) monocytes monitor endothelial cells and orchestrate their disposal. *Cell* 2013, **153**(2)**:** 362-375.

103. Cros J, Cagnard N, Woollard K, Patey N, Zhang SY, Senechal B*, et al.* Human CD14dim monocytes patrol and sense nucleic acids and viruses via TLR7 and TLR8 receptors. *Immunity* 2010, **33**(3)**:** 375-386.

104. Quintar A, McArdle S, Wolf D, Marki A, Ehinger E, Vassallo M*, et al.* Endothelial Protective Monocyte Patrolling in Large Arteries Intensified by Western Diet and Atherosclerosis. *Circulation research* 2017, **120**(11)**:** 1789-1799.

105. Hanna RN, Shaked I, Hubbeling HG, Punt JA, Wu R, Herrley E*, et al.* NR4A1 (Nur77) deletion polarizes macrophages toward an inflammatory phenotype and increases atherosclerosis. *Circulation research* 2012, **110**(3)**:** 416-427.

106. Nahrendorf M, Swirski FK, Aikawa E, Stangenberg L, Wurdinger T, Figueiredo JL*, et al.* The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. *The Journal of experimental medicine* 2007, **204**(12)**:** 3037-3047.

107. Netea MG, Joosten LAB, Latz E, Mills KHG, Natoli G, Stunnenberg HG*, et al.* Trained immunity: A program of innate immune memory in health and disease. *Science* 2016, **352**(6284)**:** aaf1098.

108. Bowman RL, Busque L, Levine RL. Clonal Hematopoiesis and Evolution to Hematopoietic Malignancies. *Cell stem cell* 2018, **22**(2)**:** 157-170.

109. Bandyopadhyay S, Zhan R, Chaudhuri A, Watabe M, Pai SK, Hirota S*, et al.* Interaction of KAI1 on tumor cells with DARC on vascular endothelium leads to metastasis suppression. *Nature medicine* 2006, **12**(8)**:** 933-938.

110. Olofsson PS, Rosas-Ballina M, Levine YA, Tracey KJ. Rethinking inflammation: neural circuits in the regulation of immunity. *Immunological reviews* 2012, **248**(1)**:** 188-204.

111. Sun C, Mezzadra R, Schumacher TN. Regulation and Function of the PD-L1 Checkpoint. *Immunity* 2018, **48**(3)**:** 434-452.

112. Cairns AP, Crockard AD, Bell AL. The CD14+ CD16+ monocyte subset in rheumatoid arthritis and systemic lupus erythematosus. *Rheumatology international* 2002, **21**(5)**:** 189-192.

113. Zhu H, Hu F, Sun X, Zhang X, Zhu L, Liu X*, et al.* CD16(+) Monocyte Subset Was Enriched and Functionally Exacerbated in Driving T-Cell Activation and B-Cell Response in Systemic Lupus Erythematosus. *Frontiers in immunology* 2016, **7:** 512.

114. Urbanski K, Ludew D, Filip G, Filip M, Sagan A, Szczepaniak P*, et al.* CD14(+)CD16(++) "nonclassical" monocytes are associated with endothelial dysfunction in patients with coronary artery disease. *Thrombosis and haemostasis* 2017, **117**(5)**:** 971-980.

115. Satoh T, Nakagawa K, Sugihara F, Kuwahara R, Ashihara M, Yamane F*, et al.* Identification of an atypical monocyte and committed progenitor involved in fibrosis. *Nature* 2017, **541**(7635)**:** 96.

7. Acknowledgements

Six (!) years ago I decided to leave the “comfort zone” of my city and Country to move to Germany and start a new PhD adventure, here in Munich. As it usually happens in such circumstances, it turned out to be a very lucky strike! During the past years I have met a great variety of people, experienced adult life for the first time and considerably grown up. I have learnt plenty, both in and outside of the lab. Sometimes, difficulties and failures have caused tears. but mostly I have had many reasons to whole-heartedly laugh, enjoying every step of the way. And for this, I want to express my gratitude to all the people who have made it possible for me to carry on this beautiful journey till the end.

First, I would like to thank Univ.-Prof. Dr. med. Christian Weber, not only for his trust in me from the very beginning, which consented me to start to work at the IPEK, but also for his interest in and support to my research throughout the whole duration of my PhD. Thank you Christian.

Many thanks to Univ.-Prof. Dr. rer. nat. Sabine Steffens, my “Doktormutter”, for welcoming me between the walls of her lab, letting me share the everyday working life (and parties!) of the AG Steffens, involving me in some of her projects and ultimately providing valuable advice and help for the finalization of this thesis.

Special thanks to Prof. Antal Rot, for involving me in his studies on ACKR1, offering the opportunity to be part of a beautiful story, which also provided the occasion for learning BM imaging. Thank you Antal, for always spending compliments on both my work and me as a person.

The biggest thank you goes to my mentors, but also dear friends, Dr. Remco Megens and Dr. Johan Duchêne, who brilliantly supervised and unconditionally supported me every single day of these past years, even at moments when I was not perceptive enough to immediately recognize (and value) it.

Dear Remco, from the very first time I set foot in freezing Munich for my interview, on that December day in 2013, I realized that you were an exquisite person and working with you couldn’t be anything but a delightful experience. Thank you for teaching me the secrets of microscopy, conveying your passion and dedication to it, and letting me pursue and develop my ideas freely, without ever imposing yours. But most of all, thank you for helping me with settling in my German life, making me feel at home in the IPEK and always, always being there whenever I have needed a pair of non-emotional/unbiased ears to listen to my problems and complaints. Thank you for keeping on doing it even now. And thank you for deciding that you still want to continue to work with me also during the first steps of my post-doc. Your trust and appreciation for everything I do mean so much to me!

Cher Johan, il n'est pas facile de décider par où commencer pour te remercier, la liste des raisons est longue. Dès que tu as accepté de travailler avec moi sur les NCMs, j'ai découvert une nouvelle façon passionnante de concevoir ce métier de "chercheurs". Grâce à toi, j'ai appris à me poser toujours des questions, à évaluer toutes les possibilités, à ne pas craindre les échecs. Tu m'as encore appris à "lire", avec un sens critique, et à "écrire", de manière rigoureuse. Tu m'as montré ce que signifie être vraiment dévoué. Et surtout, avec toi, j'ai compris que la Science est, avant tout, amusante. Merci, car au cours des (plus de) cinq dernières années, je n'ai jamais eu une journée d'ennui. Merci pour les rires, les blagues, les chansons inconnues, le français texte. Merci même pour les disputes qui me font grandir chaque fois davantage, elles aussi. Merci d'être un exemple et une inspiration, travailler avec toi est le mieux que je puisse me souhaiter.

I would like to further thank all people in the big "IPEK family", both past and present, with whom I have shared precious scientific ideas, plenty of enjoyable moments and countless liters of beer/prosecco/spirits. Thanks to Renske, for being a precious confident and a kind friend (and my favorite person to eat chocolate with). To Janina, who has always had a good word for everything and everybody. To Martina, for sharing desks, snacks and laughs and being so fond of my Christmas deco. To Ela, for teaching me German humor with memes and videos. To Aindrila, for immediate bonding and delicious dinners (and your fondness of Chienpo). To Quinte, Michael H., Holger, Norbert, Carlos and Martin, for being protagonists of some of the funniest non-sober memories. To Max for being a lighthearted half-italian gentleman. To Sarah, Raquel, Yvonne, Emiel, Sascha, Larisa, Thomas, Manu, Pati, Sanne, Ariane, Carla, Philipp, Xavier, Doro, Katrin, Michael L., Rundan, Zhen and all others. I have at least one special happy memory of each of you.

Un grazie particolare al "gruppo Italia", gli unici a capire ogni singola sfumatura di buon e cattivo umore senza bisogno di spiegazioni 😉. A Giovanna, che è stata, e tutt'ora è, un'amica ed un sostegno prezioso per ambientarmi sin dal principio. Grazie per le lunghe chiacchierate, le passeggiate, i pranzi e le merende; l'aiuto tuo e di Sven; i sorrisi e le pernacchie di Marianna. Grazie a Bartolo, per condividere opinioni, gioie e dolori di vita in terra teutonica. E per essere sempre aggiornato sui migliori concerti a Monaco. Grazie a Lucia, perché senza di te in ufficio non so come farei. Per avermi reso dipendente dal ginseng. E per essere la miglior psicologa in circolazione. E grazie a Donato, per essere stato un valido collega.

Un sentito riconoscimento al mio Paese, l'Italia, e al suo sistema di istruzione. Sottofinanziato, bistrattato, spesso deriso e denigrato. Eppure in grado di sfornare quotidianamente eccellenze riconosciute ed apprezzate in tutto il mondo. Grazie alla scuola e all'università italiana ho potuto permettermi di proseguire la mia formazione all'estero, senza mai sentirmi svantaggiata né per conoscenze o competenze, né per difficoltà linguistiche, approccio al mondo del lavoro o capacità

di affrontare le difficoltà della vita in generale. Grazie Italia, perché nonostante tutto, rimani il Paese più bello del mondo, e solo la lontananza permette di renderti realmente giustizia.

Vielen Dank an Deutschland, mein Adoptivland, und an sein Arbeitssystem, das Engagement und guten Willen auszeichnet, egal aus welchem Hintergrund man kommt. Nach fast sechs Jahren kann ich endlich sagen, dass ich hier eine zweite Heimat gefunden habe. Und darüber freue ich mich sehr.

E infine, ma in cima alla lista del mio cuore e dei miei pensieri, un GRAZIE infinito alla mia famiglia, solida base di tutti i miei traguardi di vita e testimone costante delle mille e più peripezie per raggiungerli. A mia sorella Letizia, senza la quale nessun giorno di questi ultimi vent'anni avrebbe avuto lo stesso sapore. Leti, nonostante da sei anni non dividiamo più la stessa camera, non passa giorno senza la tua simpatia e il tuo pungente umorismo. Grazie per essere la mia migliore amica, sempre e dovunque. Alla mia mamma, che mi fa iniziare ogni mattina con un "Buongiorno" e finire ogni sera con una "Buonanotte", passando per i vari "Sei al lavoro?", "Hai mangiato?", "Che fai?". Mami, grazie per essere il mio più grande punto di riferimento, la costante di tutta una vita. Se sono come sono, e ho imparato ad affrontare ogni difficoltà a testa alta, lo devo soprattutto a te e al tuo grande esempio. Al mio papà, i cui occhi pieni di orgoglio ogni volta che mi guarda, sono forse il mio traguardo più bello in quanto figlia. Grazie papo, per avermi sempre spronata ad essere me stessa, a perseguire i miei obiettivi senza paura, affiancandomi sempre all'inizio di ogni nuovo percorso: questo dottorato non ha fatto eccezione. A Chienpo, per aver portato nella mia vita un nuovo tipo di amore, uno sguardo dolcissimo e una sacco di morbido pelo ♥. Vi voglio bene!

Danke!

Merci!

Grazie!

Thank you!