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Cellular characterization of yolk sac-derived macrophages

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Berichterstatter:	Prof. Dr. Christian Schulz
Mitberichterstatter:	Prof. Dr. Christian Ries
Mitbetreuung durch den promovierten Mitarbeiter:	Dr. Christopher Stremmel
Dekan:	Prof. Dr. med. dent. Reinhard Hickel
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1 Abbreviations

ABCA	ATP-binding cassette transporter
AD	Alzheimer disease
AGM	Aorto-gonado-mesonephros
ALS	Amyotrophic lateral sclerosis
BM	Bone marrow
BSA	Bovine serum albumin
CAF	Cancer-associated fibroblast
CCL	Chemokine (C-C motif) ligand
CCR2	C-C chemokine receptor type 2
CD	Cluster of differentiation
CNS	Central nervous system
COX-2	Cyclooxygenase-2
CRP	C-reactive protein
CSF1R	Colony stimulating factor 1 receptor
CX3CR1	CX3C chemokine receptor 1
CXCL	Chemokine (C-X-C motif) ligand
D	Day
DC	Dendritic cells
E	Embryonic day
EAE	Experimental autoimmune encephalitis
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMP	Erythro-myeloid progenitors
ER	Estrogen receptor
ERBD	Estrogen-binding domain
FACS	Fluorescent activated cell sorting
FL	Fetal liver
GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage colony stimulating factor
GMPs	Granulocyte-macrophage progenitors

HDAC	Histone Acetyl Deacetylase
HIF-1	Hypoxia-inducible factor 1
HSC	Hematopoietic stem cell
Ig	Immunoglobulin
IGF-1	Insulin-like growth factor 1
IHC	Immunohistochemical
IL	Interleukin
INF-γ	Interferon gamma
iNOS	Inducible nitric oxide synthetase (iNOS)
IRF	Interferon regulatory factors
ITGb	Integrin beta
LDL	Low density lipoprotein
LT	Leukotriene
LXR	Liver X receptor
M-CSF	Macrophage colony-stimulating factor
MAC	Membrane attack complex
Mac-1	Macrophage-1 antigen
MAM	Metastasis-associated macrophage
MBL	Mannan-binding lectin
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
MPS	Mononuclear phagocyte system
MRC1	Macrophage mannose receptor C1
MS	Multiple sclerosis
MSCV	Murine stem cell virus
NADPH-oxidase	Nicotinamide adenine diphosphate oxidase
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PD-Ls	Programmed death ligands
PDGF	Platelet-derived growth factor
PECAM	Platelet endothelial cell adhesion molecule
PFA	Paraformaldehyde

PG	Prostaglandin
PPARg	Peroxisome proliferator-activated receptor-gamma
PRR	Pathogen recognition receptors
RNA	Ribonucleic acid
RT-PCR	Real-time polymerase chain reaction
RUNX1	Runt-related transcription factor 1
STAT	Signal transducer and activator of transcription
TAM	Tumor associated macrophage
TGF-β	Transforming growth factor beta
TH	T helper cell
TLR	Toll-like receptors
TMEM	Tumor microenvironment for metastasis
TNF-α	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor
YS	Yolk sac

2 Introduction

2.1 Innate immunity

Organisms have to be in permanent exchange with their environment in order to survive and are repeatedly exposed to potential pathogens and organisms. They have to distinguish endogenous from exogenous and obligatory from non-obligatory pathogenic germs. The innate immune system is mandatory to protect humans from a plethora of permanently surrounding pathogens.

As a first barrier, the human body possesses a variety of surfaces able to bar pathogens, namely the stratum corneum of the skin, intestinal mucosa, respiratory and urogenital epithelium. Mucous is considered to be essential to this barrier. It contains multiple protective substances such as defensins, lysozyme, lactoferrin and IgA. In addition, stomach acid kills diverse germs in the gastrointestinal system. In case of failure of this first barrier, phagocytic immune cells have to eradicate or attenuate potential pathogens. (Eales, 1997; Kaufmann, 2013; Joachim Rassow, 2012)

Elie Metchnikoff, a Ukrainian zoologist, is considered to be the pioneer of the innate immune system. He is known to be the first one to emphasize the impact of phagocytosis on the development, on biological balances and on infections of organisms. Together with Paul Ehrlich, the discoverer of the humoral immune system, he won the Nobel prize in 1908. Metchnikoff observed phagocytosis in transparent starfish larvae. Detecting the attacking and subsequent engulfing process following the insertion of foreign components lead him to name and define phagocytosis. Even back then he understood phagocytosis not simply as a form of ingestion, but also as a defense mechanism against foreign bodies. (Gordon, 2016a, 2016b; Rosales & Uribe-Querol, 2017; Tan & Dee, 2009; Tauber, 2003)

Phagocytosis is known as one of the oldest and most primitive processes to secure homeostasis and it occurs in all species. Protozoa regulate vital activities such as gas exchange, digestion and cellular defense by the use of phagocytosis. In vertebrates, phagocytosis is performed by cells belonging to the mononuclear phagocytic system (MPS), such as monocytes or macrophages. Phagocytosis, the

internalization and enveloping of diverse particles, is not only used against invading bacteria or other foreign bodies in the context of inflammation or infection but is likewise helpful to eliminate necrotic or apoptotic cells. (Fig. 1) (Eales, 2003)

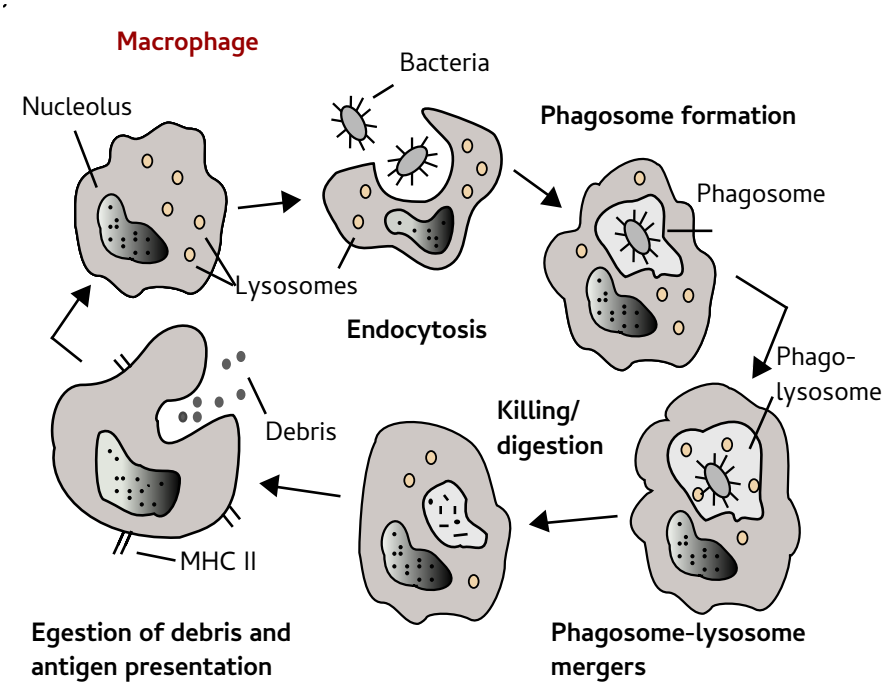


Figure 1: Phagocytosis executed by a macrophage
Bacteria are engulfed by the macrophage and subsequently enclosed by the formed phagosome. Within the merger of phagosome and lysosomes bacteria are digested, excreted in form of debris and presented to cells of the adaptive immunity (adapted and modified from Todar 2008 (Todar, 2008)).

A precise distinction between vital and apoptotic cells and an accurate detection of exogenous invading pathogens has to be preconditioned. Specific pathogen detecting proteins could be identified – so called pathogen recognition receptors (PRR) of phagocytic cells. These receptors identify characteristic prokaryotic structures, termed pathogen-associated molecular patterns (PAMPs). Endothelial cells, DCs, macrophages and other cells belonging to the innate immune system, comprise toll-like receptors (TLR) in their cell membrane. TLR contribute to bacterial detection and activate the transcription factor NFkB, initiating the expression of genes essential to effective immune responses. More than 10 different TLR could be identified until now. With their specificity against particular bacterial components, TLR are indispensable to immune defense. In addition,

formyl-peptide receptors bind to prokaryotic proteins and are fundamental to granulocytes. DCs and macrophages detect particles via the scavenger receptors before subsequent phagocytosis.

The innate immune system is responsible for a primary non-antigen specific and upon infection immediately mobilized reaction against foreign substances and pathogens. It consists in three major components: phagocytizing cells, such as neutrophils or macrophages, plasma proteins and natural killer cells. By recognizing integral components of pathogens or cells of the body, they initiate or inhibit the immune response. Subsequent to phagocytosis, neutrophils emit inflammatory proteins which lead to an intensified immune defense by blocking bacterial growth and causing fever, by increasing vessel permeability and inducing migration of more phagocytotic cells.

Released cytokines stimulate a further crucial defense mechanism; the complement system. This defense mechanism leads to a recruitment of more phagocytic cells, to a superior pathogen recognition and in total to a faster elimination of pathogens. Cytokines induce a release of acute phase proteins in the liver which activate the classical or lectin pathway ((C-reactive protein (CRP) or respectively mannan-binding lectin (MBL). Antibodies and the adaptive immune system are equally able to activate the complement system. The classic way is antibody-dependent and requires the disposability of IgM or IgG antibodies ligated to detected antigens. The Fc-part of these antibodies can bind C1q, a first protein of the complement system. CRP bound to bacterial polysaccharides can equally recruit C1q and activate the classic path of the complement system. Subsequently, a reaction cascade is induced. Therefore, C2, C3, C4 and C5 are activated. C5b, a cleavage product of C5, initiates the composition of the membrane attack complex (MAC) containing C6 to C9. This MAC is finally able to lyse invading bacteria. The complement system can operate in an antibody-independent manner, the alternative way. C3b is binding covalently to microorganisms and conversely ligated to CR1, a receptor expressed by phagocytic cells such as macrophages or granulocytes. These cells are subsequently capable of phagocytizing ligated microorganisms. In addition, C3b can recruit C5 in order to initiate the MAC. C3b is continuously produced and eliminated if no foreign surface can be detected. Endogenous cells escape from the alternative activation path via CD55 and CD45, proteins eliminating C3b. Various bacteria expose carbohydrates containing

mannose which can be detected by MBL. The structure of MBL is very similar to C1q which could be the reason why MBL is capable of complement system activation. Cleavage products C3a and C5a serve as chemokines, attracting macrophages and granulocytes. These latter likewise initiate histamine release in mast cells and depict an important part of anaphylactic reaction (Fig 2). (Eales, 1997; Kaufmann, 2013; Joachim Rassow, 2012; Robertson, 1998; Stites, Terr, & Parslow, 1994)

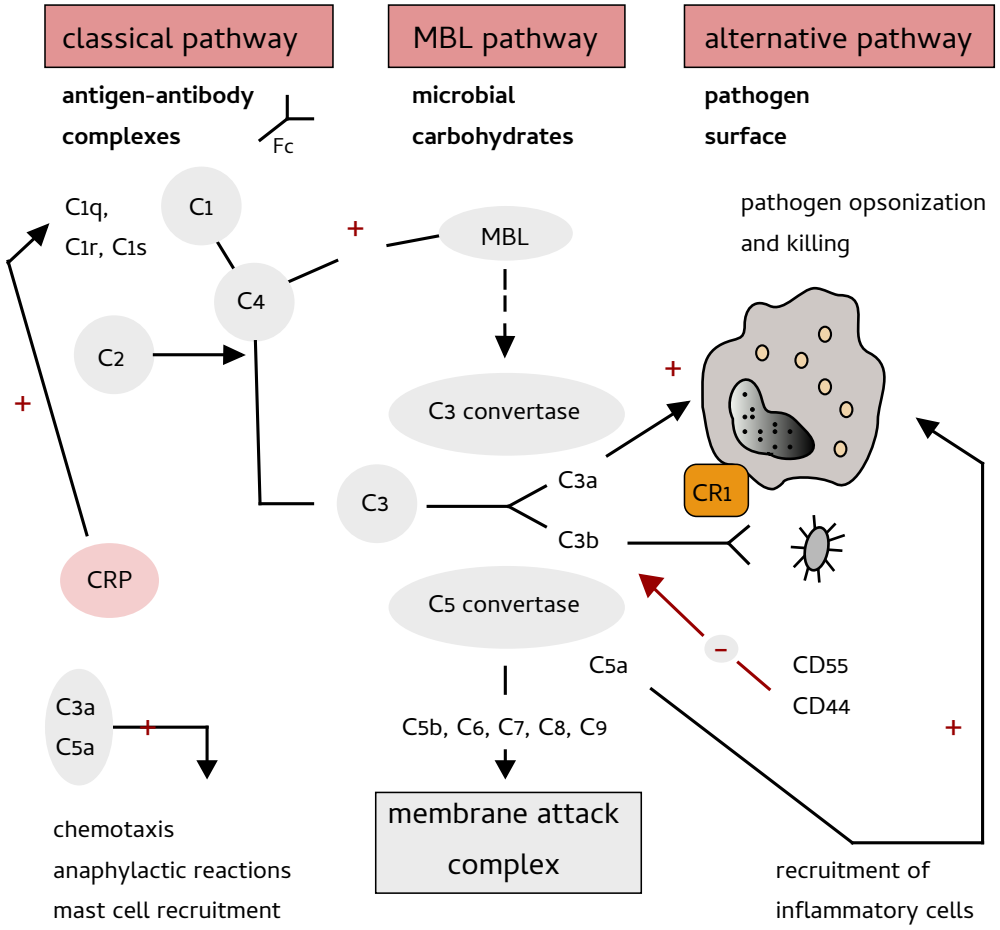


Figure 2: The complement system
Antigen-antibody complexes stimulate the creation of C1q which initiates a cascade of protein activation leading to the MAC that is responsible for pathogen lysis. This system can equally be stimulated by MBL or pathogen surfaces. C3a and C5a are chemotactic proteins that can induce anaphylactic reactions or recruit mast cells (adapted and modified from De Vriese 2015 (De Vriese, Sethi, Van Praet, Nath, & Fervenza, 2015)).

2.2 Innate and adaptive immunity

As opposed to protozoa, vertebrates developed a second defense mechanism during evolution, the adaptive immune system. This acquired immune system can be activated alongside of the established innate immune system and advantages vertebrates in comparison with other, more primitive species. Reappearing pathogens can more easily be attacked and homeostasis is considered as ensured. Compared to the innate immune system, the adaptive immune system appears in a more specific and antigen dependent manner. The acquired immune response is based on specific pathogenic surface molecules. These can be identified by particular cells, belonging to the innate immune system, and presented to diverse cells of the adaptive immune system. Through the interaction between these two defense mechanisms, specific antibodies against invading pathogens can be generated and a more effective immune response is guaranteed. Whilst the initial creation of antibodies is protracted, antibodies appear sooner after the contact with a reappearing pathogen since memory cells could be established. Initially, the innate immune reaction interacts faster than the acquired one. However, the acquired immune system is able to eliminate foreign substances exceedingly specific and more effective. (Fig. 3) (Eales, 1997, 2003; Kaufmann, 2013; Stites et al., 1994)

Adaptive immunity is the basis of vaccinations and the prevention of certain infections. In the 18th century during the cowpox epidemic, various experiments showed that the application of attenuated or dead pathogens could prevent the manifestation of the primary disease and Jenner coined the term “vaccination”. As a consequence of further research, the idea that these pathogens induced the creation of memory cells and stimulated a specific immune response, was established. (Ginglen & Doyle, 2017)

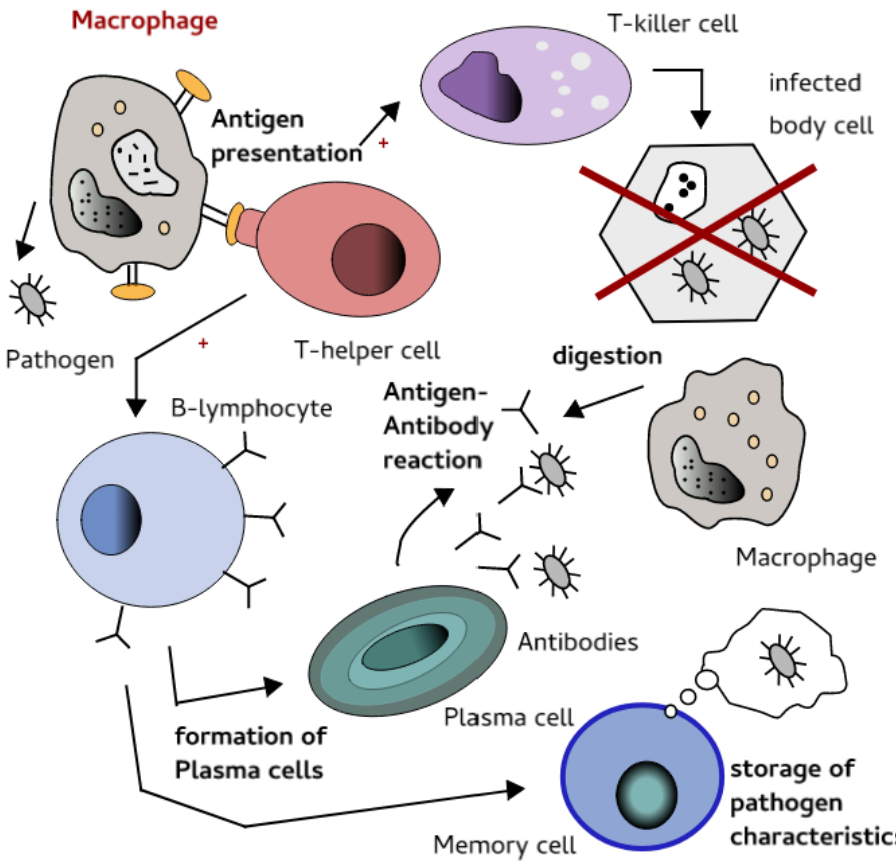


Figure 3: Innate and adaptive immune response
T helper cells recognize antigens presented by macrophages, T-killer cells are formed and kill infected body cells. B-lymphocytes, activated by T-helper cells, form Plasma cells which cause an antibody production enabling a more efficient pathogen elimination. Formed memory cells accelerate future immune responses (adapted and modified from Baumann et al. 2014 (Baumann, 2014)).

The innate immune system consists of a complex interaction between humoral and cellular components. It is the first defense mechanism of organisms and therefore essential for survival.

The following chapters will focus on the role of macrophages in the broad and complex system of immune responses.

2.3 Macrophages

2.3.1 Origin

Macrophages arise from different origins, including the yolk sac (YS) and the bone marrow (BM) hematopoiesis.

As other blood cells, certain macrophages can be replenished by hematopoietic stem cells (HSCs). Former studies emphasized the aorto-gonado-mesonephro (AGM) region as the first site where HSCs appear around embryonic day 10.5 (E10.5). Subsequently, HSCs colonize the fetal liver (FL), where fetal hematopoiesis further develops. A significant proliferation of these cells can be detected from E12.5 onwards. (Bertrand et al., 2005; Cumano & Godin, 2007; McGrath, Frame, & Palis, 2015; Orkin & Zon, 2008; Schulz et al., 2012)

Alliot and co-workers were the first to describe the YS as an origin of macrophages (Alliot, Godin, & Pessac, 1999). Recently, several independent studies confirmed this primary origin. Blood islands in the YS represent the first tissue giving rise to hematopoietic progenitor cells. These appear around E7.5 (Alliot et al., 1999), preceding the primary appearance of the first HSCs in the AGM region. (Fig. 4) (Bertrand et al., 2005; Cline & Moore, 1972; Cumano & Godin, 2007)

From E8.5. onwards, definitive erythroid (BFU-E), bipotential granulocyte/macrophage progenitor cells (GM-CFC), mast cell progenitors (Mast-CFC) and other myeloid progenitor subtypes can be perceived in the YS. These different subsets arise from the same cohort, named erythro-myeloid progenitors (EMPs), possessing a diverse myeloid potential. EMPs can be identified in the mouse bloodstream and the FL from E10.5 onwards. Thus, the emergence of EMPs and proliferation of HSCs in the AGM region is overlapping. (McGrath et al., 2015)

Two distinct subsets of myeloid cells can be distinguished in the FL. Fetal erythrocytes, macrophages and granulocytes originating from EMPs of the YS and HSCs arising from the AGM region. (Bertrand et al., 2005; Cumano & Godin, 2007; Schulz et al., 2012)

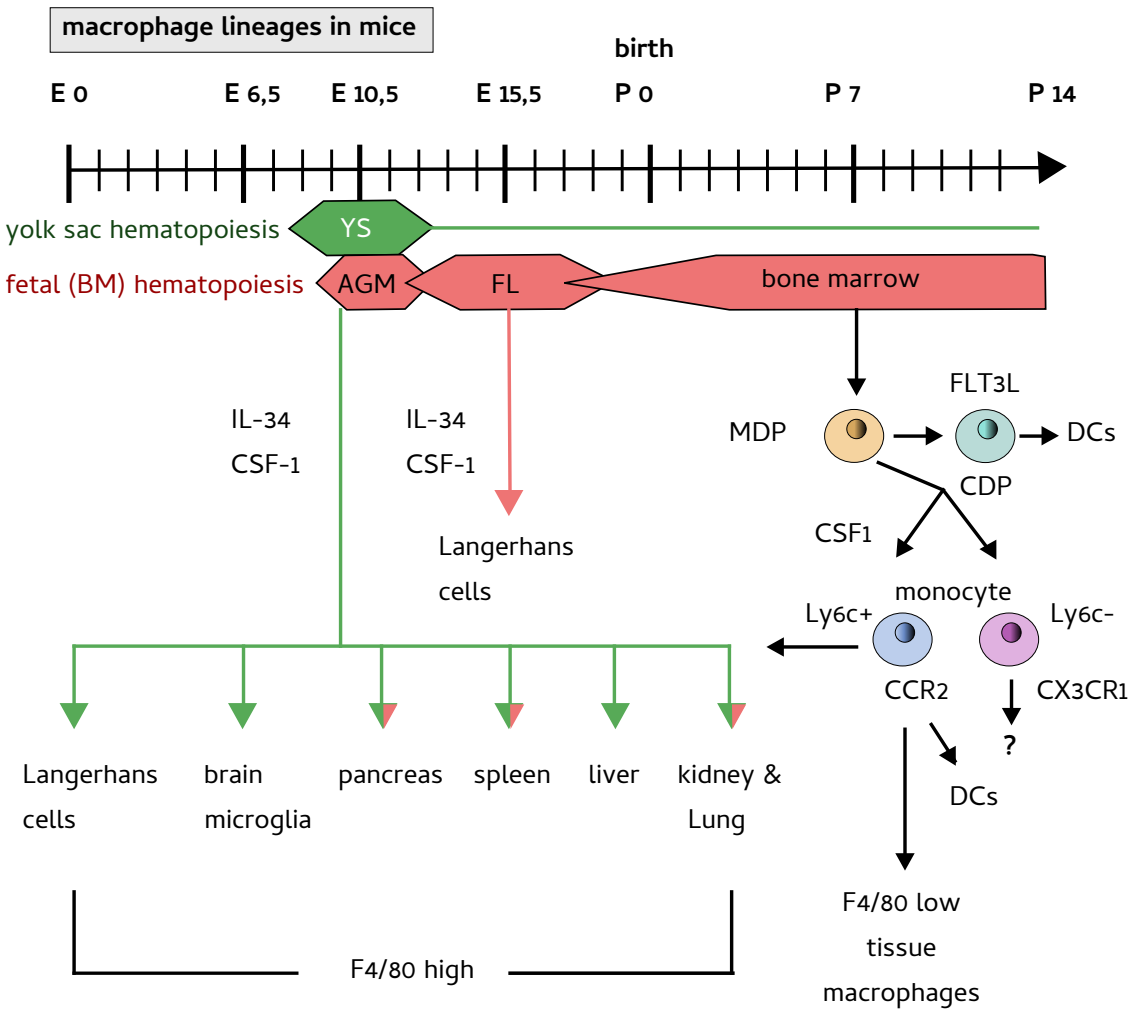


Figure 4: Macrophage lineage in mice
YS-derived cells appear from E7.5 onwards and are persistent in adult mice. Macrophage populations originating from the YS can be identified in different organs such as skin, brain, pancreas, spleen, liver, kidney and lung. Definitive hematopoiesis is initiated around E8.5, firstly in the AGM and followed by the FL and BM (adapted and modified from Wynn 2013 (Wynn, Chawla, & Pollard, 2013)).

In order to clearly identify different subsets of progenitors, reporter mouse models have been established. In adult mice, the fractalkine receptor, equally known as CX3C chemokine receptor 1 (CX3CR1), is expressed by monocytes and subsets of the MPS, in particular macrophages and DCs. Primitive macrophage precursors arising in the YS around E7.25 infiltrate the embryo once blood circulation occurs. EMPs, the principal source of tissue resident macrophages, appear around E8.25. Progenitors express receptor tyrosine kinase KIT (c-KIT, CD117) and the colony

stimulating factor 1 receptor (CSF1R), similar to the phenotype of HSCs. (Stremmel et al., 2018)

Pre-macrophages are CD45+ and express Macrophage-1 antigen (Mac-1), a heterodimeric receptor consisting of integrin alpha M (CD11b) and integrin β2 (CD18) as well as CX3CR1. Along with macrophage differentiation, an upregulation of F4/80 can be perceived. CSF1R, similarly known as CD115, is equally upregulated in differentiated macrophages and plays a key role in the chemokine-receptor-dependent colonization of the whole embryo. Specific markers permit genetic labeling and the visualization of macrophage progenitors along the development of the embryo. (Bertrand et al., 2005; Mass et al., 2016; Stremmel et al., 2018).

The CX3CR1-GFP mouse model allows to highlight and further analyze macrophages arising from the YS. As a consequence, Schulz and co-workers were the first to distinguish embryonic YS-derived macrophages and to compare these latter to macrophages arising in the BM. In contrast to BM-derived macrophages, macrophages originating from the YS show a greater F4/80 but lower CD11b expression. Additionally, they reveal - similar to BM-derived macrophages - a PU.1-dependence in their development. YS-derived macrophages are Myb-independent and show a weak to none expression of Gata2, Ccr2 and Flt3 compared to their BM-derived counterparts. (Schulz et al., 2012)

Following the colonization of the embryo, pre-macrophages transform into diverse subsets of tissue-specific mature macrophages. Tissue resident macrophages have different functions. To name some examples, microglia in the brain enable brain development and the functionality of synapses, Kupffer cells eliminate cell debris in the liver and alveolar macrophages reduce air pollutants and pathogens in the lung (Mass et al., 2016). In order to maintain their perpetual function, tissue resident macrophages are capable of self-renewal. (Gomez Perdiguero, Schulz, & Geissmann, 2013; Schulz et al., 2012)

Macrophages vary in different micro-environments regarding their differentiation. This diversity enables vice versa tissue specific functions as mentioned before. Recent studies were able to identify so-called tissue-specific transcriptional regulators contributing to micro-environment-dependent differentiation. For example, the expression of Gata6 influences the development of large peritoneal macrophages, Runx3 contributes to the differentiation of Langerhans cells, Nr1h2

participates in the emergence of splenic marginal zone macrophages, whereas SpiC is important for the development of splenic red pulp macrophages, Pparg emerged as contributing to the alveolar macrophage emergence and pre-macrophages acquire Id3 in order to differentiate into Kupffer cells. (Fig. 5) (Gordon, 2012; Mass et al., 2016)

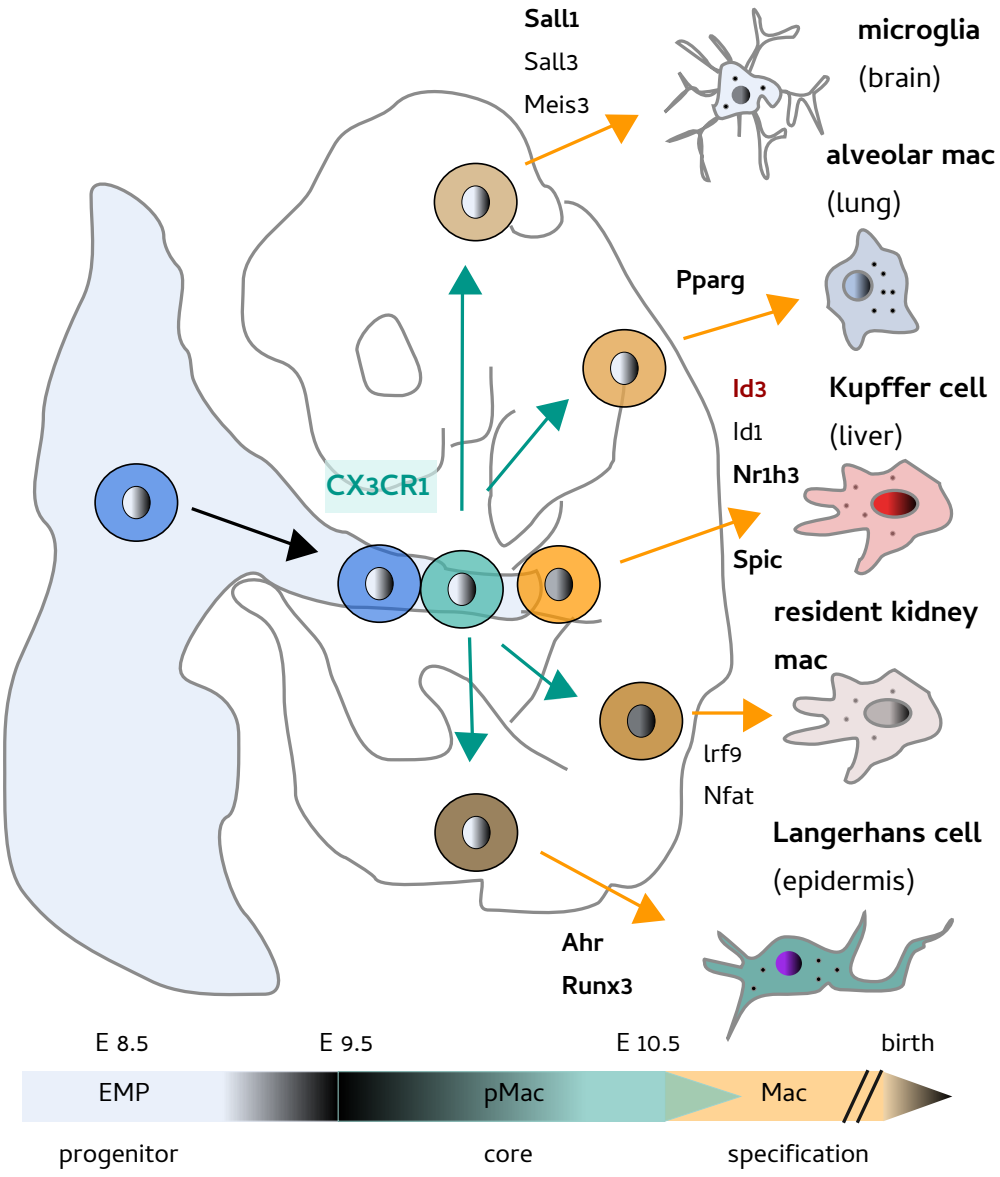


Figure 5: Specification of tissue-resident macrophages
YS-derived EMPs colonize the embryo starting with the FL around E9.5. Macrophage precursors (pMacs) are formed, initiating a core specification program in a CX3CR1 dependent manner. Respective regulators guide these transcription processes into diverse tissue-resident macrophages and are indispensable for their development and maintenance (adapted and modified from Mass 2016 (Mass et al., 2016)).

Erythroid and myeloid cells originating from the YS are partially replaced after the onset of BM hematopoiesis. This replacement varies concerning time and degree in each tissue. In the case of brain microglia, replacement of macrophages can only occur within the frame of injuries. Indeed, microglia consists solely of YS-derived macrophages. Other examples of YS-derived tissue-resident macrophages that are not replaced by cells originating from the BM are Kupffer cells in the liver, Langerhans cells in the epidermis and alveolar macrophages in the lung. Ultimately HSC-derived tissue-resident macrophages can be detected in other organs such as skin, intestine and others. (Gomez Perdiguero et al., 2015; Gomez Perdiguero et al., 2013; McGrath et al., 2015; Schulz et al., 2012)

2.3.2 Basic functions of macrophages

Macrophages are responsible for various functions such as immune defense, homeostasis and tissue repair. In cooperation with monocytes and dendritic cells, they form the MPS (Hume, 2006). Macrophages can be detected in all tissues. Since they appeared to be characteristic for certain organs, tissue-resident macrophages were consequently termed differently depending on their tissue of residence. Prominent examples are Kupffer cells in the liver, osteoclasts in bone, microglia in the brain and Langerhans cells in the skin. (Gordon, 2012; Mass et al., 2016)

A variety of experiments lead to a specific classification of macrophage subpopulations. Macrophages were firstly separated into pro- (M1) and anti-inflammatory (M2) subdivisions. Classically-activated macrophages (M1) are stimulated by T helper cell 1 (TH1) characteristic substrates such as interferon gamma (INF-γ) or tumor necrosis factor alpha (TNF-α). They secrete inflammatory cytokines, for instance IL-12 or IL-23, and perform antigen presentation via major histocompatibility complex-II (MHC-II) molecules. As a consequence, they detain invading bacteria. M1 macrophages show a high IL-12 and low IL-10 expression and express inducible nitric oxide synthetase (iNOS) in murine experiments. In contrast, M2 macrophages are indispensable to homeostasis. They are involved in vascular development, tissue repair and anti-inflammatory activities. M2 macrophages show a characteristic expression profile, namely scavenger receptors, arginase-1, e-cadherin and further substances. Compared to M1

macrophages, they show an inferior IL-12, but superior IL-10 expression. M2 macrophages can be subdivided by their respective inducement in M2a or M2b macrophages. Cytokines, as for instance IL-4 or IL-13, activate M2a, whereas M2b macrophages can be stimulated by immune complexes in liaison with TLR and IL-1 receptor antagonist. The M2c population is under the influence of glucocorticoids and IL-10. Despite the fact that even considerably more subpopulations exist and occur in the innate immune system, this simplified classification elicits a primary comprehension of various macrophage functions. These subpopulations arise from diverse precursors and differentiate in interaction with cytokines such as M-CSF or granulocyte macrophage colony stimulating factor (GM-CSF) or other transcription factors. Mentionable are for instance PU.1 or interferon regulatory factors. The final differentiation process in M1 or M2 macrophages is highly orchestrated and depends on abundant additional elements such as specific transcription factors. Members of the signal transducer and activator of transcription (STAT) protein family are for instance known to play a key role in this differentiation process, but little of these pathways have been elaborated until today. (Fig. 6) (Schmieder, Michel, Schonhaar, Goerdt, & Schledzewski, 2012; Zhang & Wang, 2014)

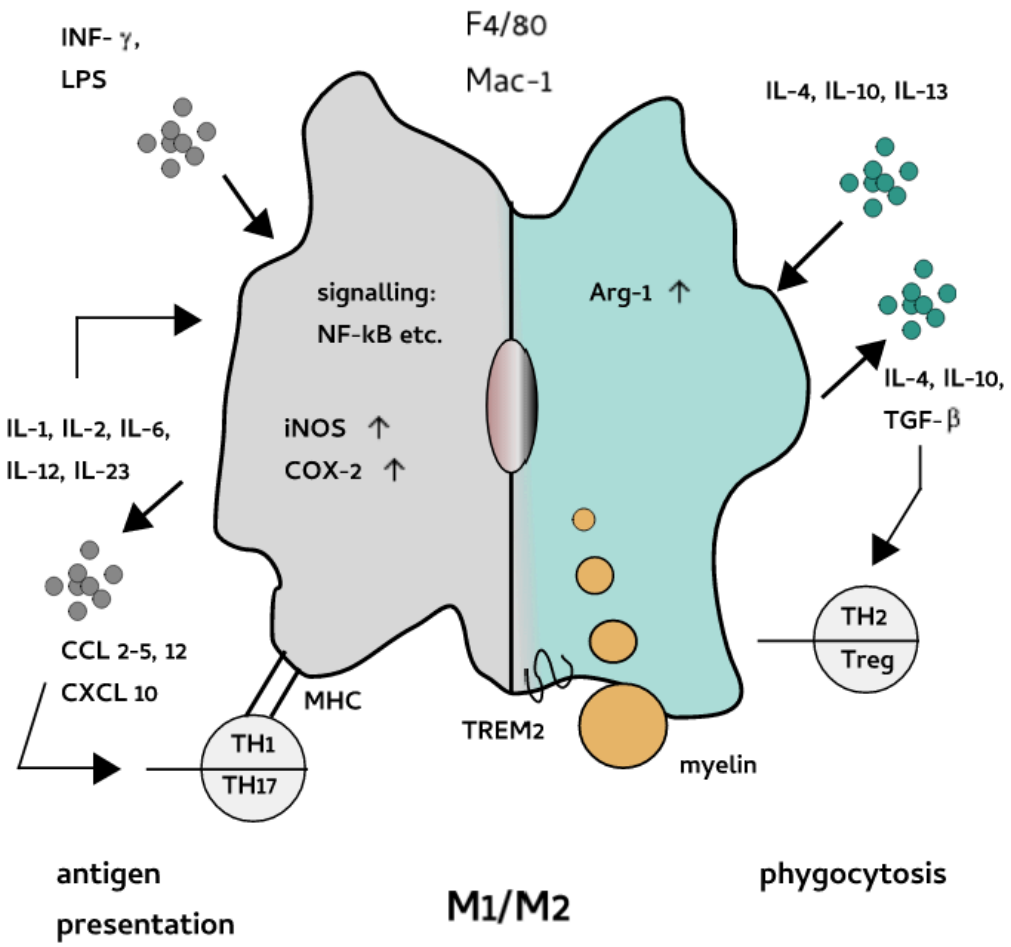


Figure 6: M1 and M2 macrophages
Depiction of pro-inflammatory macrophages, M1, versus anti-inflammatory subdivisions, M2, and their specific substrates and activation pathways (adapted and modified from Goldmann 2013 (Goldmann & Prinz, 2013)).

2.3.2. Phagocytosis and receptor

Being the main responsible components in homeostasis, macrophages constantly sample their environment by pinocytosis, meaning the formation of surface vesicles containing extracellular fluids. In this context, diverse receptors are indispensable. Macrophages are able to detect released opsonins, for instance complement components or immunoglobulins, chemotactic factors, growth factors, cytokines, hormones and other mediators. Once macrophages are in direct contact with bacteria, microorganism related endotoxin, tissue damage products, proteins resulting from the complement or blood coagulation system or other

previously mentioned ligands, they are activated and undergo a specific activation process. Consequently, macrophages enhance their metabolic capacity, motility and phagocytic processes. They show an enlarged cytoplasmic volume which leads to facilitated phagocytosis. As a result, activated macrophages phagocytize all kinds of surrounding foreign particles or cell debris. A principal initiation method is opsonization. Fragments of the complement protein C3, certain carbohydrates or immunoglobulins are detected by opsonin-specific receptors which subsequently guide their engulfment within membrane-bounded vacuoles, termed phagosomes. Other ingestion processes performed by macrophages are pinocytosis, phagocytosis by pseudopodia formation and receptor-mediated endocytosis, all of which generate phagosomes. Lysosomes, cell organelles containing digestive enzymes, merge with a created phagosome forming the phagolysosome. Before digestion occurs in the phagolysosome, engulfed particles get immediately acidified as soon as they get ingested. Thus, lactic acid and hydrogen ions accumulate, move via specific pumps into the lysosomes, reduce the present pH and initiate finally the release of acidic granule contents in order to dissolve engulfed particles. Components of these granules are defensins, lysozyme and lactoferrin. The creation of lactic acid and hydrogen ions are part of the respiratory burst, which is also known as oxidative or metabolic burst. It occurs in stimulated macrophages and causes accumulation of toxic oxygen metabolites. Indeed, highly reactive molecules are created by nicotinamide adenine diphosphate (NADPH) oxidases, a group of involved enzymes. These free radicals are capable of protein, lipid, DNA, cell and bacterial destruction. Under unstimulated circumstances, created radicals are attenuated by endogenous scavenger receptors or enzymes present in surrounding cells. In comparison to neutrophils, macrophages are less efficient regarding phagocytosis. Macrophages show lower amounts of lysosomes but contain organelles which are able to renew lysosomes as needed. As a consequence, all engulfed particles are entirely dissolved. In addition, macrophages show the capability to kill invaded germs. (Stites et al., 1994)

2.3.2.2 Chemokine and chemotaxis

At the onset of inflammation or tissue damage, inflammatory mediators are released by macrophages, mast cells, basophils and other types of surrounding cells. Mentionable chemicals are prostaglandins (PGs) and leukotrienes (LTs), both deriving from arachidonic acid. Macrophages, which are resident in the inflamed tissue, recognize these chemicals and convert into an activated form. This leads to a release of cytokines such as IL-1, TNF-α and chemokines, which in turn stimulate endothelial cells to express adhesion molecules to enable diapedesis of leucocytes passing the adjacent vessel. Inflammatory mediators initiate inflammation signals, enable vascular permeability and cause chemotaxis, the directed migration towards the inflammatory site. A possible explanation for this targeted movement could be the fact that ligated chemokines induce pseudopod formation. The interaction between numerous of these chemokines leads to a directed movement towards the damaged tissue or respectively inhibits movements in any other direction. Accompanying these chemokines, C3a and C5a, already mentioned complement proteins, play a key role as chemotactic agents. To conclude, the principal aim of chemotaxis consists in enticing phagocytic cells in order to eliminate pathogens or cell debris. Supplementary to this first chemotactic movement, recently arrived cells are able to release further chemokines themselves. Mentionable are IL-8, produced by monocytes, lymphocytes, granulocytes, fibroblasts, endothelial and other cell types, macrophage inflammatory proteins 1α and 1β, released by monocytes, macrophages, T and B cells, or monocyte chemo-attractant proteins, induced by stimulated mononuclear cells. As a result, effective immune reactions and wound-healing can occur. (Eales, 1997; Gordon, 2003; Iwasaki & Medzhitov, 2015; Wynn & Barron, 2010)

2.3.2.3 Interaction

Macrophages play a critical role regarding homeostasis and innate immunity. A main part of their functions is based on interactions with other cell types. Thus, they do not operate exclusively under the guise of the innate immune system but cooperate additionally with cells belonging to the acquired immune response.

Subsets of T helper cells (CD4+) are in direct contact with macrophages, as TH1 cells release lymphokines stimulating macrophages and cytotoxic T cells (CD8+). Vice versa, macrophages operate as antigen presenting cells. They transform digested particles originating from invaded pathogens and present them to T cells. This antigen presentation is enabled by MHC Class II molecule expression on the surface of macrophages. A main purpose of this presentation consists in defending bacteria, fungi or protozoon. Alike all other nucleated cells, macrophages express type MHC Class I molecules. Class I molecules are identified by cytotoxic T cells and conduce to an elimination of viral infections or cell debris. However, the degradation of invading bacteria via Class II molecules and the defense of viral infections via MHC Class I molecules are sometimes overlapping. Certain microorganisms can migrate from the endosome into the cytoplasm. Their bacterial proteins can be presented by Class I molecules. Vice versa, endogenous particles can be transferred into exogenous antigens and presented to T helper cells. Crosspriming ways, meaning the CD4 and CD8 stimulation, can be initiated once infected cells form antigen containing vesicles. These vesicles are subsequently taken up by dendritic cells. DCs are able to present antigens in a MHC I or II dependent manner. In addition, a conspicuous cooperation between cytotoxic killer cells and T helper cells is acknowledged. (den Haan & Kraal, 2012; Eales, 1997; Iwasaki & Medzhitov, 2015; Kaufmann, 2013)

2.3.3 Pathogen defense

As previously mentioned, macrophages are able to kill invading germs. Essential to their defense mechanism is phagocytosis. In addition, macrophages are able to detect attacking pathogens by their PAMPs with the help of special pathogen recognition receptors such as TLRs (Wynn et al., 2013). To name an example, *Salmonella typhi* and *Listeria monocytogenes* reveal this mechanism regarding their recognition by macrophages (Shaughnessy & Swanson, 2007). At the onset of detection, M1 like macrophages become activated, which leads to a subsequent release of pro-inflammatory mediators. Noteworthy are in this context TNF-α, IL-1 and nitric oxide (NO). Invading bacteria are eliminated and a further acquired immune response is primed. M2 macrophages are necessary to keep this

inflammation process under control. These latter suppress excessive reactions leading to sepsis and induce healing processes. (Liu, Zou, Chai, & Yao, 2014; Seo et al., 2015; Vannella & Wynn, 2017; Wynn et al., 2013)

Infections caused by viruses can be attenuated with the help of macrophages. Phagocytosis, in particular exerted by M2 macrophages, is intensified by influenza virus stimuli (Hoeve, Nash, Jackson, Randall, & Dransfield, 2012). In addition, these macrophages hamper pulmonary fibrosis during the progress of the severe acute respiratory syndrome (SARS) (Dagvadorj et al., 2015; Page et al., 2012). Macrophages contribute to the development and maintenance of chronic inflammations. Kupffer cells release IL-1β once they get in touch with hepatitis C virus (HCV) and facilitate the formation of fibrosis (Negash et al., 2013).

Macrophages are also involved in parasitic infected tissues. To take the example of *Taenia crassiceps*, M1 macrophages show an initial typical pro-inflammatory behavior, whereas M2 subpopulations occur via IL-4 hereinafter and cause a healing status (Sica & Mantovani, 2012). Concomitantly, macrophages attenuate helminth infections. Regarding the *Leishmania infantum* infection, macrophages initiate a IL-1β dependent inflammatory response and create reactive oxygen species (ROS) (Lefevre et al., 2013).

Taken together, macrophages play a key role concerning the defense of imminent infections since they diminish reactions associated with invading bacteria, viruses or parasites. (Liu et al., 2014; Vannella & Wynn, 2017; Wynn et al., 2013)

2.3.4 Organ injuries and human diseases

In the course of several studies the idea of various macrophage populations with respective different functions could be established. At the onset of inflammation and organ destruction, macrophages enable migration of inflammatory cells by releasing chemotactic factors. With the help of matrix metalloproteinases (MMPs) they demolish already damaged basement membranes and support cell movement. Macrophages do not only phagocytize apoptotic cells or invading bacteria, but also initiate a first inflammatory response. Following this inflammation initiation, macrophages can switch from injury-inducing to repair-promoting populations. Subsequently, they release growth factors such as platelet-

derived growth factor (PDGF), insulin-like growth factor 1 (IGF-1), vascular endothelial growth factor alpha (VEGF-α), transforming growth factor beta (TGF-β) or wnt proteins in order to cause cell proliferation, differentiation and angiogenesis. TGF-β also induces vessel contraction and wound closure by stimulating tissue fibroblasts to differentiate into myofibroblasts. Surrounding stem cells play an important role in this inflammation response as they complete the wound healing process. Essential to tissue repair, arising anti-inflammatory macrophages act in response to IL-10 and TGF-β secretion, express programmed death ligands (PD-Ls) receptors, reduce immune responses in order to ameliorate inflammatory responses and replace - if necessary - tissue resident macrophages. The phenotypic switch from injury-inducing into wound repair macrophages is very important to orchestrate the various parts of fibrosis development and initiate a complete tissue repairing process. These diverse macrophage functions can be observed in several organs and diseases. (Vannella & Wynn, 2017; Zhang & Wang, 2014)

Macrophages play a key role in various pathologies, for example neuropathologies and the development of the central nervous system (CNS) or the maintenance of cancer.

2.3.4.1 Central nervous system – development and neuropathologies

Microglia are phagocytic cells and occur in the brain during organogenesis, where they contribute essentially to the development of the CNS. In the adult brain, they show a homeostasis function and are capable of self-renewal. Besides homeostasis, microglia can physically bind ruptured endothelium with the help of filopodia and lamellipodia (Cybulsky, Cheong, & Robbins, 2016; Kierdorf, Prinz, Geissmann, & Gomez Perdiguero, 2015; Vannella & Wynn, 2017). Microglia are capable of phagocytosis and play a key role in the functionality of synapses. They perform synaptic pruning, distinguish essential from dispensable synapses and eradicate the dispensable ones. Taken together, microglia ensure the efficient proceeding of the neuronal network and the olfactory system. (Paolicelli et al., 2011; Vannella & Wynn, 2017)

Microglia secrete neurotrophic cytokines, like proteins belonging to the neurotrophin family or growth factors such as activin A and IGF-1, which ensure targeted synapse formation and the persistence of neuronal conjunctions (Bogie, Stinissen, & Hendriks, 2014; Parkhurst et al., 2013). On the onset of brain injuries, microglia show inflammatory and repairing activities. Activated microglia proliferate increasingly, intensify their phagocytosis capacity and release of inflammatory cytokines and enhance their antigen presentation capacity. These processes can have counter-productive effects as they can prime neuropathologies. Various studies revealed this phenomenon using the example of multiple sclerosis (MS) or the experimental autoimmune encephalitis (EAE) in mice and demonstrated the effect of macrophage activation on the development of these diseases. Vice versa chimera experiments showed less inflammatory lesions in the CNS by inhibiting macrophage activation. It remains unclear if this macrophage stimulation has to be understood as a secondary effect that occurs subsequent to cytodegeneration or if macrophage activation processes contribute directly to the development of neuropathologies. On the contrary, microglia have a neuroprotective function as EAE affected mice with inhibited microglia activation show a delayed recovery process. Experimental accelerated microglia activation at the onset of EAE showed an enhanced recovery. Further studies highlighted the neuroprotective functionality of macrophages as they are capable of inhibitory myelin debris elimination and production of neurotrophic proteins as mentioned before. Studies revealed the fact that oligodendrogenesis and neurogenesis occurs once microglia are activated by IL-4 and INF-γ. This bi-functional activity of microglia has also been demonstrated in further neuropathologies such as Alzheimer disease (AD), Amyotrophic lateral sclerosis (ALS), Parkinson's disease or after spinal cord injury. (Bogie et al., 2014; Vannella & Wynn, 2017)

2.3.4.3 Cancer

Previous studies emphasized essential characteristics of malignant cells, namely independent proliferation, untouchability by apoptosis or negative growth signals, unlimited cell replication, angiogenesis initiation and capability of tissue invasion.

Macrophages induce vascular development, contribute to tissue elimination and enable tissue invasion and subsequent metastasis. (Hanahan & Weinberg, 2000)

Macrophages have a major impact on the maintenance of chronic inflammation. Several studies accentuated chronic inflammation as a promoter of tumorigenesis. For instance, Helicobacter pylori is known to prime the development of stomach cancer, whereas Schistostoma hematobium is identified to be associated with bladder cancer. (Coussens & Werb, 2002)

Within the frame of tumorigenesis, macrophages act similarly as at the onset of inflammation or tissue damage. They are influenced by tumor associated microenvironments and are stimulated to release growth and angiogenesis factors in order to establish a perfect proliferation environment for the respective tumor. Tumor associated macrophages (TAMs) eliminate apoptotic cells and enable tumor cells to move through surrounding tissues and furthermore into recently created vessels. This facilitated migration and intravasation is essential to metastasis development and preservation of tumor functionality. (Condeelis & Pollard, 2006)

Further studies characterized the involved TAMs and depicted these latter as M2-like macrophages. Primarily installed M1-like macrophages release inflammatory substances, for instance IL-6, IL-12 or tumor necrosis factor alpha (TNF-α), iNOS and cyclooxygenase-2 (COX-2) and establish a tumor suitable environment. Ablation experiments elucidated NF-κB as essential to tumor instruction and development.

M1-like macrophages undergo a phenotypic switch into an anti-inflammatory phenotype and present a characteristic M2 expression profile, namely macrophage mannose receptor C1 (MRC1), low MHC II complex, stabilin-1, arginase-1 and IL-10 and TGF-β. COX-2 has been illustrated to be essential to this phenotypic switch, which points out why COX-2 inhibitors could play a key role in preventing carcinogenesis and have already shown a chemoprotective behavior regarding the development of intestinal cancer. Low oxygen availability causes hypoxia-inducible factor 1 (HIF-1) which in turn facilitates vessel development, metastasis and cell proliferation. Further studies showed a jointly migration by macrophages and tumor cells under the influence of EGF and CSF-1. Moreover, macrophages induce VEGF-secretion by releasing MMP9. EGF or CSF-1 blocking diminishes migration of both, phagocytic and tumor cells. Concomitantly, pharmacological

blocking of EGF reduces cell movement into blood vessels. Enzymes such as arginase-1, iNOS creating nitric oxygen (NO), cysteine, cathepsin and proteases enable tumor progression. Higher quantities of TAMs are associated with poorer cancer outcomes and show an enhanced metastasis and lymphangiogenesis development. Therefore, TAMs are regularly attracted by chemokines, in particular cytokines of the CC chemokines. (Condeelis & Pollard, 2006; Schmieder et al., 2012; N. Wang, Liang, & Zen, 2014)

TAMs reveal different origins, in particular circulating monocytes and embryonically derived tissue-resident macrophages. Zue et al. depicted the meaning of dissimilar origins and their specific functions. In pancreatic cancer models, monocyte-derived TAMs show a more significant role regarding antigen presentation, whereas YS-derived TAMs appear more important concerning the initiation and maintenance of fibrosis as they generate remodeling molecules in the extracellular matrix (ECM). (Pollard, 2017)

TAMS are essential to the initiation of angiogenesis and metastases. TAMs can be activated by CSF-1 and ANG2, which leads to an inhibition of anti-angiogenetic factors and to a release of vascular endothelial growth factor A (VEGFA) or WNT7B which stimulate angiogenesis. Concerning the contribution to an increased invasiveness of cancer cells, TAMs release EGF which stimulates the corresponding receptor. In cooperation with cancer-associated fibroblasts (CAF) and their derived factors, TAMs create a perfect tumour microenvironment for metastasis (TMEM). CCL2 is equally important to TAM activation and metastasis initiation. (Fig. 7) (Kitamura, Qian, & Pollard, 2015; Kitamura, Qian, Soong, et al., 2015; Noy & Pollard, 2014)

Regarding this context, Cassetta et al. investigated the role of TAMs in breast cancer metastases. By using Class IIa Histone Acetyl Deacetylase (HDAC) inhibitors, the invasiveness of cancer cells was disabled as metastasis-associated macrophages (MAMs) and monocytes were reprogrammed and T-killer cells and INF-γ were activated. Adding a conventional chemotherapy or immunotherapy, the anti-tumorous effect was even more significant. (Cassetta & Pollard, 2017)

Taken together, TAMs play a key role regarding tumor initiation and expansion. Macrophages cannot be influenced by tumor associated mutation and contain a stable genome. Being essential to tumor development, TAMs could be a

worthwhile study object regarding targeted therapies. (Condeelis & Pollard, 2006; Schmieder et al., 2012; Wynn et al., 2013)

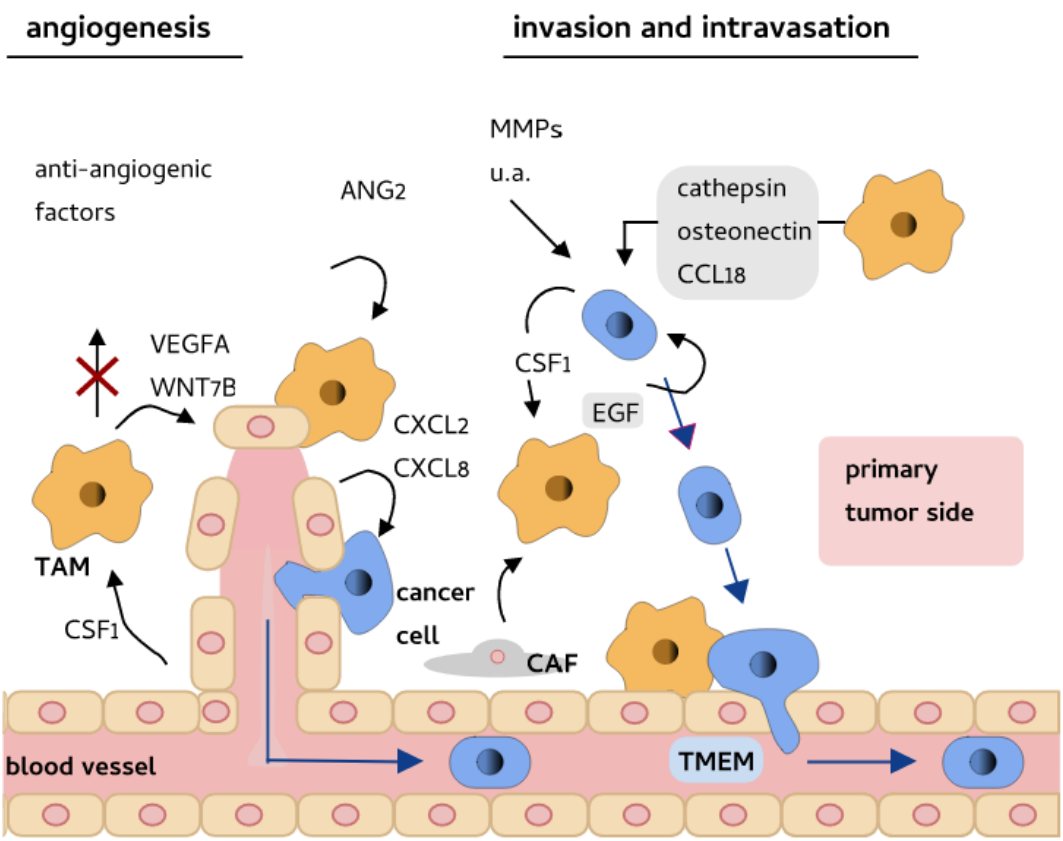


Figure 7: TAMs in the context of angiogenesis and intravasation initiation
TAMs being activated via CSF1 or ANG2 can diminish anti-angiogenic factors and stimulate vice versa angiogenesis indispensable factors. They form the TMEM with the help of CAFs and initiate tumor invasiveness and intravasation with the help of EGF. MMPs and other factors released by TAMs such as cathepsin, osteonectin and CCL18 accelerate these processes (adapted and modified from Kitamura 2015 (Kitamura, Qian, & Pollard, 2015)).

3 Aims of the thesis

3.1 Cellular characterization of isolated YS-derived macrophages

The dual origin of macrophages has been recently identified, namely the yolk sac and bone marrow dependent emergence. However, YS-derived macrophages have not been characterized in detail until now.
CX3CR1-GFP-labeled macrophages will be characterized by fluorescence microscopy, FACS, RT-PCR, immunohistochemical (IHC) staining and cell culture-based experiments.

3.2 Generation and analysis of immortalized yolk sac macrophages

In order to create stable cell culture conditions, an immortalized YS-derived macrophage-committed progenitor cell line shall be established. Subsequent to macrophage differentiation via estrogen removal and M-CSF supplement, created macrophage populations are supposed to be illustrated with the help of bright field images, Giemsa and IHC staining, FACS and RT-PCR. Ultimately, the phagocytosis capability of immortalized YS-derived macrophages will be elaborated via phagocytosis assays consisting in zymosan-ligated particles, detectable after successful engulfment.

4 Methods

4.1 Mouse strains

4.1.1 CX3CR1$^{GFP/GFP}$ mouse

CX3CR1-GFP knock-in mice were kindly provided by Steffen Jung, The Weitzman Institute, Rehovot Israel. (Jung et al., 2000)
Genomic fragments of the murine CX3CR1 locus were replaced by a cDNA encoding eGFP (enhanced Green Fluorescent Protein). With the CX3CR1-eGFP targeting vector mice expressed the reporter protein eGFP instead of the fractalkine receptor and visualization of cells expressing CX3CR1, such as monocytes, DCs, natural killer cells or macrophages, was enabled. During early embryonic development, however, CX3CR1 expression is mostly restricted to monocytes and macrophages (Schulz et al. 2012).

4.1.2 Cmyb$^{-/-}$ mouse

Myb-deficient mice were kindly provided by Jon Frampton, University of Birmingham, United Kingdom. (Mucenski et al., 1991)
In order to interrupt the endogenous c-myb gene, the neo gene from pMCIneo/polyA+ (Thomas and Capecchi, 1987) was used with the aim to create a replacement vector. Because Myb-deficient mice die during late embryonic development, heterozygous mice were bred and used for timed matings.

All animal studies were approved by the Regierung von Oberbayern.

4.2 Antibodies

The following antibodies were used for immunofluorescence.

Antibody	Company (Clone)	Catalogue #	Dilution
Rat anti-F4/80	abcam	ab6640	1:200
Rabbit anti-CX3CR1	abcam	ab8021	1:200
Hoechst	invitrogen	H3570	1:2000
Rabbit anti-Ki-67	NOVUS Bioloicals	NB500-170	1:200
Rat anti integrin beta 2	abcam	ab185723	1:200
Alexa Fluor™ 488 Phalloidin	ThermoFisher	A12379	1:200

The following antibodies were used for fluorescence activated cell sorting.

Antibody	Company (Clone)	Catalogue #	Dilution
F4/80 – BV421	BioLegend	123132	1:200
KIT – APC-Cy7 CD117	BioLegend	105826	1:200
CD11b - PE	BD Pharmingen	553311	1:200
CD45.2 – APC-Cy7	BD Pharmingen	560694	1:200
CX3CR1 - FITC	BioLegend	149020	1:200
Fc Block CD16/CD32	eBiosience	553142	1:200
CD115 - PE	eBiosience	12-1152-82	1:200

4.3 Epifluorescence microscopy

Epifluorescence microscopy is based on fluorochromes which emit light once they get electronically activated. This activation occurs when fluorochromes are radiated by light waves. Once fluorochromes get back into their initial state, the thereby released energy is set free as light with a specific wavelength. A xenon lamp has been used as light source. The light emitted by fluorochromes is focused by collimator lenses and narrowed by a dichroic excitation filter which is impermeable for shorter wavelengths. The emitted light with a longer wavelength gets separated from the one caused by the light source and is perceived by the emission filter which generates a distinct signal to visualize the specimen. (Mulisch, 2010)

To capture the signal of the GFP-labeled macrophages, the NIBA filter was used, the excitation filter selected wavelengths between 510 and 550nm and the emitted light was detected by a CCD camera (Charge Coupled Device, ORCA-ER, Hamamatsu, Japan) and sent to the computer. Images were created via the cellR Imaging Software (Olympus Deutschland GmbH, Hamburg).

4.4 Embryo isolation and preparation of cryo-slides

For histological analysis, staged embryos (E9.5 to E16.5) were dissected in ice-cold phosphate-buffered saline (PBS) (Gibco®, Thermo Fisher). Subsequently, embryos were fixed in 4% paraformaldehyde (PFA) (Thermo Fisher) at 4°C (E9.5 and E12.5 embryos were incubated for 1hour, E.16.5 embryos overnight).

Samples were washed with PBS and snap-frozen on dry ice. Therefore, Tissue-Tek® cryomolds (15 x 15 x 5 mm and 25 x 20 x 5 mm) and Tissue-Tek® O.C.T.™ Compound (embedding medium of glycol and rosin) were used.

Interim storage was at -80°C. Cryo-slides were performed with the cryostat (Thermo Scientific CryoStar™ NX70, Thermo Fisher), 10µm cutting width and 15°C knife temperature. The slides were thawed for 10 minutes at room temperature and subsequently stained.

4.5 Immunofluorescence

For immunostaining, we used either cryo-slides or cultured cells on 8 chamber-slides (Nunc™ Lab-Tek™ II Chamber Slide™ System, Thermo Fisher). Cells were fixed with 4% PFA/PBS for 20 minutes at room temperature, washed with PBS and permeabilized with 0,1% Triton X (Sigma Aldrich)/PBS for 5 minutes on ice. Cells were once again washed with PBS and blocked with 3% bovine serum albumin (BSA) (Roth)/PBS for 30 minutes followed by incubation with the primary antibody in 1% BSA/PBS for 1 hour at room temperature. After several washes with PBS, the secondary antibody was used for the incubation of the cells for 1 hour at RT. Cells were then incubated with 0,1% Hoechst/PBS for 10 minutes and washed with PBS. The slides were subsequently embedded.

During every step, the slides were protected from ultraviolet light by using aluminum foil.

Pictures of each slide were collected by fluorescence microscopy (AxioImager Z1 microscope from Zeiss) with a 63x/ 1.4 oil objective.

4.6 Fluorescence-activated cell sorting

By fluorescence-activated cell sorting (FACS), cell populations can be distinguished by their size, granularity and fluorescence and can be quantitatively recorded. Quantities of YS-derived macrophages can be compared in different embryonic organs of different genotypes. FACS allows a specific isolation of cell populations and their investigation.

The targeted tissue was incubated for 20 minutes in a mixture of enzymes containing 100U/ml DNAse 1 (Sigma), 2,4mg/ml dispase (Invitrogen) collagenase D 1mg/ml (Roche) and 5ml PBS at 37°C (Eppendorf ThermoMixer® comfort).

To analyze cells originating from cell culture, adherent differentiated cells were detached with Accutase (StemPro™ Accutase™, Thermo Fisher) and incubated for 10 minutes at 37°C during the dissociation process.

Tissue cells were transferred to a single-cells suspension with a 100µm filter (Falcon® cell strainer) and washed with a 4°C cold 1% BSA solution (PAA Laboratories, Pasching, Austria). The cell suspension was centrifuged for 5 minutes and 400g. Cell pellets were solved with 1% BSA and distributed onto a 96 well plate (96 well U-Bottom, Falcon®). Cells were stained with a 50µl solution of a FACS antibody for 20 minutes at 4°C and under protection from ultraviolet light. Subsequently, cells were washed with 1% BSA and transferred in 1% BSA into FACS tubes with a total volume of 400µl. The analyzation of the cells was performed with the flow cytometer (Beckman Coulter MoFLow Astrios).

Detected cells are visualized by histograms or so-called scatter plots. The histogram represents a tool to depict the quantity of a specific marker of a cell population. Whereas the x-axis shows the intensity of the detected cells, the y-axis represents the cell quantity. Scatter plots compare two different parameters and enable the identification of different cell subpopulations.
The acquired data was analyzed by the software FlowJo® 10.07 (Tree Star). CD45 and CX3CR1-GFP positive cells were selected. F4/80 and CD115 (CSF1R) positive macrophages were visualized.

4.7 Quantitative real-time polymerase chain reaction

Ribonucleic acid (RNA) isolation and purification were performed using the RNeasy Mini Kit (Qiagen). 2µg of the isolated RNA was transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time polymerase chain reaction (qRT-PCR) assays were performed with SYBR® green (QantiTect, Qiagen) and CX3CR1, CSF1R, CD45, KIT and actin primers (QantiTect, Qiagen).

Primer	Catalogue#
Mm_Cx3cr1_1_SG QuantiTect Primer Assay	QT00259126
Mm_Csf1r_1_SG QuantiTect Primer Assay	QT01055810
Mm_Ptprc_1_SG QuantiTect Primer Assay	QT00139405

Mm_Kit_va.1_SG QuantiTect Primer Assay	QT01753507
Mm_Actc1_1_SG QuantiTect Primer Assay	QT00322434

4.8 Immortalization of yolk sac macrophages

Whole yolk sacs from mice at E9.5 were transferred into a single cell suspension. YS-derived pre-macrophages were cultivated in a RPMI (Gibco®, ThermoFisher) medium. Cells were transferred onto a Ficoll-Plaque (Pharmacia) and centrifuged. A pre-stimulation with the use of 50ng/ml SCF, 25ng/ml IL3 and 25ng/ml IL6 for a duration of 48 hours followed. In collaboration with the working group from Prof. Dr. rer. nat. Barbara Walzog and with the help of the protocol of Hans Häcker purified cells were infected with an ER-Hoxb8 retrovirus. Progenitor cells were cultivated in a "myeloid cell medium" (RPMI 1640 with 10% FBS, 1% PSQ, 1% GM-CSF–conditioned medium from B16 melanoma expressing the Csf2 cDNA (10 ng/ml GM-CSF) and 1mM estrogen). The infection efficiency was approximately 10%. Immortalized progenitors were separated by removing nonadherent cells every three days and cultivating them on a new six-well culture plate. This process was repeated for three weeks. Immortalized progenitors were distinguished by their G418 resistance, encoded by MSCV. (G. G. Wang et al., 2006)

Hoxb8-immortalized macrophages that derived from YS progenitors were compared to BM-derived macrophages (provided by the Walzog group) in vitro.
Progenitor cells, either YS- or BM-derived, were cultivated in a proliferation medium (500ml RPMI, 50ml FCS (FBS SUPERIOR, #S0615, Merck), 5ml Penicillin/Streptomycin (Thermo Fisher), 20ml SCF medium (homemade, from CHO-SCF cells), 55µl estradiol (#E4389, Sigma-Aldrich), 277µl β-Mercaptoethanol (Gibco®, ThermoFisher)). Cells acclimated after around six days to their new environment which allowed the subsequent differentiation. The cell suspension was centrifuged, washed with RPMI medium and subsequently cultivated in a differentiation medium (500ml RPMI, 50ml FCS, 5ml Penicillin/Streptomycin, 20ml SCF medium, 277µl β-Mercaptoethanol) and with a M-CSF (Invitrogen) supplement (1:10.000 dilution regarding the differentiation medium). The

ongoing differentiation process was depicted by bright field images. Mature macrophages could be identified after about six days which enabled further investigations concerning the characterization of YS- versus BM-derived macrophages.

4.9 Phagocytosis assay

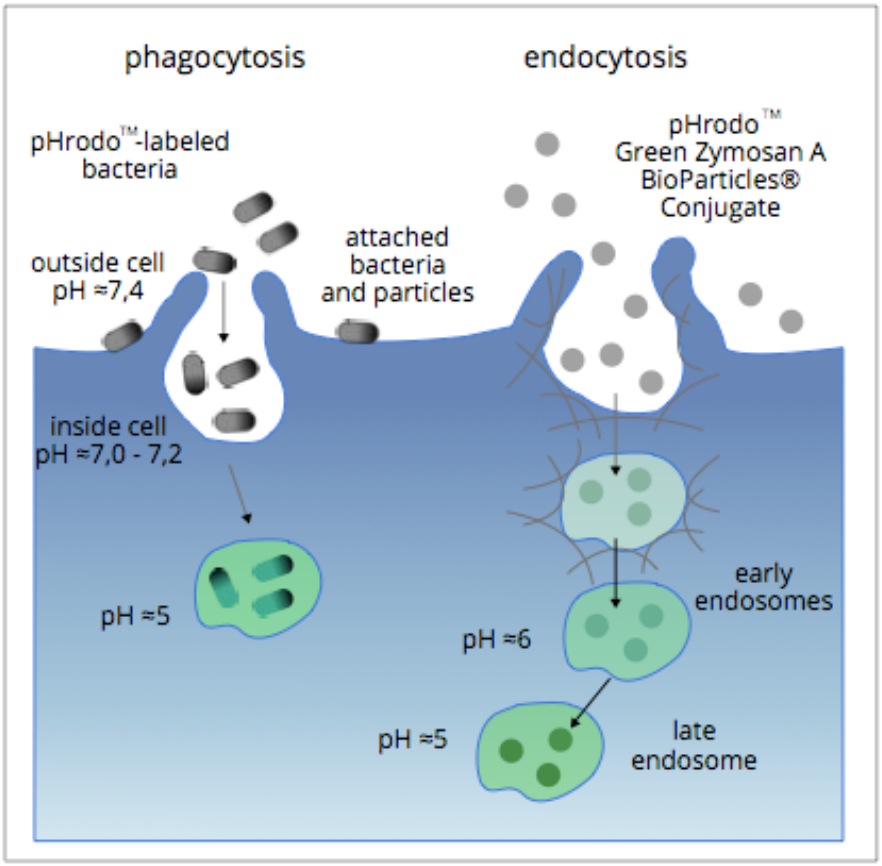


Figure 8: Illustration of the phagocytosis assay
Phagocytized zymosan-conjugated particles emit a green fluorescent signal once they are exposed to an acidic pH-value (adapted and modified from ThermoFisher 2018("Schematic of pHrodo™ dye-based Detection of Phagocytosis and Endocytosis,")

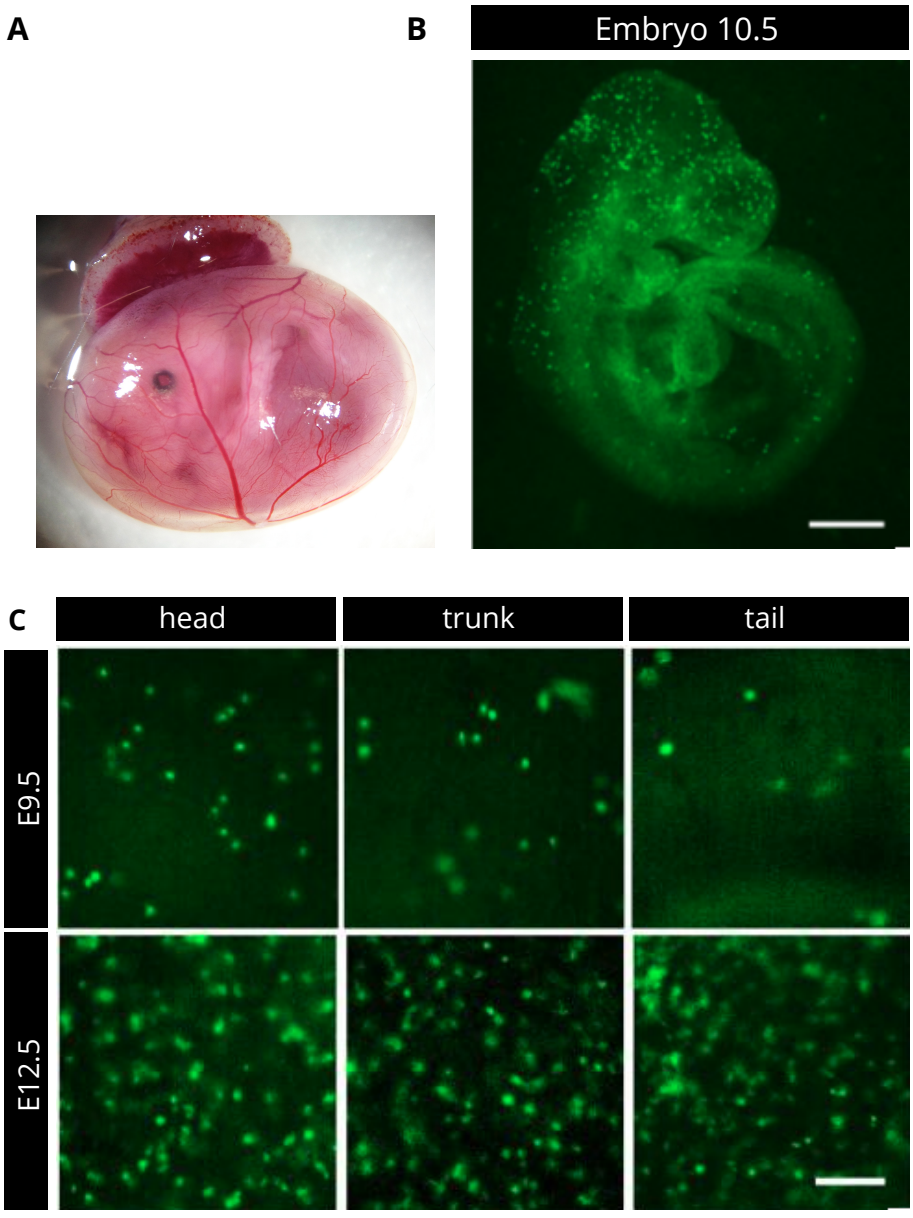
To illustrate the functionality of created macrophages, a phagocytosis assay was performed. Hox B8 immortalized cells were cultivated and differentiated in an 8-chamber slide. pHrodoTM Green Zymosan A BioParticles® (life technologies) were resuspended in 2ml DMEM (Gibco®, Thermo Fisher). Subsequently, the medium of the 8-chamber slide was replaced by the BioParticles® suspension.

Macrophages engulfing Zymosan particles emit a green fluorescent signal due to a more acidic pH in the endosome. Following 1 hour of incubation at 37°C, cells were stained with Hoechst. Phagocytizing cells were detected by their green fluorescent signal and collected using a fluorescence microscope (AxioImager Z1 microscope from Zeiss).

5 Results

5.1 Characterization of YS-derived macrophages

5.1.1 Epifluorescence microscopy with the CX3CR1-GFP mouse model



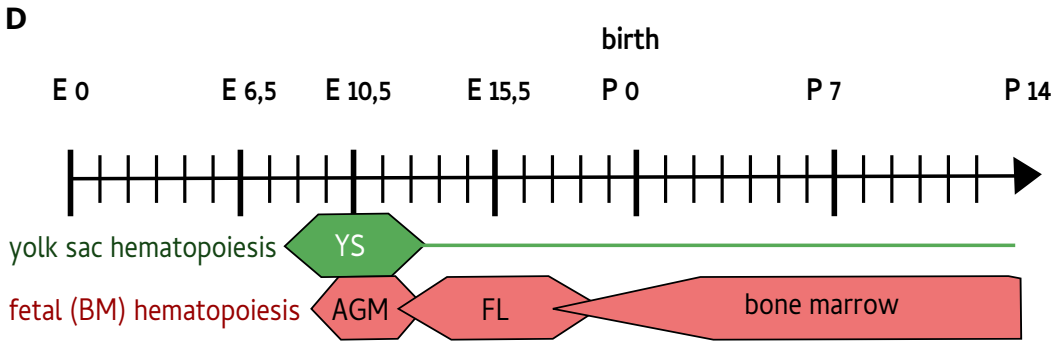


Figure 9: In vivo epifluorescence microscopy with the CX3CR1-GFP mouse model
(A) overview depicting the embryo and YS in toto, (B) overviewing image of the embryo at E10.5 illustrating the GFP-labeled macrophages, scale 1mm, (C) diverse parts of the embryo (head, trunk, tail) at E10.5 and E.12.5, scale 100 µm (with friendly approval of C. Stremmel and R. Schuchert (Stremmel et al., 2018)) (D) timeline of macrophage lineages in mice (F. Wagner)

YS-derived macrophages appear earlier than macrophages arising from the BM (Alliot et al., 1999). In the mouse model the YS encases as a membranous structure the whole embryo (Fig. 9a). With the purpose of illustrating cells originating from the YS, a specific mouse model was used, allowing to label macrophages created in the YS. Fractalkine (CX3CR1) is a chemokine, expressed by monocytes, DCs, T-killer cells and macrophages. A direct and indirect ligation of GFP and the fractalkine receptor enabled the visualization of macrophages. Green fluorescent macrophages arising from the YS were detected with the help of an Olympus BX51W1 epifluorescence microscope (Fig. 9b). Early macrophages could be identified in the YS and the embryo. Zooming sections of the head, trunk and tail of the embryo illustrate YS-derived macrophages distributed in the entire embryo. Compared to the trunk and tail region, a higher density of green fluorescent macrophages could be detected in the brain. During the development of the embryo, the density of macrophages equalized in the different parts of the embryo (Fig. 9c). YS-derived, GFP positive macrophages were visualized before the onset of the main BM hematopoiesis (Fig. 9d).

5.1.2 Cellular characterization of YS-derived macrophages

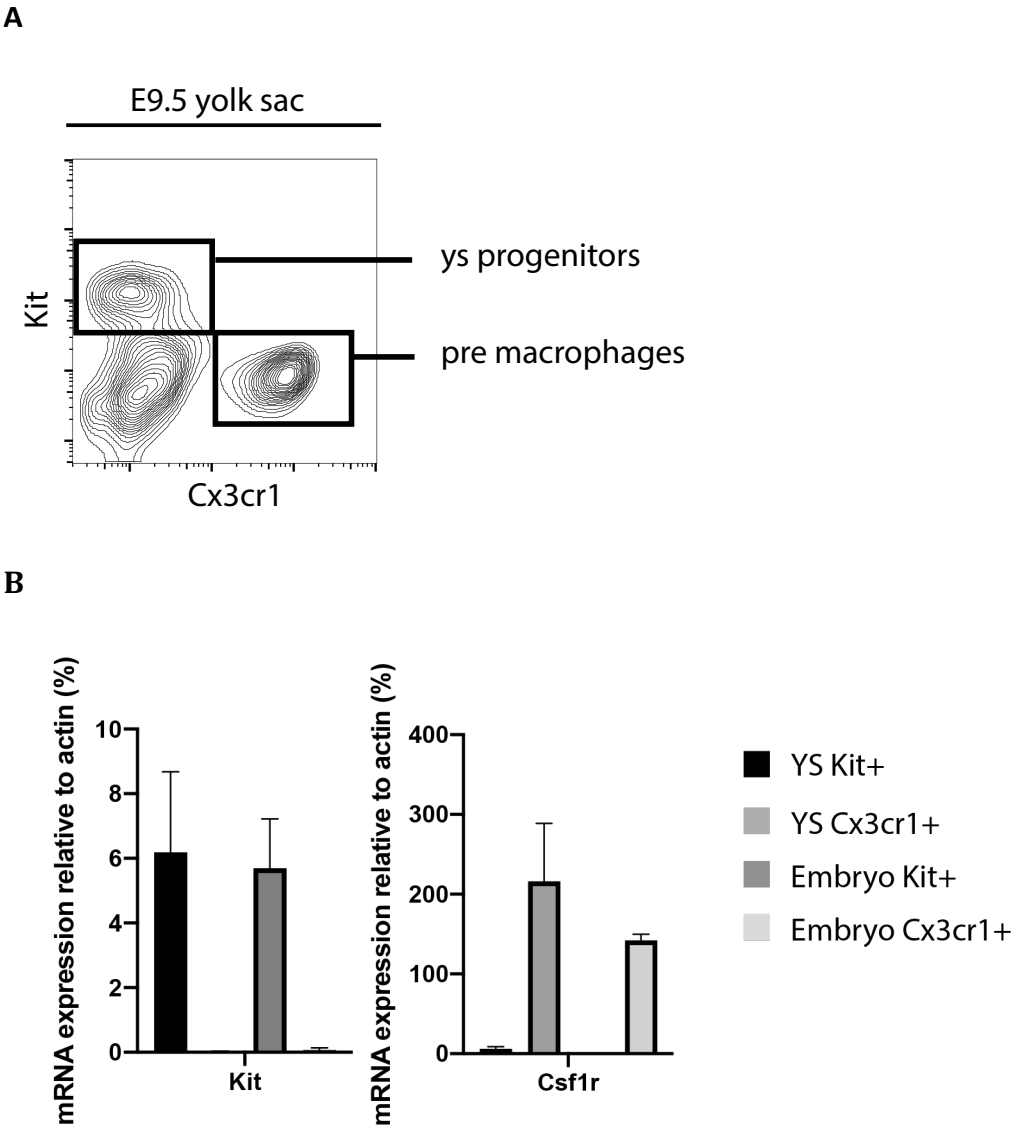


Figure 10: Cellular characterization of YS-derived macrophages
(A) selection of YS progenitors and pre-macrophages via KIT- and CX3CR1 expression (B) RT-PCR

Cell sorting of mice at E9.5, before the onset of BM hematopoiesis, showed two different populations; KIT+ CX3CR1- progenitors and KIT- CX3CR1+ pre-macrophages (Fig. 10a).

RT-PCR quantifications showed that KIT+ progenitors lack Csf1r expression, whereas per macrophages expressed significant levels of Csf1r as an early macrophage marker. These findings were true for cell isolations from the YS as well as from the embryo (Fig. 10b).

5.1.3 Cultivation of YS-derived macrophages

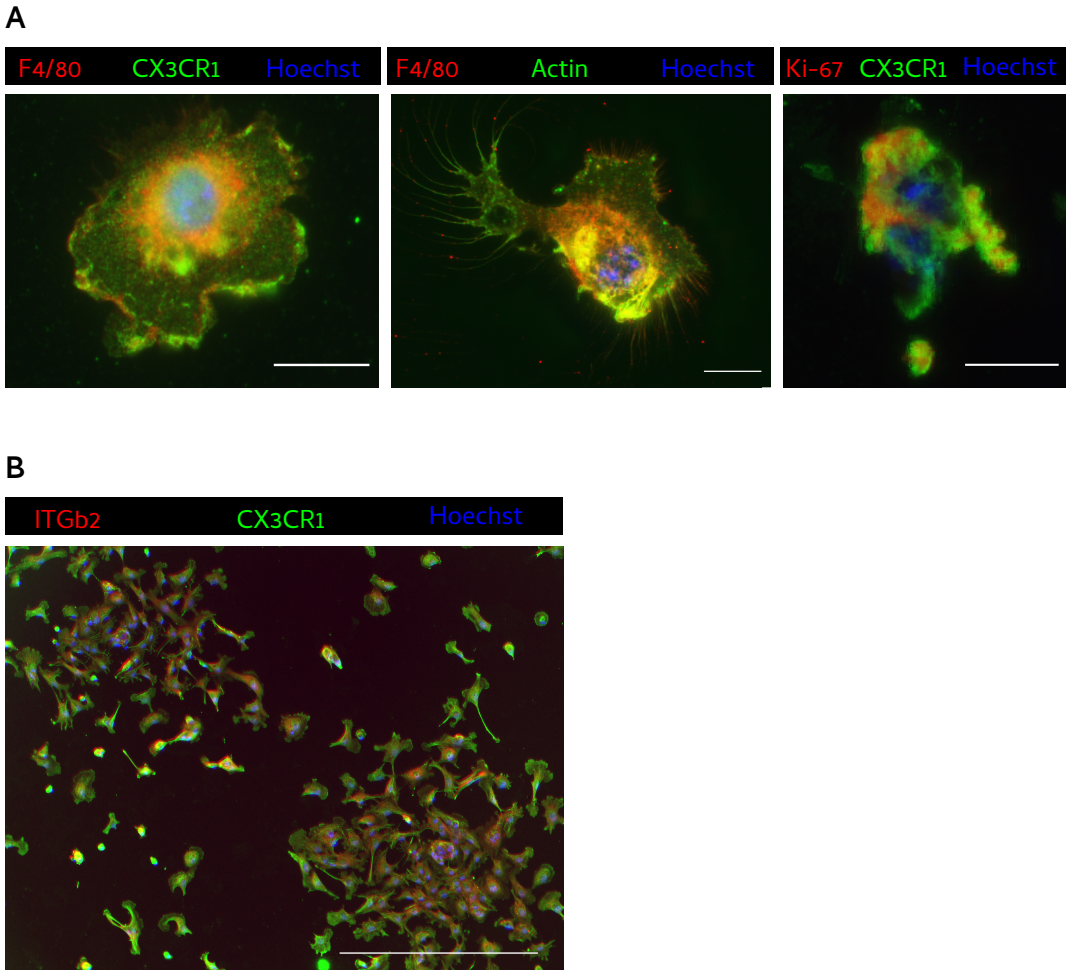
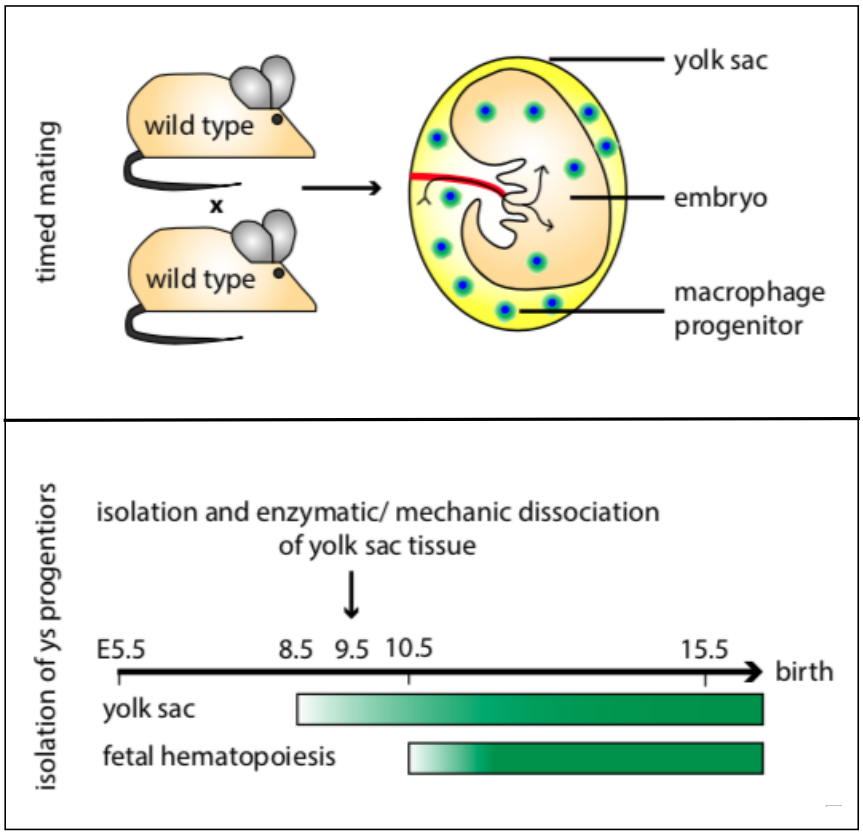


Figure 11: IHC staining profiles of cultivated YS-derived macrophages sorted via FACS
(A) staining profile of YS-derived macrophages, (F4.80/ CX3CR1; F4.80/ Actin; Ki-67/ CX3CR1; benchmark of 50µm), (B) clonal expansion of YS-derived macrophages (ITGβ2/ CX3CR1; benchmark of 500µm)

YS-derived macrophages from 12 to 14 days old embryos (E12.5 and E.14.5) were separated via FACS and subsequently stained. In accordance with previous experiments, they show characteristic macrophage surface antigens. YS-derived sorted macrophages show apart from their GFP-labelled CX3CR1 expression F4/80, a protein which is used as a macrophage marker. Their cytoskeleton is typically actin-based. In order to proof their high proliferation rates, sorted macrophages were stained with antibodies detecting Ki-67, a protein mainly appearing in the interphase of proliferating cells (Fig. 11a).

Enabling higher quantities of YS-derived macrophages, a cell culture was applied. After the removal of their typical environment and other surrounding cells, sorted cells died few hours after having been cultivated. As a consequence, a cell culture containing the whole extracted yolk sac was established. As the signal of ligated CX3CR1-GFP did not show an enduring persistence, attached cells were subsequently stained and distinguished by their F4/80 expression. In the course of clonal expansion, stained macrophages appear clustered (Fig. 11b).

5.3 Generation of an immortalized, YS-derived progenitor cell line



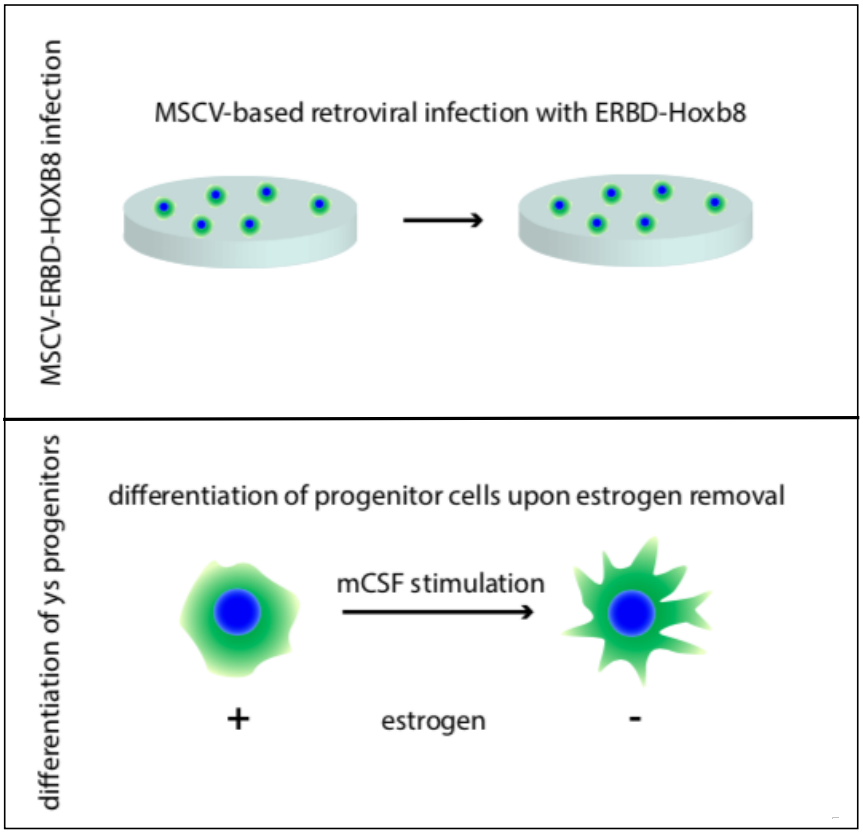


Figure 12: Creation of immortalized progenitor cells
Creation of an immortalized progenitor cell line: isolation of CX3CR1-GFP positive cells before the onset of the hematopoiesis in the AGM around E9.5, MSCV-based retroviral infection with ERBD-Hoxb8, cultivation of progenitor cells and differentiation upon estrogen removal

To enable a long-term cultivation, the aim of creating a more durable cell line was pursued. A specific retrovirus conduced to transfect YS originating cells. A murine stem cell virus (MSCV) retroviral expression vector introduced subsequent to an infection of cells cDNA, and generated a fusion between the estrogen-binding domain (ERBD) of the estrogen receptor (ER) and the N-terminus of Hoxb8. Hoxb8 is a protein which is used as a marker for stem cell. By presence of estrogen and under the influence of Hoxb8, ER-related transcription processes were accelerated and created progenitor cells acquired an unlimited proliferation. By use of this transfection method immortalized progenitor lines were created. These latter preserved their infinite proliferation potential whilst being exposed to a medium containing 1µM estrogen (Fig. 12a). (G. G. Wang et al., 2006)

These generated cells are the first YS-derived and immortalized progenitor cells existing hitherto. Once estrogen is removed from the applied medium and growth

factors are added, progenitors differentiate but lose their immortality. Supplementing M-CSF leads to differentiated YS-derived macrophages.

5.4 Characterization of YS HoxB8 macrophages

5.4.1 Visualization of differentiating progenitors and cellular characterization

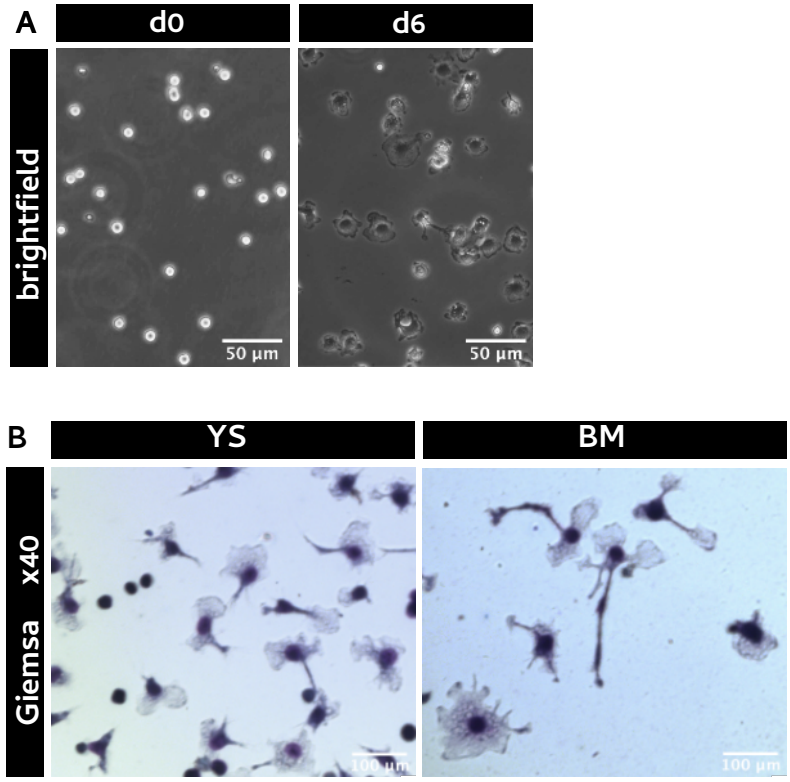


Figure 13: Morphological and phenotypical transformation of cultivated progenitor cells and differentiated YS-derived macrophages
(A) differentiation process of pre-macrophages visualized by bright field images: comparison between progenitor cells (day 0) and differentiated macrophages (day 6), (B) Giemsa staining of YS- and BM-derived differentiated macrophages

In order to depict proliferation and differentiation processes of initially immortal progenitor cells, bright-field images of cultured cells were recorded at different time points. Cultivated progenitor cells show a roundish shape and reveal a notable proliferation capability. Once estrogen is removed and M-CSF is supplemented,

these progenitor cells start to form clusters, differentiate and lose their immortality.

Zooming images illustrate a dendritic shape of differentiated macrophages. Images taken from day zero (d0) to day six (d6) of the differentiation process, emphasize the ongoing transformation (Fig. 13a).

Giemsa staining permitted to highlight the typically dendritic shape of the created differentiated macrophages after their pseudopodia formation (Fig. 13b).

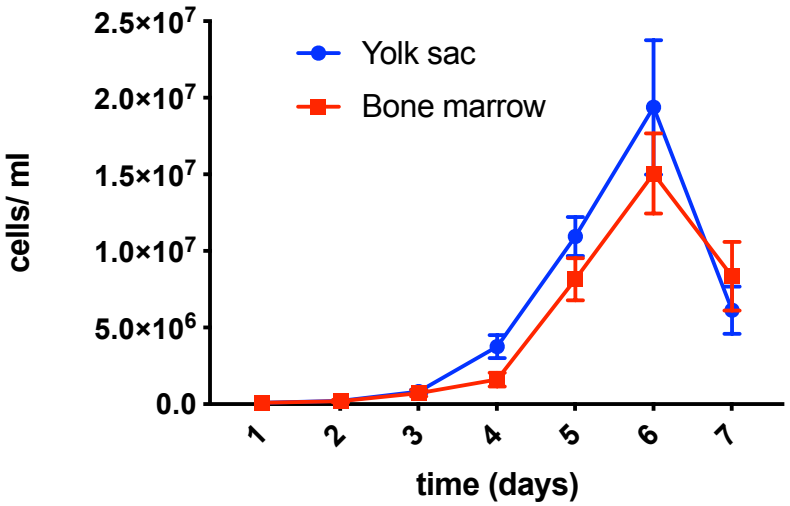


Figure 14: Differentiation of YS- and BM-derived progenitor cells
Rate of differentiated YS-derived macrophages vs. macrophages originating from the BM from day 0 to day 7

Visualizing progenitor cells and recording them from day 0 to day 7 after embedding them in a specific proliferation medium lead to a demonstration of the proliferation potential of YS- and BM-derived progenitor cells. From day 0 to day 3 both progenitor cells show a slight proliferation. A linear proliferation to differentiated macrophages occurs from day 4 to day 6 and is diminished afterwards. YS-derived macrophages appear in a slightly higher amount than BM-derived macrophages (Fig. 14).

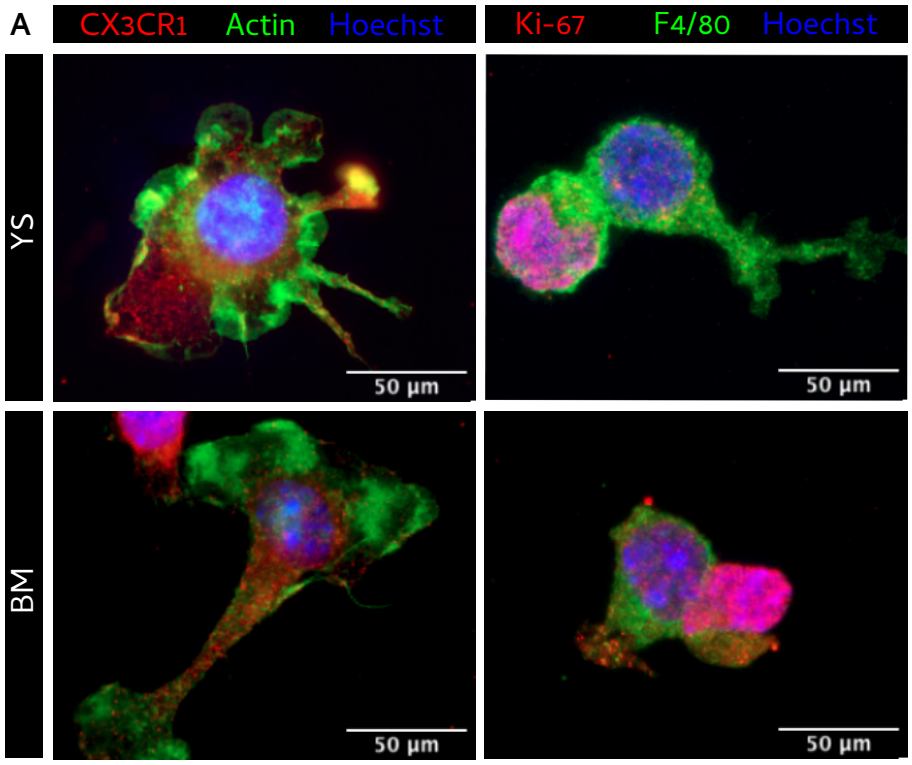


Figure 15: Characterization of HoxB8 macrophages
(A) IHC staining of Hoxb8 YS- and BM-derived macrophages (CX3CR1/ Actin/ Hoechst; Ki-67/ F4.80/ Hoechst)

IHC staining underlined the expression profile of the created macrophages. The depicted expression of F4/80 and CX3CR1, as well as the characteristic actin-based cytoskeleton, confirms the development of macrophages. The proliferation potential of previously created macrophages is pointed out by Ki-67 detection. Ki-67 expression can be perceived in cells currently dividing and already showing two nucleuses (Fig. 15a).

5.4.2 Flow cytometry analysis

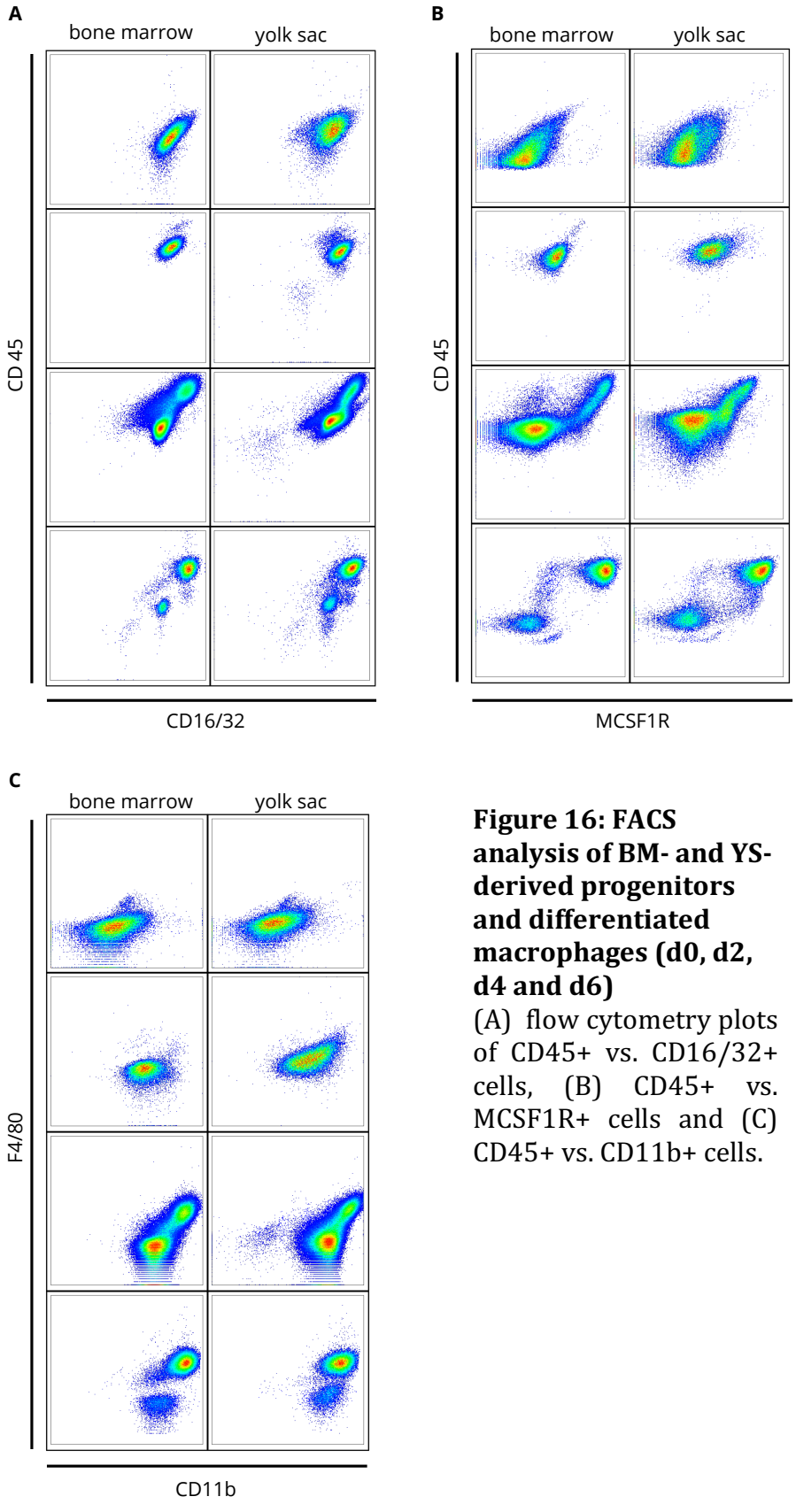
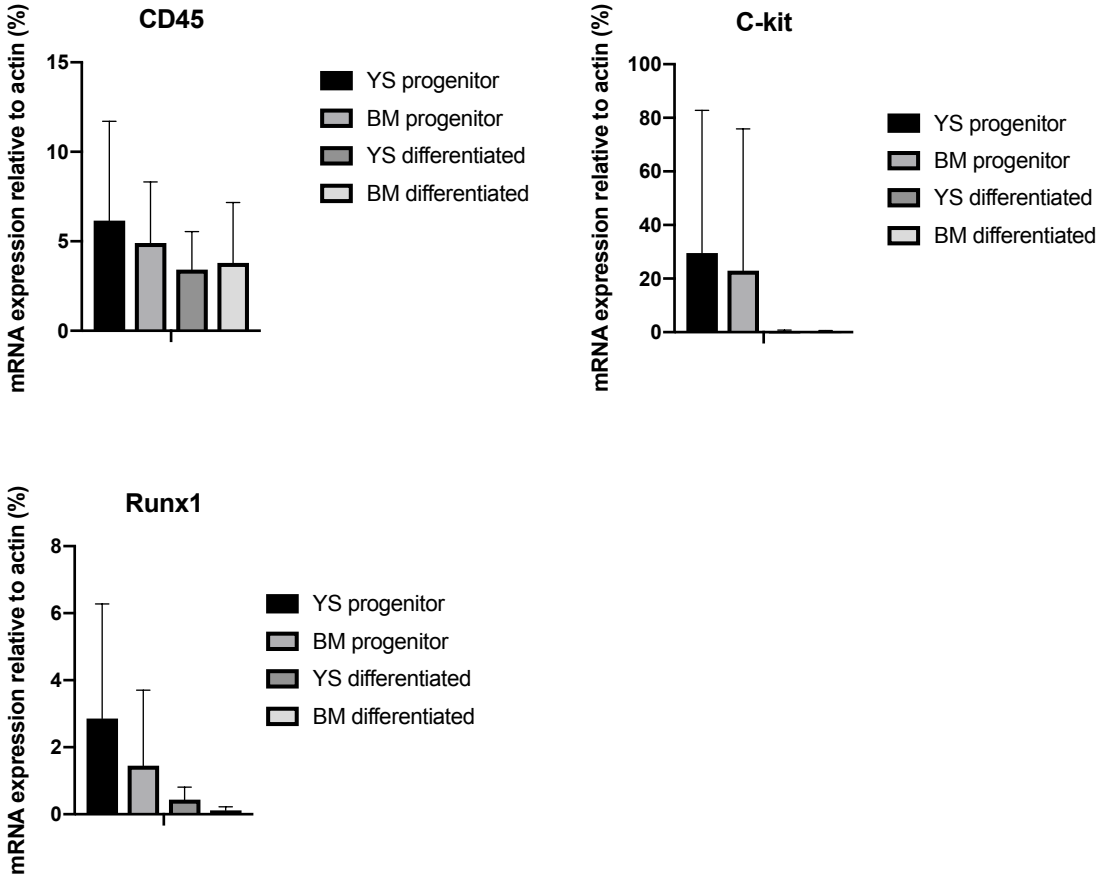


Figure 16: FACS analysis of BM- and YS-derived progenitors and differentiated macrophages (d0, d2, d4 and d6)
(A) flow cytometry plots of CD45+ vs. CD16/32+ cells, (B) CD45+ vs. MCSF1R+ cells and (C) CD45+ vs. CD11b+ cells.

A further purpose was to characterize YS-derived macrophages via flow cytometry. Targeted cell surface antigens were labeled using fluorophores which were detected by the flow cytometer BD LSRFortessa™ (BD Bioscience). FACS analysis was performed with progenitor cells and differentiated YS- and BM-derived macrophages. Differentiated YS- and BM-derived macrophages show a typical profile by expressing CD45, F4/80, CD11b and M-CSF. Compared to progenitor cells arising from the BM or the YS, differentiated cells show a higher expression of CD45, F4/80, CD11b and M-CSF. CD16 and CD32, both Fc receptors expressed by macrophages, are similarly expressed in progenitor and mature cells (Fig. 15 a-c).

5.4.3 Gene expression profiles

A



B

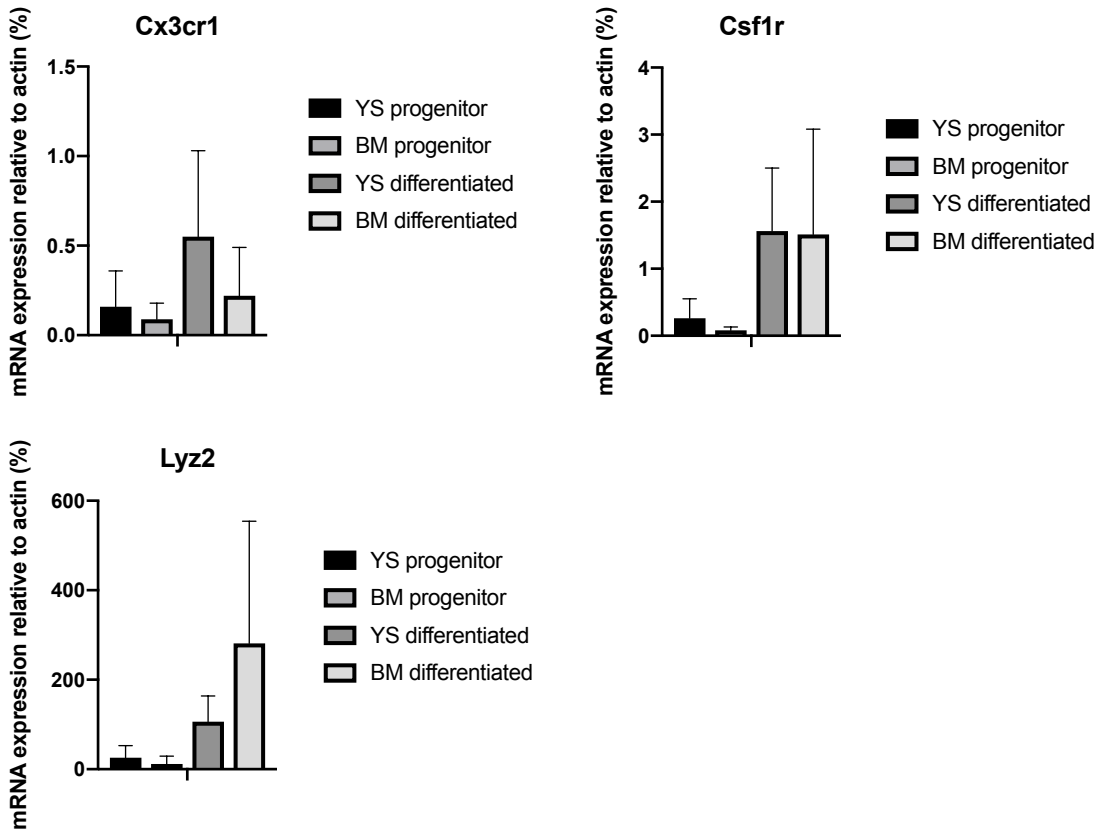


Figure 17: RT-PCR depicting the gene expression profile of YS- and BM-derived Hoxb8 progenitor cells and differentiated macrophages
(A) percentage of expression of CD45, c-KIT, Runx1 (B) CX3CR1, CSF1R, and Lyz2 compared to actin.

To investigate mRNA expression patterns, RT-PCR was performed. CD45 is expressed in all progenitor and differentiated cells whereas c-KIT and Runx1 expression can only be perceived in both progenitor cells. An increased expression of CX3CR1, Lyz2 and CSF1R can be perceived in YS- and BM-derived differentiated macrophages compared to their progenitors. (Fig. 16a).

5.4.4 Phagocytosis and functionality

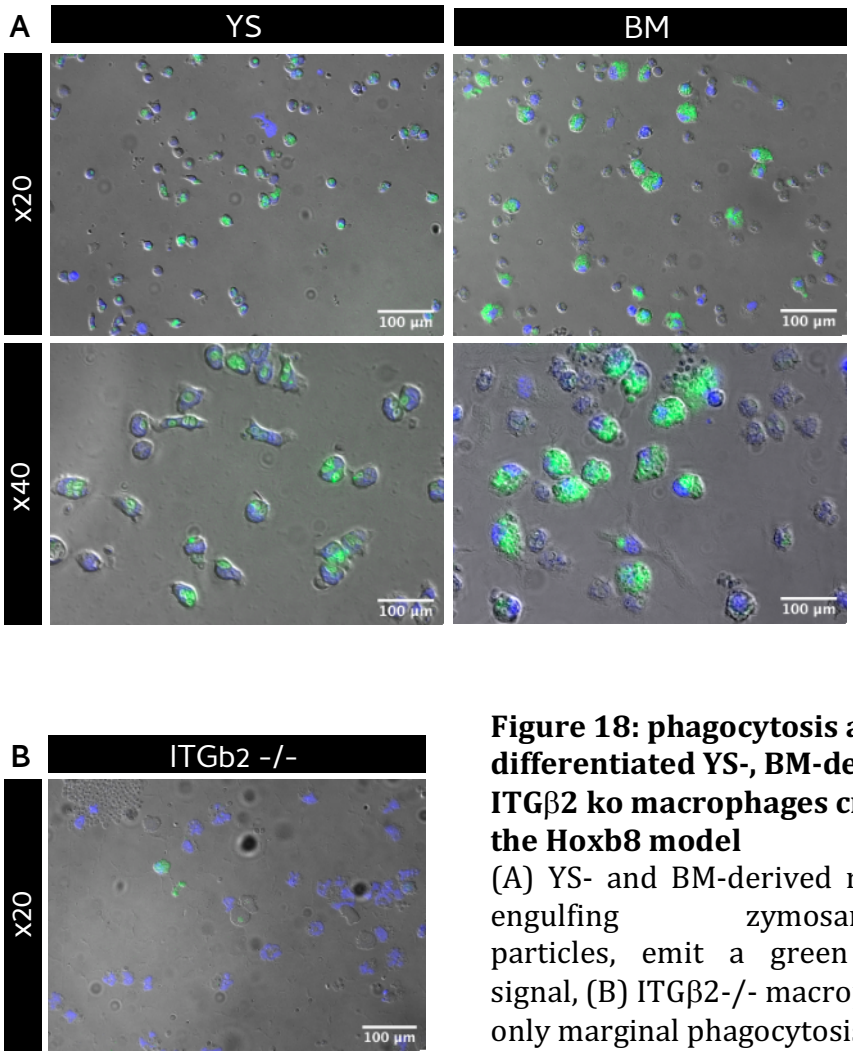


Figure 18: phagocytosis assay with differentiated YS-, BM-derived and ITGβ2 ko macrophages created with the Hoxb8 model
(A) YS- and BM-derived macrophages engulfing zymosan-conjugated particles, emit a green fluorescent signal, (B) ITGβ2-/- macrophages show only marginal phagocytosis

Overlays of bright field and fluorescent images enable the detection of cultivated macrophages exerting phagocytosis. In comparison to quiescent surrounding cells, phagocytizing YS- and BM-derived macrophages emit a green fluorescent signal (Fig. 17a).

Various studies have shown the indispensable role of beta 2 integrin to the phagocytosis process. (Aderem & Underhill, 1999; Wiedemann et al., 2006) Patients with leukocyte adhesion deficiency type I (LADI) lack functional β2 integrin and show a diminished anti-bacterial defense because of the associated phagocytosis deficiency. (Bunting, Harris, McIntyre, Prescott, & Zimmerman, 2002; Hogg et al., 2002)

With the aim of confirming the phagocytosis capability of Hoxb8 differentiated macrophages, an ITGβ2 knockout cell line was used as a negative control. They were likewise exposed to zymosan-linked particles. In comparison to non-mutated macrophages, ITGβ2 deficient cells showed an inferior phagocytosis capability (Fig. 17b).

6 Discussion

Macrophages can arise from different origins, for instance the YS and the BM. The first origin of macrophages and their progenitors is the YS. (Alliot et al., 1999; Ginhoux et al., 2010; Schulz et al., 2012). BM-derived macrophages are described precisely hitherto. They express surface markers such as CD45, CD11b, F4/80, or CX3CR1 and show a characteristic dendritic shape. Before the onset of the hematopoiesis in the BM, HSCs arise in the AGM region around E10.5, infiltrate the fetal liver and seed the embryo. (Bertrand et al., 2010; Bertrand et al., 2005)

Various studies aimed to investigate alternative sources of tissue resident macrophages. First primitive progenitor cells arise in the YS around E7.25, whereas EMPs, the major principal source of tissue resident macrophages, can be perceived from E8.25 onwards. (Stremmel et al., 2018)

Progenitor cells resemble HSCs regarding their expression profile, in particular the c-KIT and CSF1R expression (Stremmel et al., 2018). Colonizing pre-macrophages undergo varying differentiation processes depending on the surrounding microenvironment (Gordon, 2012; Mass et al., 2016). The importance of this specific environment is even more highlighted by the differing functionality of macrophages. One popular classification is the differentiation between M1, pro-inflammatory, and M2, anti-inflammatory macrophages (Zhang & Wang, 2014). This distinction is similarly shown by the development of diverse diseases.

Various diseases originate in the disfunction of the homeostasis capability of macrophages. One famous example is the genesis of cardiovascular diseases. Vessels are regularly exposed to mechanical stress. Endothelium damages provoke the invasion of monocytes into the arterial wall. These monocytes differentiate into macrophages and exhibit an inflammatory response. Oxygen radicals and hydrogen peroxide is produced which in turn modifies low density lipoprotein (LDL) particles. Macrophages phagocytize these adapted lipids by recognizing them with their scavenger receptors. Due to a lack of complete phagocytosis capability they transform into foam cells. Fatty streaks are established in the

intima and thrombosis and local plaque hemorrhage occur. Inflammatory mediators are released, smooth muscle cells invade the arterial wall and extracellular matrix proteins are accumulated. So-called atheromata, aggregates composed of macrophages and cell debris, are generated. They get calcified and cholesterol crystals are installed. The surrounding tissue undergoes a diminished perfusion and thrombus particles can form and partially detach from the damaged endothelium which subsequently cause stroke or (cardiac) infarction. (Naito, 2008; J. Rassow, Hauser, Netzker, & Deutzmann, 2016) The pathogenesis of atherosclerosis represents a famous example of a defective inflammatory macrophage response. Macrophages have on the contrary a positive impact on heart injuries as they can attenuate inflammation processes by secreting IL-10 or TGF-β. This reparative macrophage phenotype expresses a higher amount of MertK receptors and appears essential to cardiac recovery. M2-like macrophages, being activated via IL-4, have been proved to be necessary to orchestrate fibrosis after cardiac injury. However, they can act in a protective or pathogenic manner. (Vannella & Wynn, 2017) In this context a precise characterization of YS-derived macrophages is indispensable. Further investigations could permit a modification of the pro-inflammatory response of macrophages and lead to an establishment of more advanced therapeutic strategies regarding heart injuries and atherosclerosis.

YS-derived macrophages are either replaced by macrophages originating from the BM or persist in the tissues. Tissue resident macrophages arising from the YS are capable of self-renewal. The difference regarding the origin of the macrophages has to have an impact on the homeostasis capability. This could also lead to different pro- or anti-inflammatory responses. Further investigations concerning the activation of YS-derived macrophages could play a key role regarding the pathogenesis of multiple sclerosis as microglia are tissue resident macrophages originating from the YS and their disfunction initiates neuropathologies (Vannella & Wynn, 2017).

Due to the importance of macrophages concerning immunity and the development and persistence of diseases, further analyses of YS-derived macrophages are

crucial. Performed investigations illustrated in this thesis, followed the principal aim of a primary characterization of YS-derived macrophages.

Pre-macrophages arising from the YS express CX3CR1 (Bertrand et al., 2005). A significant quantity of cells originating from the definitive hematopoiesis appear initially from E10.5 onwards. Thus, we selected YS-derived cells by CX3CR1-GFP labeling before this point in time and the onset of the hematopoiesis in the AGM region. These labeled macrophages can be detected in the YS as well as in the whole embryo. By the use of epifluorescence microscopy, we enabled a first visualization of in vivo YS-derived macrophages. At E10.5 we detected higher densities of green fluorescent macrophages in the brain compared to the trunk and tail region. This difference regarding the cell density was equalized from E12.5 onwards. Macrophages are indispensable to the development of the brain and its homeostasis. Former studies already elucidated the fact that microglia, originating from the YS, are not replaced by BM-derived macrophages and have a self-renewal capability. The fact that YS-derived macrophages are initially recruited by the brain tissue underlines the importance of the microglia to the embryonal brain development.

Regarding the selection of YS-derived macrophages via CXCR1-GFP labeling, one could conjecture that our investigated cells are BM-derived. Dispelling these concerns Stremmel et al. used the *myb* knockout mice (Stremmel et al., 2018). Schulz and co-worker already showed a *myb*-independent proliferation of YS-derived macrophages (Schulz et al., 2012). CX3CR1-GFP labeled macrophages could still be shown in the YS of mice lacking definitive fetal hematopoiesis and their cell number and density did not vary (Stremmel et al., 2018).

Schulz and co-workers were the first ones to distinguish YS- and BM-derived macrophages via their antigen profile. Investigations characterized macrophages originating from the YS with a major F4/80 and inferior CD11b expression compared to BM-derived macrophages ($CD11b^{low}$, $F4/80^{bright}$) (Schulz et al., 2012). We confirmed the existence of YS-derived progenitors and macrophages via showing two different populations in cell sorting experiments with mice at E9.5 and before the onset of BM hematopoiesis. Progenitors were characterized by their

KIT expression, macrophages showed a superior CX3CR1 expression. This expression profile of the two different populations could be confirmed with RT-PCR analysis.

With the intention of detecting further expression markers we created cryo-slides from frozen embryos and tissues. We used immunofluorescence staining in order to visualize specific expression profiles. As the immunofluorescence staining with cryo-slides appeared to be non-specific, we used whole mount samples. The size of the embryos required a long timeframe of epifluorescence microscopy which lead to a significant reduction of the GFP signal and a non-specific staining profile. Pursuing the objective of characterizing the expression profile of YS-derived macrophages we performed cell culture experiments.

By the immunofluorescence staining of cultivated macrophages originating from the YS we did not only underline the expression of the macrophage markers F4/80, CX3CR1 and the characteristic actin-based cytoskeleton, but illustrated a cluster formation of YS-derived macrophages and the proliferation potential by detecting Ki-67-labeled separating cells.

The cultivation of via FACS distinguished YS-derived macrophages lead to various difficulties. Specific time mating with CX3CR1-GFP mice was required in order to be aware of the respective timepoint of embryogenesis. Caesarean sections and cell sorting were necessary to obtain the investigated cells which lead to a certain dependency on mice. Sorted macrophages appeared to depend on a specific microenvironment in the cell culture experiments. Only by adding the whole yolk sac, macrophages seamed to survive and further investigations were enabled. Beside unstable cell culture conditions, the quantities of the investigated macrophages were non-specific and the repeatability and reproducibility were limited.

By the use of the ER-Hoxb8 model, we created immortalized YS-derived progenitor cells which were unrestrictedly available and ensured reproducibility and stable cell culture experiments. During the ongoing differentiation process of YS-derived progenitors, we used bright field images and Giemsa staining to demonstrate the

proliferation capability of progenitor cells. We showed a specific proliferation potential varying over the timeline and a transformation from a roundish to a typically dendritic shape of differentiated macrophages.

With FACS and immunofluorescence staining we confirmed the expression profile of earlier analyzed YS-derived macrophages by showing an expression of CX3CR1, F4/80, CD45, CD11b, and macrophage specific markers such as M-CSF, CD16 and CD32. The proliferation potential was underlined with the help of Ki-67 detection. By the use of RT-PCR we validated the mRNA expression patterns of Hoxb8 YS-derived macrophages by confirming their CD45, CX3CR1, CSF1R positive and KIT negative expression profile.

Phagocytosis is known as essential to immunity and the homeostasis capability of macrophages. (Eales, 2003; Gordon, 2016b; Stites et al., 1994; Wynn et al., 2013) To further analyze the functionality of Hoxb8 generated YS-derived macrophages, we implemented a specific phagocytosis assay. We used integrin beta2 knockout cells as a negative control. Both, YS- and BM-derived macrophages emitted green fluorescent light after having engulfed zymosan-conjugated particles, which proved their phagocytosis capability. This result could lead to the supposition that macrophages arising in the YS represent a primary compound of the innate immune system of the unborn embryo. To further characterize impact factors on this phagocytosis capability additional phagocytosis essays with different knockout models, progenitor and differentiated macrophages or various tissue resident macrophages (e.g. sorted microglia or Kupffer cells) should be pursued. As mentioned before some particular tissue resident macrophages originating from the YS are not replaced by BM-derived macrophages, are capable of self-renewal and are indispensable to the tissue homeostasis. This leads to the assumption that YS- and BM-derived tissue resident macrophages vary regarding their phagocytosis capability. Analyzing the variation in quality of phagocytosis could result in new targeted therapies.

In order to understand the complete function of YS-derived macrophages further investigations will be necessary. Regarding the role of macrophages in infections one possible experiment could be the injection of sorted macrophages originating

from the YS into infected mice or cell culture-based analysis with germs and cultured macrophages.

Macrophages play a key role in tumorigenesis. So-called Tumor associated macrophages (TAMs) create the perfect micro-environment for tumor cells and are essential to metastasis development (Condeelis & Pollard, 2006). One crucial part of the establishment of TAMs is the phenotypic switch of M1, pro-inflammatory, into M2, anti-inflammatory macrophages which is initiated by tumor cells (Pollard, 2017). To further analyze the role and function of YS-derived macrophages a cancer model could be a possibility. The injection of sorted macrophages originating from the YS into mice with cancer and the subsequent analysis of the impact of the injected cells on the tumor development could be a way to complete the characterization of YS-derived macrophages.

Concerning above mentioned pathogenesis migration is indispensable to macrophages. Without migration the response of macrophages on the onset of inflammation or tissue damage would be limited. To further characterize YS-derived macrophages the installation of migration experiments and the comparison between BM-derived macrophages or knock-out models are essential.

The yolk sac is a two-layered membranous sac composed of an endoderm layer arising from the hypoblast and an ectoderm layer originating from epiblast cells. In mice the yolk sac surrounds the whole embryo whereas it is situated in humans ventral of the embryo and attached to it via the omphalomesenteric duct. (Carter & Enders, 2016; Kaufman, 1991) In mammals the yolk sac contains a metabolic function and first stem cells appear. However, findings of early progenitor cells occurred in mice. Investigations with human counterparts would be of huge interest in order to transfer the results.

Diagnosing an intact early pregnancy, the yolk sac has to be round-shaped and in a specific size, corresponding to the gestational age. Varying sizes or irregular forms of the yolk sac represent early signs of an abnormal pregnancy development (Rath, 2010). Macrophages being responsible for homeostasis could have a crucial impact on the development and maintenance of the yolk sac and the early

pregnancy. The placenta is an organ that has to function particularly at the end of its lifetime. Malfunction of the placenta leads to severe diseases. The growth of the fetus and the vascular nourishment can be restricted or preeclampsia or HELLP syndrome can occur because of a lack of trophoblast migration and invasion through the decidua and maternal uterine spiral arteries (Weyerstahl, 2013). Additionally, diseases as preeclampsia have a main impact on later appearing maternal or fetal diseases. Studies proved that preeclampsia is associated with a higher risk for maternal cardiovascular diseases (Orabona et al., 2017) and that low fetal birth weight is related to a later appearing coronary heart disease (Alexander, 2006; Barker, Osmond, Golding, Kuh, & Wadsworth, 1989). Dong et al. currently described a correlation between yolk sac vasculopathies and embryonic malformation rates. It is known that maternal diabetes mellitus is associated with the risk of structural birth defect. Hyperglycemia injures the yolk sac in vitro and in vivo and damages cells and organelles significantly. Vasculopathies lead to abnormal nutrient transportation and have an impact on the amount of embryonic malformation. (Dong et al., 2016) Macrophages are a primary element of the development of vasculopathies. Their early appearance in the yolk sac plays an important role for homeostasis and the function of the embryo nourishing structures. Malfunctioning could lead to a higher risk of abortion, intrauterine growth retardation, pregnancy induced hypertension, increased calcification in the placenta, preeclampsia and postpartal occurrence of cardiovascular diseases. Further investigations regarding YS-derived macrophages in human counterparts are indispensable for the comprehension of the pathogenesis of above-mentioned diseases and the development of future therapeutic strategies.

In summary, it can be stated that the yolk sac depicts the first origin of mature macrophages and that YS-derived macrophages show a close resemblance to those arising in the bone marrow. Performed investigations in this thesis such as immunofluorescence staining, RT-PCR or FACS analysis illustrated the similarity of YS- and BM-derived macrophages regarding their antigen profile. Similar to BM-derived macrophages, macrophages originating from the YS express typical markers such as CX3CR1, F4/80, CD45 and CD11b. Cell culture experiments and bright field images showed the transformation of a roundish shape of progenitor cells to a typical dendritic shape of differentiated macrophages. The ER-Hoxb8

model enabled a first analysis of immortalized YS-derived progenitors and differentiated macrophages and lead to a significant repeatability and reproducibility. Alike BM-derived macrophages, YS-derived macrophages reveal a similar proliferation potential and phagocytosis capability.

YS-derived macrophages remain persistent as tissue-resident macrophages in adult mice. They cannot be distinguished on a molecular basis from those arising from the BM so far and require temporary fate mapping analysis. A precise description of macrophages originating from the YS could play a key role for potential future therapeutic strategies. A detailed characterization with proteomics, RNA sequencing or metabolic analysis and the illustration of the impact of the surrounding matrix to the development of tissue-resident macrophages will be addressed to future studies. Beside the placenta, the unborn embryo does not dispose of a substantial immune defense system. Before the onset of hematopoiesis in the BM, YS-derived macrophages could depict an important compound of the innate immune system. Further investigations concerning the defense-mechanism of YS-derived macrophages in the embryo have to be pursued in the future. In order to trace the migration and defense capability of YS-derived macrophages, mature macrophages arising from the HoxB8 cell culture, could be injected in adult mice and further in infected mice. Additionally, experiments with human counterparts should occur in order to transfer the results to the human system.

7 Summary

This thesis confirms the yolk sac as the first source of macrophages and analyzes macrophages originating from the mouse yolk sac. In vivo investigations such as epifluorescence microscopy depicted yolk sac-derived macrophages and their migration towards specific parts of the embryo.

Further analysis revealed the expression profile and proliferation potential of YS-derived macrophages. Similar to macrophages originating from the bone marrow, these latter show an expression of F4/80, CX3CR1, CD45 and CD11b. Additionally, YS-derived macrophages appear in a clonal expansion pattern.

For the first time, an enduring cell culture with YS-derived progenitor cells could be established. In vitro investigations visualized the transformation of the roundish shape of progenitor cells to a typical dendritic shape of differentiated macrophages. Macrophages arising from the yolk sac, show a similar proliferation capacity as those originating from the bone marrow. Cell culture experiments enabled a higher quantity of cells and confirmed the above-mentioned expression profile. The phagocytosis capability of YS-derived macrophages could be proved via specific phagocytosis assays.

The collected data gives first insights in the development, molecular profile and functionality of yolk sac-derived macrophages. A close resemblance to macrophages arising from the bone marrow could be shown. A more detailed characterization of YS-derived macrophages regarding their migration and immune defense potential as well as investigations concerning the role of these cells in humans have to be pursued in the future.

8 Zusammenfassung

Die vorliegende Arbeit bestätigt den Dottersack als ersten Ursprung von Makrophagen und analysiert Makrophagen, welche aus dem Dottersack der Maus hervorgehen. In vivo Experimente, wie beispielsweise die Epifluoreszenzmikroskopie, stellten vom Dottersack abstammende Makrophagen, sowie deren Migration in spezifische Regionen des Embryos dar.

Weitere Untersuchungen zeigten das Expressionsprofil und Proliferationspotential der aus dem Dottersack entsprungenen Makrophagen auf. Ähnlich wie Makrophagen, welche aus dem Knochenmark hervorgehen, exprimieren diese F4/80, CX3CR1, CD45 und CD11b. Zudem erscheinen Makrophagen, welche aus dem Dottersack entspringen, in Zellkulturversuchen in einem klonalen Expansionsmuster.

Zum ersten Mal konnte eine fortwährende Zellkultur mit aus dem Dottersack hervorgehenden Vorläuferzellen etabliert werden. In vitro Versuche veranschaulichten die Transformation der rundlichen Form der Vorläuferzellen hin zu einer typischen Form mit Ausläufern der differenzierten Makrophagen. Makrophagen, welche aus dem Dottersack hervorgehen, zeigen eine ähnliche Proliferationsfähigkeit wie die, die aus dem Knochenmark entstammen. Zellkulturversuche ermöglichten eine höhere Zellzahl und bestätigten das oben genannte Expressionsprofil. Zudem konnte die Fähigkeit der Phagozytose der aus dem Dottersack entsprungenen Makrophagen mittels spezifischer Versuche bestätigt werden.

Die gewonnen Daten geben erste Einblicke in die Entwicklung, das Profil auf molekularer Ebene und die Funktionalität der aus dem Dottersack entstandenen Makrophagen. Eine große Ähnlichkeit zu den Makrophagen, die aus dem Knochenmark entspringen, konnte gezeigt werden. Eine noch detailliertere Charakterisierung der untersuchten Makrophagen hinsichtlich der Migration, des Immunabwehrpotentials, sowie deren Rolle im Menschen, sollten in der Zukunft verfolgt werden.

9 Table of figures

10 References

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