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Characterization of two promising experimental tools to further improve the functional analysis of neutrophils

Ph.D. thesis

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LIST OF ABBREVIATIONS

ACK	Ammonium-Chloride-Potassium lysing solution
ADCC	Antibody-dependent cellular cytotoxicity
ANOVA	Analysis of variance
APL	Acute promyelocytic leukemia
ATP	Adenosine triphosphate
ATRA	All-trans retinoic acid
BALB/C	Albino, laboratory-bred strain of the house mouse
Bcl-2	B-cell lymphoma 2
BoyJ	C57/Bl6 congenic mouse strain expressing CD45.1 pan leukocyte marker
Card9	Caspase recruitment domain-containing protein 9
CD	Cluster of differentiation
CD11a	αL integrin, also known as LFA-1
CD11b	αM integrin, also known as Mac-1
CD18	Integrin β chain-2
CHO	Chinese hamster ovary cell
CMP	Common myeloid progenitor
COPD	Chronic obstructive pulmonary disease
CXCL	CXC-chemokine ligand
CXCR	CXC-chemokine receptor
DAMP	Damage associated molecular pattern
DMSO	Dimethyl sulfoxide
ELANE	Gene of neutrophil expressed elastase
ELISA	Enzyme-linked immunosorbent assay
EML	Erythroid-myeloid-lymphoid cell
ER-BD	Estrogen-binding domain of estrogen receptor
EVs	Extracellular vesicles
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FCγR	Fcγ-receptor
FITC	Fluorescein isothiocyanate

fMLP	N-Formylmethionine-leucyl-phenylalanine
Foxo3	Forkhead box O3
FSC	Forward scatter
G-CSF	Granulocyte colony-stimulating factor
Gfi-1	Growth factor independent 1 transcription repressor
GMP	Granulocyte-monocyte progenitor
GPI	Glucose-6-phosphate isomerase
Gy	Gray
HBSS	Hank's Balanced Salt Solution
H&E	Haemotoxylin and eosin dye
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffering agent
HoxB8	Homeobox protein B8
HSA	Human serum albumin
HSC	Hematopoietic stem cell
Hsp 90	Heat shock protein 90
IC	Immune complex
ICAM-1	Intercellular cell adhesion molecule 1
IFN-γ	Interferon-γ
KO	Knock-out
LTB_4	Leukotriene B4
LTB_4R	Leukotriene B4 receptor
Ly6G	Lymphocyte antigen 6 complex, locus G
Ly6C	Lymphocyte antigen 6 complex, locus C
Lyz2	Lysozyme C-2
Mcl-1	Myeloid cell leukemia-1 protein
M-CSF	Monocyte/macrophage colony-stimulating factor
MFI	Mean fluorescence intensity
MHC I/ II	Major histocompatibility complex I or II
MPO	Myeloperoxidase
MRP8	Myeloid-related protein 8
MSCV	Murine stem cell virus

mTmG	tdTomato (mT – red) and EGFP (mG- green) fluorescent protein reporter mouse strain
NADPH	Nicotinamide adenine dinucleotide phosphate
Ncf1	Neutrophil cytosol factor 1
NE	Neutrophil elastase
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cell
NK-cell	Natural killer cell
NLR	NOD-like receptor
NOD	Non-obese diabetic mouse
Nox2	NADPH oxidase 2
OVA	Ovalbumin
PAD4	Protein arginine deiminase
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PLCγ2	Phospholipase C gamma 2
PMA	Phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear leukocyte
PNPP	p-Nitrophenyl phosphate
PRR	Pattern-recognition receptor
PSGL-1	P-selectin glycoprotein ligand 1
RA	Rheumatoid arthritis
ROS	Reactive oxygen species
Rpm	Rounds per minute
SCF	Stem-cell factor
SLE	Systemic lupus erythematosus
SPF	Specific pathogen-free
SSC	Side scatter
SYK	Spleen tyrosine kinase
TCR	T-cell receptor
TLR	Toll-like receptor

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TNF	Tumor necrosis factor
YPD	Yeast Peptone Dextrose Broth
WT	Wild type

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1 INTRODUCTION

Neutrophilic granulocytes (neutrophils) are the most abundant circulating leukocytes in humans and a prominent leukocyte population in mice (1-3). Neutrophils are essential players in innate defence mechanisms against invading pathogens, but they have also long been known to play an important role in the formation of acute inflammatory responses. Since their inappropriate activation often leads to various diseases like autoimmune and inflammatory diseases or even cancer development (3, 4), it is essential to gather as much information on their biology as possible.

Effector mechanisms of neutrophil responses to pathogen burden have been targets of pharmacological manipulation in the recent years with promising results (3). However, great scientific progress in the field is very much limited by the fact that neutrophils are short-lived, terminally differentiated cells. These characteristics of the neutrophils make their long-term maintenance, culture, expansion, and genetic manipulation in vitro nearly impossible. Although there are several myeloid leukemia cell lines with a potential to differentiate into neutrophil-like cells, most of these lack very important features of neutrophils (e.g.: they cannot carry out phagocytosis or they do not release granule proteins, etc.) so they cannot be used in extensive functional studies. Instead of neutrophil-like cell lines researchers can use primary neutrophils freshly isolated from blood or bone marrow, but these neutrophils are only viable for a couple of hours and can be used only in short-term experiments. The most widely used tool to test the effect of genetic manipulations on neutrophil function is to use transgenic mice carrying germline mutations. However, generation and maintenance of these mouse strains is very difficult and highly expensive, not to mention that studies on these mouse models in vitro are also limited by the small number of neutrophils that can be obtained from these animals.

We were hoping to overcome the above-mentioned obstacles of neutrophil research, therefore I was focusing on characterising two different approaches and tools that could be useful to study neutrophil cell functions using both in vitro and in vivo techniques.

One of the approaches is the so-called HoxB8 myeloid progenitor cell line (5). HoxB8 cells are murine progenitor cells immortalised via retroviral transduction, such that they retain their differentiation potential to develop into various myeloid cell types.

In my thesis, I am going to describe the HoxB8 cell line focusing on how the so-called HoxB8 neutrophils can differentiate from the immortalised HoxB8 progenitors. Then I am going to characterise the effector functions of HoxB8 neutrophils in various in vitro experimental conditions. After that I am going to explain the possibility of using the HoxB8 cells in vivo by producing so-called HoxB8 chimeras. Utilizing these chimeric mice in various acute and chronic inflammatory mouse models I would like to demonstrate that HoxB8 neutrophils can play crucial role in disease development just like normal neutrophils do.

Apart from the characterisation of the HoxB8 system, I am also going to describe some aspects of the *Mcl1*$^{\Delta Myelo}$ transgenic mouse model, which is a very promising neutropenia mouse model that can also be a useful tool in neutrophil-related in vivo studies.

1.1. Overview of neutrophil biology

1.1.1. Development and phenotype of neutrophils

Neutrophils are one of the most important phagocytic cells of the innate immune system, developing from the common myeloid progenitor (CMP) cells (6). Their developmental program (called granulopoiesis) takes place in the bone marrow and progresses through many different steps (GMP-myeloblast-promyelocyte-myelocyte-metamyelocyte) whereby oligopotent progenitors lose their self-renewal capacity while turning into unipotent, functionally active mature granulocytes (7-9). During the last stages of their development, the nucleus becomes highly segmented, resulting in the classical neutrophil phenotypes. This is the reason why they are also called polymorphonuclear cells (PMN) together with basophils and eosinophils (Fig. 1.).

Another classical neutrophil phenotype is that the cytoplasm contains neutrophilic granules which carry various proteins that take essential part in inflammation and pathogen elimination (10).

They are also very abundant in the circulation: in humans 50-70%, in experimental mice approximately 20% (11) of leukocytes are neutrophils. Upon infection, the number of circulatory neutrophils can be highly elevated, due the mobilization of the neutrophil pool from the bone marrow. These neutrophils reside in the marrow as a result of the

connection between CXCL12 ligand expressed on bone marrow stromal cells and the neutrophils' CXCR4 receptor (12, 13).

1.1.2. Roles and functions of neutrophils

As part of the innate immune system, neutrophils have a major role in eliminating pathogens like bacteria and fungi. They are one of the most important phagocytic cells and contribute to the immune defence by several effector mechanisms (3) as depicted in Fig. 1. Via releasing cytokines, chemokines, and lipid mediators, they play an important role both in maintaining the immune homeostasis of the body and keeping the autoimmune responses under control (14, 15).

While in the circulation, mature granulocytes can sense emergency signals like cell surface molecules of microorganisms, cell debris, cytokines produced by other immune cells, etc. They are also able to leave the vessels and migrate to the place of microbial invasion through a process called extravasation (1, 16). Extravasation includes numerous steps, controlled by various selectin and integrin molecules on the surface of both neutrophils and endothelial cells (17-19).

Once inside the interstitial space, neutrophils follow chemoattractant molecules (e.g.: CXC and CC chemokines, formyl peptides, LTB_4, complement fragments) which guide them towards the inflammation (17, 20, 21). Here they carry out many different effector mechanisms to promote immune defence. As a first step, they can recognise the pathogens using either PRR, Fcγ-receptors or complement receptors and carry out phagocytosis. They can also release granular proteins from their four different types of cytoplasmic granules and secretory vesicles. Primary granules release antibacterial proteins into the phagosome, while the other types release their content mainly into the extracellular space. As a result of degranulation (22), neutrophils are able to kill pathogens both inside and outside the phagosome. Some of these released granular proteins can further promote the movement of neutrophils in the tissues, thereby enhancing anti-microbial actions. Secondary and tertiary granules, and secretory vesicles also express various kinds of proteins in their membrane (e.g.: formyl peptide-receptor, LPS-receptor etc.) which further activate neutrophils and promote neutrophil-related host defence mechanisms. Neutrophils are also able to produce reactive oxygen and nitrogen species, in part via their NADPH oxidase Nox2. The superoxide anions released into the

phagosomes can lead to the degradation of engulfed pathogens (23, 24). PMNs both at rest or during activation by pathogens can release extracellular vesicles (EVs), which are thought to contain antimicrobial peptides and can activate other innate immune cells, as well (25).

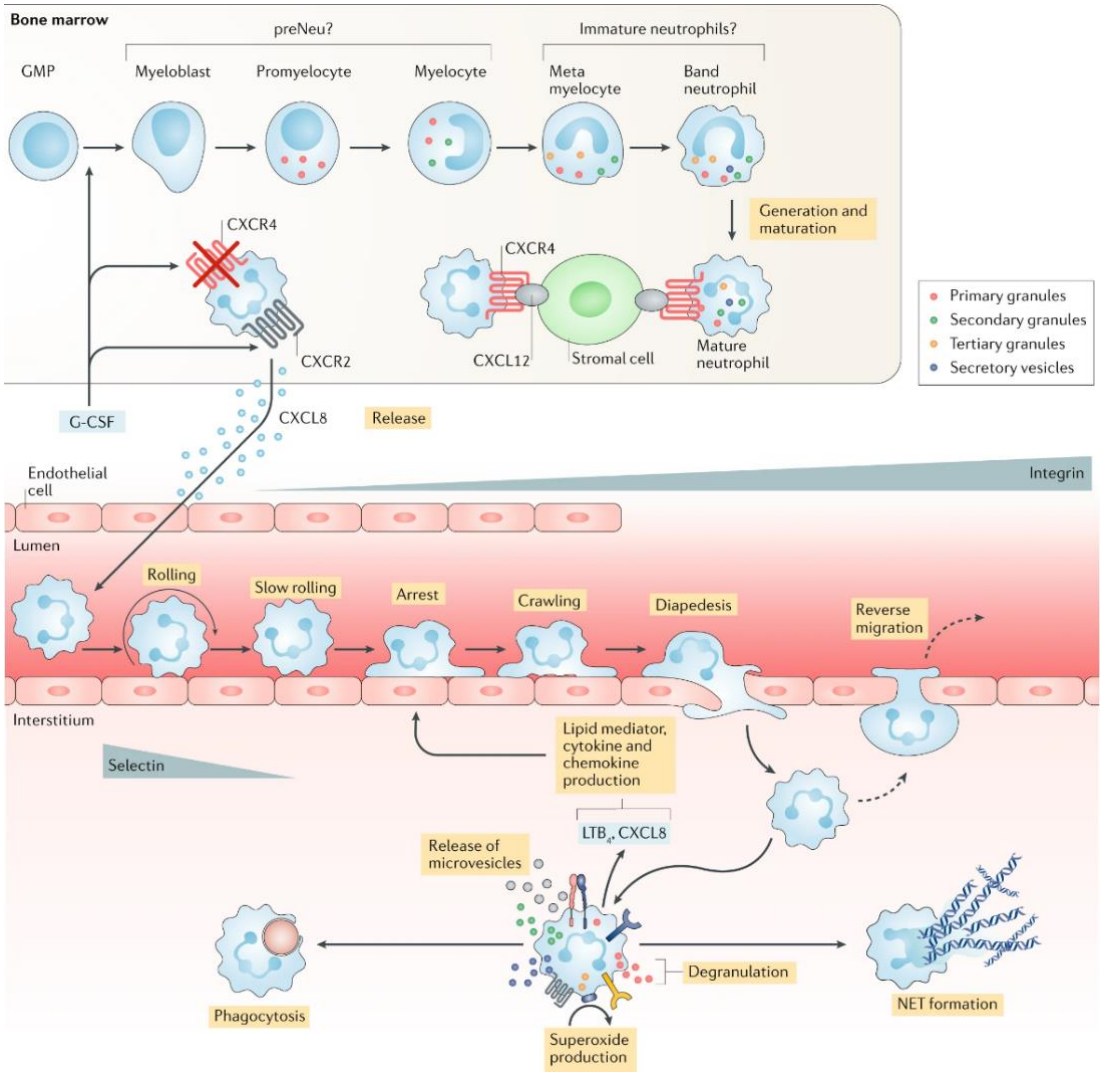

Figure 1. Neutrophil development and function *(From Németh et al., Fig. 1 (3)). Neutrophils develop in the bone marrow from granulocyte-monocyte progenitor (GMP) cells into neutrophils with highly segmented nucleus through many different steps. Neutrophils reside in the bone marrow because of CXCL12-CXCR4 connection. Upon infection neutrophils are mobilized from the marrow and they can leave the circulation through numerous steps of extravasation. In tissues neutrophils help to fight pathogens via superoxide production, phagocytosis, degranulation, NET formation, cytokine and chemokine production.*

Neutrophils are also known to create extracellular traps (NETs) as a result of NETosis. The function of these NETs is rather controversial as more and more types of NETs are discovered. But it is primarily thought to trap pathogens inside a mixture of DNA and histone proteins released from the cells' nucleus, also accompanied with different types of proteases, elastase, defensins, etc., to kill evaders more effectively (26, 27). Moreover, neutrophils are also capable of producing cytokines and chemokines (i.e.: IL-1α, IL-1β, IL-6, LTB_4, IL-8/MIP-2, etc.) that activate and recruit other immune cell types to the site of inflammation to participate in the immune response (1, 28).

1.1.3. The role of neutrophils in various diseases

Even though neutrophils play critical roles in the host defence mechanisms via their several effector functions (listed in section 1.1.2.), quite often their abnormal activation leads to disease formation of various kind.

According to Németh et al. (3), there are three major ways neutrophils can contribute to disease development. One occurs because of the poor activation of neutrophils which leads to insufficient effector functions. This particular form can cause systemic spread of infectious agents, like bacteria and fungi.

Overactivation of neutrophils can be just as dangerous as their insufficient activation. Uncontrolled production of ROS, degranulation, NET formation and release of proteolytic enzymes can lead to severe pulmonary diseases like COPD (29) or asthma (30), atherosclerosis (31), multiple sclerosis (32) Alzheimer's disease (33) SLE (4, 34), rheumatoid arthritis (4, 35, 36), etc.

The third possible mechanism leading to neutrophil-dependent disease formation happens when neutrophils are active, but instead of carrying out their regular effector functions they show pathogenic behaviour. This seems to play a role in cancer development and metastasis, but the complex nature of this disease formation is yet to be completely understood (3, 37).

1.2. Experimental tools in neutrophil research

Based on their role in the pathogenesis of diverse human diseases, there has always been a major interest in understanding neutrophil function from a biological, medical, and pharmacological perspective. Even though there are several effector

functions of these cells (Fig.1.) that can be tested by numerous experimental procedures, the inaccessibility of effective neutrophil-targeting conventional genetic manipulation techniques in vivo and the lack of neutrophil cell lines that could be successfully used in vitro have been great challenges in the field.

One of the main obstacles of neutrophil research is that these are short-lived cells which can circulate in the body only for a short period of time (current estimates range from a couple of hours to up to 5 days) (3, 4, 38). The aging neutrophils start to express CXCR4 instead of CXCR2 which promotes their homing towards peripheral tissues (39). In tissues they undergo apoptosis and are finally cleared by macrophages. Their short lifespan is most likely related to an intrinsic proapoptotic program controlled by antiapoptotic members of the Bcl-2 protein family, with a predominant role for Mcl-1 (Myeloid cell leukemia-1 protein) (36, 40). Mcl-1 is expressed mostly in cells of the hematopoietic system. It seems to have an important role in the development of many cell types (41). Mcl-1 is essential for the maturation of B-and T cells (42), survival of hematopoietic stem cells (43) and synovial fibroblasts (44). Deletion of the protein coding gene *Mcl1* results in embryonic lethality in very early stages in mice (45). Lack of Mcl-1 in CMP and GMP cells resulted in severe and fast cell death (43). Mcl-1 carries out its antiapoptotic effect by supressing proapoptotic proteins such as Bim, Bak and Bid (46, 47).

The other main problem which makes neutrophil research difficult, is that neutrophils are also terminally differentiated cells: they are unable to proliferate and have a transcriptionally silent genetic machinery. Therefore, it is impossible to further culture neutrophils that were freshly isolated from bone marrow or human blood. It is at present also not possible to expand, maintain and genetically manipulate these isolated neutrophils in vitro. Although primary neutrophils can be isolated relatively easily for short-term experiments, our tools for more complex interventions are very limited.

Therefore, we are in constant need of new and effective tools that could be useful to study the biological functions and effector mechanisms of neutrophils in various diseases both in vitro and in vivo.

1.2.1. Experimental tools to study the role of neutrophils in vivo

1.2.1.1. Pharmacological depletion of neutrophils in vivo

One commonly used method to study the role of a certain cell type is to deplete the cell type of interest (e.g.: neutrophils) in vivo in order to characterize the resulting phenotype. Neutrophil depletion can be achieved by administration of different pharmacological agents in vivo, like cyclophosphamide (47) or vinblastine (48).

Various depleting antibodies (e.g.: anti-Ly6G antibody in mice) can also be administered to achieve depletion of neutrophils (49-51). However, pharmacological depletion does not provide a sustained reduction of circulating neutrophils, and as freshly differentiated neutrophils from the bone marrow enter the bloodstream the effect of the depleting agents get diluted.

1.2.1.1.1. Cyclophosphamide

Cyclophosphamide is a commonly used drug in cancer therapies (52). Its main function is to cause cell death via the inactivation of the cell cycle machinery by inducing DNA crosslinks and DNA strand lesions (53). After cyclophosphamide treatment neutrophils completely disappear from the circulation of mice. However, treated animals seem to be more susceptible to infections most likely because cyclophosphamide induces the death of the hematopoietic stem cells, and it blocks the proliferation and differentiation of other leukocytes as well (54, 55). The biggest disadvantage of cyclophosphamide is that apart from neutrophil depletion it greatly reduces the number of monocytes, T and B-cells as well (56, 57).

1.2.1.1.2. Vinblastine

Vinblastine is an alkaloid from *Catharanthus roseus* that can cause depletion of neutrophils, monocytes, and lymphocytes alike (48, 58). The cell depletion is achieved via blocking the formation of the mitotic spindle in the metaphase of the cell cycle (59, 60). Even though it is a quite effective neutrophil depleting agent it is rarely used in vivo, mostly because of its non-neutrophil-specific nature.

1.2.1.1.3. Anti-Gr-1 antibody

Anti-Gr-1 (RB6-8C5) monoclonal antibody is specific for Ly6G and Ly6C proteins on the surface of neutrophils and monocytes (49, 61). For many years it was believed to be a highly neutrophil-specific antibody (62), however, it is clear now, that it also effects monocytes and $CD8^+$ memory T-cell counts as well (63). However, as a result of anti-Gr-1 antibody treatment various side effects (such as damage in the capillaries and respiratory system) were also assessed, along with incompatibility with different genetic backgrounds or mutations (49, 64).

1.2.1.1.4. Anti-Ly6G antibody

Anti-LyG antibody successfully replaced the anti-Gr-1 antibody in neutrophil-specific depletion studies, as the Ly6G protein is only present on the surface of neutrophils in mice (61).

There are many monoclonal Ly6G antibodies on the market. For example, the 1A8 rat anti-mouse IgG2 antibody completely depletes neutrophils from the circulation and 1A8 treatment results in approximately 80% reduction of neutrophil counts in the liver as well (50, 65). Even though it is more specific than the anti-Gr-1 RB6-8C5 antibody, but for a sustained neutrophil depletion multiple and high doses of antibody treatment is necessary (50). 1A8 however, was not effective in C57Bl/6J mice older than 24 weeks of age, and it could not deplete neutrophils from the bone marrow of BALB/C mice either (66).

NIMP-R14 monoclonal antibody also recognises the Ly6G protein, and it can deplete approximately 95% of neutrophils from the circulation and spleen (51, 67, 68). However, there are various publications arguing that NIMP-R14 is not specific to neutrophils, and this antibody treatment decreases the number of circulating and also the $Ly6C^{high}$ monocytes in the spleen (69).

1.2.1.2 Genetically neutropenic mouse models in neutrophil research

Even though there are effective neutrophil depleting agents, but they are not suitable tools for sustained reduction in neutrophil numbers. However, as a result of genetic modifications several neutropenic transgenic mouse strains have been developed which can be useful to study the function of neutrophils in various experimental

approaches in vivo. Unfortunately, most of these neutropenic mouse strains have very serious limitations to them. For example, cell types other than neutrophils can also be affected, they can be difficult to breed or have a limited lifespan.

1.2.1.2.1. G- CSF receptor knock out and G-CSF knock out mice

G-CSF (Granulocyte colony stimulating factor) and its receptor G-CSFR plays an important role in the proliferation, differentiation, and survival of granulocytes. By the deletion of the G-CSFR coding *Csf3r* gene, the neutrophil cell counts in the transgenic animals decreased by 80% in the circulation and approximately 50% in the bone marrow (70, 71). Partial deletion of the neutrophil compartment, together with multiple side effects in osteoblast function and bone homeostasis as well as in endothelial cell proliferation (72, 73) are the main reason for unfrequented use of this mouse model. It is also important to mention, that this mutation is lethal in a homozygous form, which complicates the breeding of the animals (74). The deletion of the G-CSF ligand coding gene resulted in very similar neutrophil deficiency compared to the G-CSFR knockouts (75).

1.2.1.2.2. Cxcr2 knock out mice

CXCR2 is responsible for the mobilization of the bone marrow neutrophil pool, as it causes the release of mature neutrophils from the bone marrow into the circulation during pathogen invasion of the body. If CXCR2 is deleted, a nearly 60% reduction in the circulating neutrophil number can be observed in the peripheral blood of the animals (76). However, since CXCR2 is also expressed in monocytes, macrophages, mast cells and endothelial cells, the deletion of the gene might alter the function of these cells as well, which complicates the interpretation of the immune response in these transgenic mice. Not to mention, that the *Cxcr2* knockout animals can become seriously immunocompromised, which is a big limitation of this model as well and needs to be taken into consideration when using these animals (76).

1.2.1.2.3. Gfi-1-deficient mice

Gfi1 (Growth factor independent-1) gene encodes a nuclear zinc finger protein that functions as a transcriptional repressor. It plays important a role in diverse

developmental processes like haematopoiesis and oncogenesis. In its absence, mature neutrophils are missing from the circulation, as are transcripts for neutrophil-specific secondary and tertiary granule proteins. The exact mechanism of its action however is unknown (77). *Gfi1* gene deletion causes almost complete eradication of neutrophil population; however, these mice seem to have a wasting phenotype with a high rate of mortality (77, 78). Mature neutrophils are practically missing from the circulation, spleen, and bone marrow of Gfi-1-deficient mice. However, an atypical myeloid population appears in these animals with both granulocytic and macrophage-like features, which can produce reactive oxygen species and carry out phagocytosis as well (77). At the same time, dendritic cells, B-and T-cells of these mice show abnormal differentiation. And since Gfi-1 is also highly expressed in the brain, severe behavioural changes can also be observed in Gfi-1-deficient mice (79). Due to all of the above-mentioned drawbacks, Gfi-1-deficient mice are rarely used in neutrophil-related research in the recent years.

1.2.1.2.4. Genista mouse model

In the so-called Genista mice, the previously (1.2.2.3.) mentioned Gfi-1 protein is not completely missing but rather the DNA binding property of the protein is compromised. It seems that the neutrophils in these mice are unable to reach a completely mature state, resulting in increased susceptibility to pathogens (80, 81). Therefore, Genista mice were successfully used in studies related to neutrophil-mediated bacterial elimination (81). However, neutrophil deletion is not complete in these animals either, so these mice are not quite suited to extensive studies on neutrophil biology.

1.2.1.2.5. Foxo3a-deficient mice

Proteins in the Forkhead (FOXO) transcription factor subfamily are important molecules in the regulation of immune cell proliferation and apoptosis. Upon Foxo3a deletion, the number of neutrophils in the periphery seem to be normal, however, Foxo3a-deficient mice are protected in various PMN-mediated inflammatory experiments (e.g.: immune complex-mediated joint inflammation or thioglycollate-induced peritonitis) (82). This is most likely due the increased apoptosis of neutrophils in inflammatory conditions, leading to overall reduced neutrophil numbers at the site of inflammation (82, 83).

Therefore, Foxo3a-deficient mice are most used when the role of neutrophils are studied in inflammatory processes.

1.2.1.2.6. Mcl1$^{\Delta PMN}$ (Mrp8-Cre Mcl1$^{flox/flox}$) mice

Our research group has generated and characterised the *Mcl1*$^{\Delta PMN}$ mice as a possible neutropenia mouse model (36). The Mrp8-Cre*Mcl1*$^{flox/flox}$ mice, also called as *Mcl1*$^{\Delta PMN}$ is a neutropenic mouse strain which is generated via mating *Mcl1*$^{flox/flox}$ mice with MRP8-Cre transgene expressing animals. As a result of the mating, *Mcl1* is deleted in the MRP8 expressing cells, which are the neutrophils (84), initiating the apoptosis of mature neutrophils in these mice. The apoptosis of neutrophils is achieved by the lack of the Mcl-1 protein in the neutrophil compartment since Mcl-1 is an antiapoptotic protein known to be important in the development of various cell types, including neutrophils. It exerts its anti-apoptotic effect in neutrophils via blocking the pro-apoptotic proteins Bim, Bak, and Bid (85).

Mcl1$^{\Delta PMN}$ mice show a drastic (99,1%) neutrophil deficiency, without having major effects on the number of other immune cell types. However, these mice have a very poor survival rate: by 6 months of age 42%, at 12 months of age 70% of the animals succumbed to death. *Mcl1*$^{\Delta PMN}$ mice seem to be unhealthy compared to their wild-type littermates. Upon homozygous mating, pairs show strongly reduced fertility, meaning that only 14% of mating pairs have offspring. Due to the difficult breeding and poor survival of *Mcl1*$^{\Delta PMN}$ mice, extended experiments or even the maintenance of the colonies are hard to be executed with these animals, therefore we concluded that this model has only limited potential for neutrophil-related research in vivo (36).

1.2.1.2.7. Mcl1$^{\Delta Myelo}$ (Lyz2$^{Cre/Cre}$ Mcl1$^{flox/flox}$) mice

In search of a good neutropenia mouse model which secures a significantly reduced neutrophil cell count while maintaining good fertility and survival rate, our group has developed the *Mcl1*$^{\Delta Myelo}$ mouse strain (36). In these mice the *Mcl1* gene is selectively deleted via the Cre recombinase's activity in Lysozyme M-expressing cells. These cells are known to be the myeloid cells.

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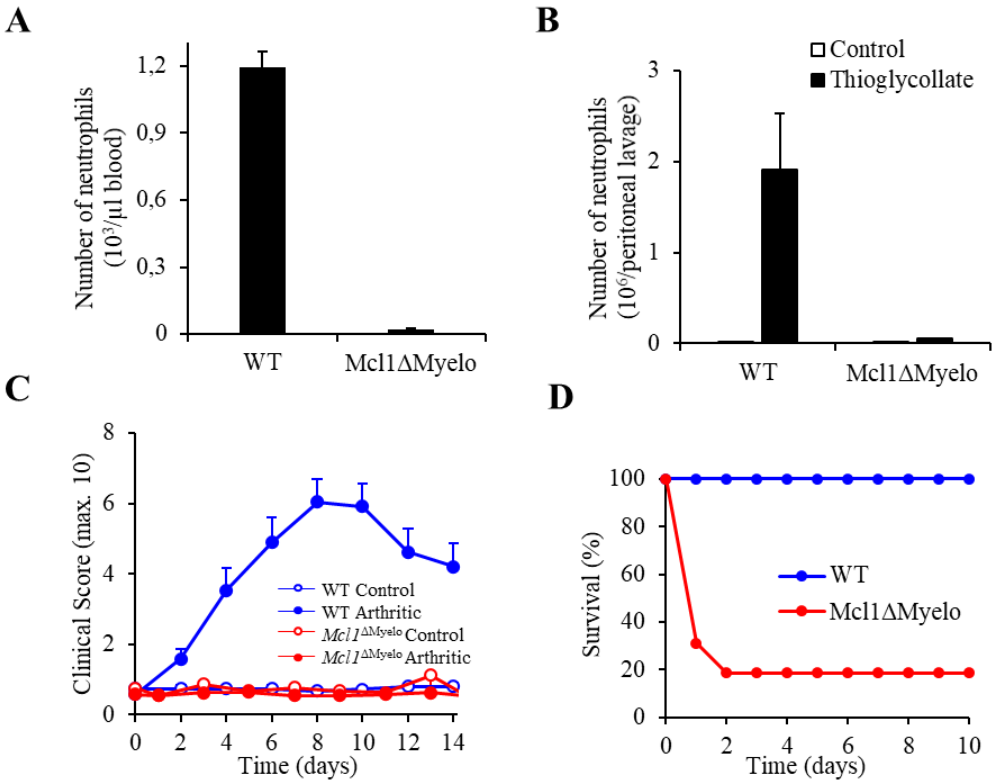

Figure 2. Characteristics of the Mcl1$^{\Delta Myelo}$ mice model *(Based on Csepregi et al. (36)). (A) The number of circulating neutrophils is reduced by 98% in the Mcl1$^{\Delta Myelo}$ mice compared to WT controls. (B) Neutrophils of the Mcl1$^{\Delta Myelo}$ mice fail to migrate into the peritoneum upon inflammation. (C) Mcl1$^{\Delta Myelo}$ mice are protected in autoantibody-induced inflammatory arthritis. (D) Mcl1$^{\Delta Myelo}$ mice are highly susceptible to Staphylococcus aureus infection.*

Through extensive experiments we have demonstrated that the Cre/lox–mediated myeloid-specific deletion of *Mcl1* leads to very severe neutropenia without affecting other hematopoietic lineages. The overall PMN count in the *Mcl1$^{\Delta Myelo}$* mice showed 98,1% reduction compared to their wild-type littermates (36, 86, 87).

This severely neutropenic phenotype of the *Mcl1$^{\Delta Myelo}$* mice is also noticeable in inflammatory conditions, as thioglycollate injection fails to evoke neutrophil accumulation in the peritoneum of the animals (36).

Surprisingly, we have found that *Mcl1$^{\Delta Myelo}$* mice can survive and breed in homozygous form under specific pathogen-free conditions for a prolonged time. The survival rate and number of offspring are comparable to those of the wild-type animals. This means that the *Mcl1$^{\Delta Myelo}$* mouse strain could provide a high number of apparently healthy animals for various in vivo experiments related to neutrophil research (36).

We have also found that the *Mcl1*$^{\Delta Myelo}$ mice are completely protected in two separate types of supposedly neutrophil-mediated autoantibody-induced inflammation models, namely the autoantibody-induced arthritis and in the autoimmune skin inflammation model of epidermolysis bullosa acquisita as well (36).

Nonetheless, *Mcl1*$^{\Delta Myelo}$ mice seemed to be highly susceptible to infectious challenges by bacterial or fungal pathogens, such as *S. aureus* or *C. albicans*, most likely because of the defect in the neutrophil mediated elimination of the pathogens (36).

1.2.1.3. Genetically inactivated models of neutrophil research

Important functions and roles of the neutrophils in various diseases were revealed by studying genetically modified mice. Especially common method is to knock out genes that are thought to be key regulators in the effector mechanisms of neutrophil immune response and study the effects of gene deletion in vivo.

Many knock out models of key enzymes in neutrophils have been developed in the recent years (83), which provide great tools for neutrophil research. However once using these mice we must be aware that the gene deletion is most likely not only affecting the cellular functions of neutrophils, but functions of other cell types might also be disturbed as well. Some of the most commonly used knock out models are listed below, highlighting both their advantages and drawbacks in relation to neutrophil studies.

1.2.1.3.1. Myeloperoxidase knock out mice (MPO$^{-/-}$)

Myeloperoxidase (MPO) is an abundant enzyme in myeloid cells that catalyses the reaction between chloride and hydrogen peroxide to generate hypochlorous acid, which is essential in pathogen elimination of these cells.

MPO$^{-/-}$ mice have normal neutrophil counts both in the circulation and in the periphery. ROS production of neutrophils in these mice is increased, however the PMA (Phorbol 12-myristate 13-acetate)-induced NET formation is impaired. Since MPO is expressed in monocytes, macrophages, and B-cells as well, the function of these cells might also be altered in addition to neutrophils' effector functions, which might complicate the interpretation of the experimental results with these animals (83, 88, 89).

1.2.1.3.2. Neutrophil elastase knock out mice (ELANE$^{-/-}$)

Neutrophil elastase (NE) is a serine proteinase which is expressed in neutrophils during myeloid development. It is stored in azurophil granules and is released when neutrophils are activated to fight invading pathogens.

Neutrophil elastase gene (*ELANE*) mutant mice show normal circulating leukocyte counts; however, the effector functions of neutrophils are severely impaired. Neutrophils fail to migrate into the peripheral tissues, they cannot carry out phagocytosis, cytokine production and NET formation either. *ELANE*$^{-/-}$ mice are more susceptible to intraperitoneal infection with Gram negative bacteria like *Klebsiella pneumoniae* and *Escherichia coli* as well (83, 90). This mouse strain is mostly used to uncover the effects of granule-originating enzymes in neutrophil-related pathogen elimination.

1.2.1.3.3. Protein arginine deiminase 4 (PADI4) knock out mice

PAD4 catalyses the citrullination/deimination of arginine residues of multiple intracellular and extracellular proteins. Dysregulated PAD4 activity can lead to the onset and progression of rheumatoid arthritis (RA), as deiminated proteins within the RA synovium are incredibly abundant. High levels of these citrulline-containing proteins correlate with increased joint destruction and inflammation (91). Important information about the pathogenesis of rheumatoid arthritis were primarily assessed by using the *PADI4*$^{-/-}$ mouse model.

Neutrophils of *PADI4*$^{-/-}$ mice show defective NETosis and bacterial killing (92, 93), however PAD4 is also expressed in macrophages, therefore the susceptibility of these mice to bacterial infection might not come from the decreased function of neutrophils only (94). This latter aspect complicates the usage of *PADI4*$^{-/-}$ animals in neutrophil-specific experiments.

1.2.1.3.4. Neutrophil cytosolic factor 1 (Ncf1) knock out mice

Ncf1 is a 47 kDa cytosolic subunit of neutrophil NADPH oxidase. Since the mutation disrupts the NADPH oxidase machinery, which is essential in neutrophil superoxide formation, neutrophils in KO mice are unable to produce reactive oxygen

species. NETosis of neutrophils is also impaired in Ncf1$^{-/-}$ mice (83). Moreover, phagocytic activity of macrophage and cytotoxicity of T-cells seem to be also reduced in these animals (83), which impair the neutrophil-specificity of this model.

1.2.2. Functional knockout mouse models of neutrophil research

The most widely used approach to test genetically manipulated neutrophils is the analysis of neutrophils from transgenic mice carrying germline mutations. Even though it is a feasible method, the major costs, and efforts of generating and maintaining germline mutations are quite significant. It is also very difficult to manipulate the developmental program of the neutrophils, meaning that every effective mutation must be introduced in an early developmental stage (e.g., germline) making the mutational process quite difficult with a low success rate.

I have listed the most commonly used knock out mice models to uncover the role of neutrophils in various in vivo mechanisms above, but several other knock out strains have also been developed in the last decade. These models are important in understanding how exactly neutrophils execute their most important role, namely resolving and in case of their overactivation, promoting inflammation.

However, since in most of these mouse models the gene deletion does not only effect neutrophils, but the function of other cell types might also be altered as well, there was a great need to develop neutrophil-specific knockout mouse models. Neutrophil-specificity was achieved via generating conditional KO mouse models using the Cre-Lox recombinase system (95, 96). MRP8-Cre mediated gene deletion yielded several new transgenic mouse models. In these animals the targeted genes are deleted strictly in neutrophils, due to the sole expression of MRP8-Cre recombinase in the neutrophil compartment.

For example, MRP8-Cre^{+}*Card9*$^{flox/flox}$ animals have the Card9 (Caspase recruitment domain-containing protein 9)-coding gene deleted in their neutrophils. Card9 is an adaptor protein which mediates signals from PRR receptors and activates cytokine production of neutrophils. MRP8-Cre^{+}*Card9*$^{flox/flox}$ animals fail to release proinflammatory chemokines and cytokines therefore these mice seemed to be protected in heavily neutrophil-mediated autoantibody induced experimental arthritis and skin blistering (97).

A very similar approach confirmed that Syk (Spleen tyrosine kinase) expression in neutrophils, but not in platelets or mast cells is essential for the development of autoantibody-induced arthritis as well (98).

Also, Futosi and all confirmed the important role of another protein in neutrophils' function using another conditional KO mouse strain. Neutrophil-specific deletion of PLCγ2 (Phospholipase C gamma 2), an enzyme critically important in neutrophil signalling pathways, inhibited the development of autoantibody-induced arthritis, most like due to a reduced generation of cytokines by neutrophils in the inflammatory microenvironment (99). Neutrophils in these MRP8-Cre^{+}*PLCg2*$^{flox/flox}$ animals failed to produce IL-1β, MIP-2 and LTB4 in synovial tissues, which reduced the secondary infiltration of macrophages into the inflamed tissues (99).

1.2.3. Myeloid leukemia cell lines

Apart from an in vivo approach, there is a need to study the functional responsiveness of neutrophils in vitro as well. For this purpose, either primary neutrophils can be isolated from bone marrow and blood samples, or myeloid leukemia cell lines can be used. However, both of these techniques have their own limitations. For example, freshly isolated neutrophils are only suitable for short-term experiments as these neutrophils cannot be cultured in vitro after isolation. And although there are many myeloid leukemia cell lines that can be differentiated towards the neutrophil lineage (100, 101), those cell lines lack several important features of mature neutrophils which hinders the possibility of using them for extensive experiments. Most of the time they cannot be considered near-primary neutrophil-like cells. Moreover, their function cannot be tested in vivo either, due to their malignant nature and their mostly human origin which is usually not compatible with studies in experimental mice. The characteristics of the most widely used myeloid leukemia cell lines are described further below, and their most important features are also listed in Table 1.

Table 1. Characteristics of the most widely used myeloid leukemia cell lines in neutrophil research.

	Origin	Maturity	Fate	Differentiating agent	Neutrophil-related functional activity	Drawbacks
HL-60	Human	Promyelocyte	Neutrophil, Monocyte and Macrophage-like cells	DMSO, butyric acid, vitamin-E	Phagocytosis	Incomplete differentiation, Multipotency
PLB-985	Human	Myelomonoblast	Granulocyte, Monocyte	DMSO, vitamin-E, PMA	Degranulation, ROS production	Difficult to maintain, Low differentiated cell number
K-562	Human	Myelomonoblast	Monocyte, Granulocyte, Erythrocyte	Deacetylase activation	-	Multipotency
NB4	Human	Promyelocyte	Neutrophil	ATRA	-	They do not have detectable effector functions
32Dcl-3	Mouse	Promyelocyte	Neutrophil	IL-3 withdrawal + G-CSF	Adhesion, Degranulation, Phagocytosis	No ROS production, Difficult to maintain
EML/EPRO	Mouse	Early multipotent progenitor	Myeloid cells, Lymphoid cells, Erythrocytes, Megakaryocytes, etc.	ATRA+GM-SCF	Ly6G expression, Gelatinase and Lactoferrin containing granule formation	Multipotency, Difficult to maintain
MPRO	Mouse	Promyelocyte	Neutrophil	ATRA+GM-SCF	Granular protein expression	Difficult to maintain
WEHI-3B	Mouse	Myelomonocyte	Neutrophil	-	Phagocytosis, Lysozyme production	Week ADCC activity

1.2.3.1. HL-60 human promyelocytic leukemia cell line

The most commonly used cell line for neutrophil-related research is a promyelocytic cell line derived by S.J. Collins and colleagues, known as HL-60 cells (102). HL-60 cells were grown from peripheral blood leukocytes obtained from a 36-year-old Caucasian female with acute promyelocytic leukemia. Administration of HL-60 cells into nude mice resulted in cancer formation, which proves the cell line's leukemic origin (103). The initial growth of the cells required conditioned media from whole human embryonic fibroblast cultures, but for the sustained growth, HL-60 cells do not require other exogenous factors.

The addition of DMSO to the culture induces HL-60 cells to differentiate into neutrophil-like cells. Several other compounds (e.g., butyric acid, antioxidants and

vitamin E (104)) can also induce analogous maturation of the HL-60 cells (101, 103), while other compounds can also cause monocyte/macrophage-like cell differentiation (105).

With the Wright-Giemsa staining, differentiated HL-60 cells seem to be mostly myeloblasts and promyelocytes, with the occasional emergence of more mature myeloid cells. Lymphoid cells are completely missing from the culture. Once differentiated, HL-60 neutrophil-like cells can carry out phagocytosis of *C.albicans* in vitro (103, 106). However, there are serious issues with this specific cell type: in many respects, their differentiation seems to be incomplete and does not fit into the myelomonocytic scheme that we know. Moreover, many of the differentiated cells show more monocyte/macrophage like characteristics than previously thought (105). So, they cannot really be considered a purely neutrophil-specific cell line.

1.2.3.2. PLB-985 cell line

DNA fingerprinting showed that the PLB-985 cell line is a subclone of HL-60 cells (107). It means that they are not original cells, but rather the effect of poor cell culturing methods resulting in the cross contamination of the original cell culture with other cell types. This is likely what happened with HL-60 cells, resulting in the now so called PLB-985 cell line. The genome of these two cell lines is so much alike, that it is highly likely that they are originally from the same cell cultures. This easy cross-contamination is a hidden disadvantage of all leukemia–lymphoma (LL) cell lines (107).

PLB-985 cells are primarily myelomonoblasts with a clearly leukemic origin. It is a diploid cell line where cells have both granulocytic and monocytic differentiation capacity in the presence of inducing agents similar to the ones used in case of HL-60 cells. Other agents like PMA induce their monocytic differentiation (108). However, even when the cells reach their maximum differentiation stage, they fail to contain primary and secondary neutrophilic granules (108). A new modified differentiation protocol has been reported recently which claims that upon ideal conditions, PLB-985 cells can be cultured into neutrophil-like cells with granulated cytoplasm and delayed apoptosis (109). PLB-985 cells that were differentiated in optimized conditions showed degranulation and ROS production upon formyl-peptide stimulation (110). Nonetheless, they still require very

meticulous culturing protocols with questionable success rate as far as the number of mature, functionally active neutrophils are concerned.

1.2.3.3. K-562 cell line

K-562 was the first human immortalised myelogenous cell line to be established. The cells were derived form a 53-year-old female with chronic myelogenous leukemia in terminal blast crisis (111, 112).

These cells can spontaneously develop features similar to early-stage monocytes, granulocytes and erythrocytes (111). The multipotent progenitors in the cell culture start their differentiation by the initiation of the histone deacetylase activity (113). During differentiation, progenitors change their phenotype and morphology, decrease their growth rate, and turn into major erythroid cells, monocytes, or granulocytes alike (113). However, their multipotent nature is one of the biggest limitations of these cells in neutrophil studies.

1.2.3.4. NB4 cell line

NB4 is an acute promyelocytic leukemia (APL) cell line of human origin (114). It was derived from the bone marrow of a patient with relapsing APL after chemotherapy. These cells are in the promyelocyte stage of neutrophil development. This cell line can undergo morphologic maturation upon ATRA (all-trans retinoic acid) administration (115). However, the cells fail to produce ROS or express secondary granule proteins (116), making them only suitable to study neutrophil development but not function (100).

1.2.3.5. 32Dcl3 cell line

This is a murine bone marrow derived IL-3 dependent cell line, which originates from long term culture of bone marrow cells from C3H/Hej mouse (117, 118). C3H/HeJ mice, commonly called C3H, are used as a general-purpose strain in a wide variety of research areas including cancer, infectious diseases, sensorineural, and cardiovascular biology research. A spontaneous mutation in Tlr4 occurred in C3H/HeJ mice at the lipopolysaccharide response locus (mutation in toll-like receptor 4 gene, Tlr4Lps-d) making these mice more resistant to endotoxin (117, 118).

32Dcl3 cell line is grown in IL-3 containing media. It is one of the few cell lines that can terminally differentiate into neutrophil-like cells once IL-3 is withdrawn and G-CSF is added to stimulate the differentiation (100). A subclone (32Dcl3C) of the original cell line shows neutrophil-like phenotype and effector functions such as adhesion, degranulation, and phagocytosis, but fails to produce reactive superoxide species (119).

One of the main problems with this cell line is that during their neutrophilic differentiation many of the cells undergo apoptosis, yielding low number of mature cells after a quite complicated culturing procedure.

1.2.3.6. EML/EPRO cell line

It is a unique multipotent murine progenitor cell line. It is originally a bone marrow-derived cell of a male B6D2F1 (also known as B6D2) mouse, known to be a useful background for transgenic manipulation (120-122). This cell line was generated by transduction of marrow progenitor cells with a retrovirus carrying a dominant negative retinoic acid receptor gene (RARa403). Myeloid differentiation is blocked in EML due to the expression of RARa403, but the block can be overcome by treatment with supraphysiological concentrations of all-trans-retinoic acid. The RARs are members of the steroid/thyroid hormone receptors that function as ligand-inducible transcription factors. RARs play important roles at the promyelocyte stage of neutrophil differentiation, as normal retinoid signalling is required for complete maturation of neutrophils (120-122).

EML culture contains progenitors that can differentiate into B-lymphocytes, erythrocytes, neutrophils, macrophages, mast cells, and megakaryocytes in the presence of appropriate cytokines. They are the only known SCF-dependent cell line with both lympho and myelo-erythroid potential (123, 124).

Once stimulated with ATRA and GM-SCF, they undergo complete morphological differentiation into neutrophil-like cells and show increased cell surface expression of the neutrophil-specific marker Ly6G (100). They can produce neutrophil-specific granule proteins like gelatinase and lactoferrin (125). Also EML cells can produce ROS upon PMA stimulation (126).

However, EML cells are very sensitive to cell density in the culture and require strict passage regimen. They can easily undergo apoptosis if the culturing conditions are not perfect (100).

1.2.3.7. MPRO cell line

Like EML cells, cells of the MPRO cell line also express a dominant negative retinoic acid receptor alpha (127), which causes developmental blockade at certain stages of the myelopoiesis (128). While EML cells have a multipotent phenotype, and nearly all types of immune cells can be differentiated from them, the MPRO cell line is mainly promyelocytic. Upon ATRA and GM-CSF treatment, they can rapidly (in 72 h) differentiate towards the neutrophil fate (100).

These cells can undergo neutrophil differentiation similar to EML cells. They can differentiate into CD11b/CD18 expressing mature neutrophil-like cells, with strong neutrophil-like morphology and granular protein expression (125).

The major problem with these cells is very similar to the previously mentioned cell type: they do not tolerate imperfect culturing conditions, meaning that their culturing requires meticulous protocols (100).

1.2.3.8. WEHI-3B cell line

WEHI-3B is a murine myelomonocytic leukemia cell line originating from peripheral blood cells of a BALB/c mouse (129). WEHI-3B cells are derived from a BALB/c mouse which has undergone mineral oil injections intended to induce plasma cell tumour development. Cell lines were adapted from ascites or solid forms of lymphomas without the use of reducing agents, in some cases by repeated cycles of culture and mouse passages. WEHI-3B cells were spontaneously developed, and their IL-3 secretion is also spontaneous (130, 131). This cell line can be used to study hematopoietic differentiation and proliferation in vitro while comparing normal and leukemic cell differentiation (132).

It was described that WEHI-3 cells produce lysozyme and can carry out phagocytosis. But they show only weak effector activity in antibody dependent cell mediated cytotoxicity (131, 133), so they are not the most accurate tools for extensive neutrophil studies regarding neutrophil cell functions.

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1.3. ER-HoxB8 progenitor cell line

Neutrophils have been in the focus of research related to inflammation and autoimmune diseases in the recent decade. And as I have explained in detail in the previous sections, there are two major approaches to study the molecular mechanisms and the genetic machinery behind the different roles of neutrophils. Some research focus on whether neutrophils have a role in certain diseases at all, while others would like to uncover the detailed molecular machinery behind diseases that are already known to be neutrophil-mediated. No matter whether researchers opt for pharmacologic depletion, diverse genetic manipulation techniques or leukemic cell lines to answer the biological questions, they all face the major difficulties of neutrophil research. Namely it is expensive and time consuming to generate gene modified animals, it is troublesome to isolate enough biological material for in vitro experiments and after isolation cells are only viable for a short period of time without the possibility of further culturing them in vitro.

However, we believe that the development of a very elegant approach might address all the different limitations of culturing and manipulating the neutrophil linage by conditionally expressing the HoxB8 transcription factor in myeloid progenitor cells (5).

1.3.1. The Hox family of transcription factors and their function

The *Hox* genes are a set of transcription factor genes that are widely known to specify the so-called segment identity of the maturing embryo. These are a cluster of genes whose order in the genome is highly homologous throughout the different species from *Drosophila* all the way to mammals. Basically, the order of the Hox genes from 3’-5’ on the chromosomes determine whether a certain segment of the embryo develops into head, thorax or abdomen (134). A common feature of these proteins is the homeodomain (Figure 3.), which is a highly conserved 60-amino-acid-long motif that is responsible for binding to DNA at specifically recognised binding sites resulting in transcriptional regulation of their target genes (135). Hox proteins can act as both transcriptional activators and repressors at the same time (136).

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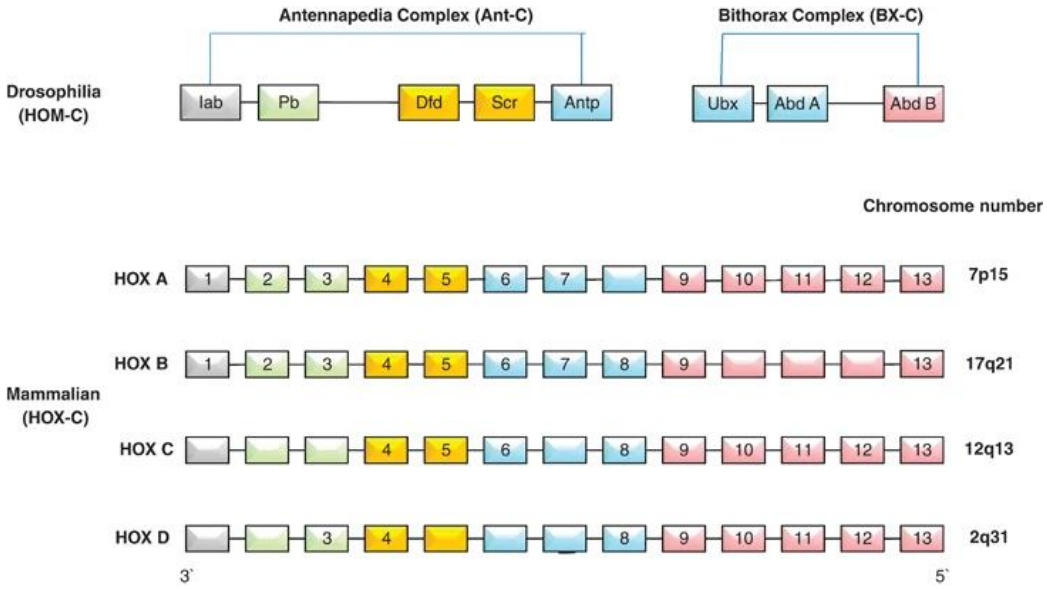

Figure 3. Cluster and homology of Hox genes in Drosophila and mammals shown by colours. *Blank squares show missing genes. (From Alharbi et al., Figure 1. (137)).*

Mammals have 39 *Hox* genes, located on four different chromosomes (135, 138). They are homologous to those of the fly. In mice there are four major cluster of *Hox* genes: *HoxA, HoxB, HoxC and HoxD* (Fig. 3.). It seems that the final morphology of the animals during embryogenesis can be attributed to the so-called Hox code, meaning that not one, but all genes in the four cluster and the combinatorial expression of them define the final phenotype of the animal. The development of each embryonic segment can be determined by all four of these genes at the same time. Genes in the four cluster show similar sequence and functional redundancy (134).

It was suggested very early, that Hox proteins are not only master regulators of the embryonic segmentation, but they also play a significant role in controlling cellular identity, cell division, adhesion and migration, morphological differentiation, and apoptosis of various cell types as well (139). Target genes of the Hox transcription factors are expressed all over the body (e.g., neurons, cells of the reproductive tract, endothelial cells, etc.) making Hox proteins likely important in numerous biological processes like angiogenesis, bone remodelling, renin-angiotensin system or even inflammation (139). Hox-family transcription factors are also critical for various stages of hematopoiesis and their excessive activation contributes to immortalization and arrested differentiation of hematopoietic cells in various forms of acute leukemia (137).

Hox genes are highly expressed in the $CD34^+$ hematopoietic cells, which are the HSCs (Hematopoietic Stem Cells) and progenitors (140), while their expression is almost completely missing from differentiated bone marrow cells which lose CD34 marker expression during their development (141, 142). Generally, it can be said that the overexpression of any *Hox* gene leads to proliferation of stem and progenitor cell populations while blocking further differentiation of these cells. This is true both in case of cells of the lymphoid and myeloid potential (143-145).

One of the important findings in case of the Hox transcription factors was, that overexpression of particular *Hox* genes, like HoxA9, leads to acute myeloid leukemia with poor prognosis in mice (146). This suggests that *Hox* genes might be important in the prolonged, uncontrolled proliferation of progenitor cells.

A less well studied *Hox* transcription factor gene, the HoxB8 (also known as Hox-2.4) is not associated with leukemia, however its deletion from the hematopoietic compartment leads to microglia-related behavioral changes in mice (147), suggesting a possible role for HoxB8 in the hematopoietic compartment. The overexpression and rearrangement of the HoxB8-encoding gene might have caused the generation of the original WEHI-3B cells, a mouse myelomonocytic leukemia cell line (mentioned in section 1.2.4.8.) (148, 149), which implies a possible link between HoxB8 overexpression and immortalization of myeloid cells. Other experiments revealed that HoxB8 overexpression in murine bone marrow cells results in the emergence of strongly IL-3-dependent myeloid cells which proliferate in the presence of high concentrations of IL-3 but undergo myeloid differentiation upon IL-3 withdrawal (150, 151). HoxB8 overexpression also led to the suspension of granulocyte differentiation (152). Altogether, these results imply that if HoxB8 overexpression is attained combined with high concentrations of IL-3, it may result in the immortalization of myeloid progenitors and blockage of their further differentiation towards mature granulocytes. Once HoxB8 is absent and IL-3 is in a low enough concentration, progenitors may start their differentiation in the usual pathway leading to differentiation of mature myeloid cells.

1.3.2. The concept of the ER-HoxB8 progenitor cell line

The above-mentioned findings led to the development of the ER-HoxB8 progenitor cell line (5). This is a myeloid progenitor cell line with conditional expression

of HoxB8 transcription factor, leading to the formation of immortalized myeloid progenitor cells.

The idea of the cell line was established 15 years ago (5), based on the ability of HoxB8 protein to block myeloid differentiation as mentioned previously. In order to achieve arrest in progenitor differentiation, HoxB8 expression by the myeloid progenitor cells had to be increased extrinsically. Moreover, since the goal was to create a progenitor cell line that is not only immortalised but could also be induced to differentiate into myeloid cells, the expression of the HoxB8 transcription factor inside the cells needed to be strictly regulated. It means, that if expansion and proliferation of the progenitor cells are required the HoxB8 transcription factor must be switched on, however, if the goal is to initiate myeloid cell differentiation of the progenitor cells, the activity of the HoxB8 protein has to be switched off.

This conditional feature of HoxB8 expression inside the progenitor cells mentioned above was achieved using retroviral transduction of the myeloid progenitor cells (5). The estrogen-binding domain of the estrogen receptor (ERBD) was fused to the N terminus of HoxB8, and this fusion protein was expressed inside the myeloid progenitor cells using the murine stem cell virus (MSCV) retroviral expression vector.

In this construct, estrogen acts as a reversible switch (153): estrogen receptor-fusion proteins are inactive in the absence of the estrogen ligand, because they are complexed with heat shock proteins (e.g.: Hsp90) of various kind intracellularly. Upon ligand binding, the fusion protein (in this case the HoxB8) is released from the inhibitory complex of heat shock proteins and becomes functionally active (Fig. 4.).

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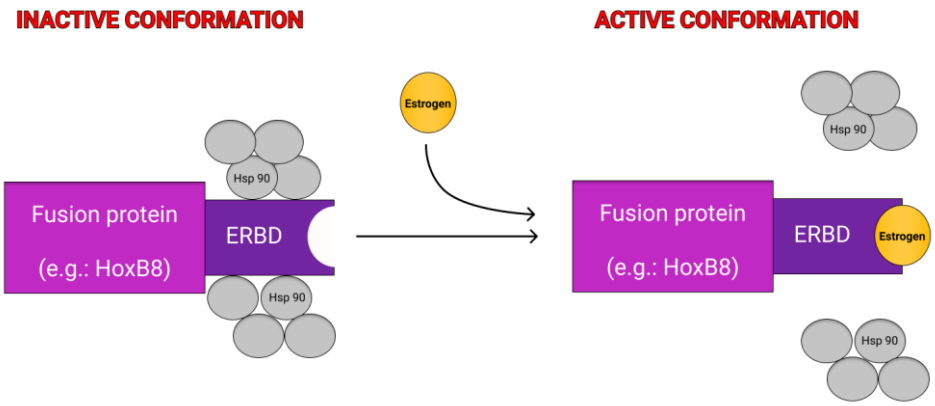

Figure 4. The switch-activity of the estrogen receptor- fusion protein system. *The schematic drawing shows the mechanism for the switch activity of the protein (e.g. HoxB8 transcription factor) fused to the ERBD (estrogen-binding domain of estrogen receptor). If the protein-ERBD is complexed with various intracellular proteins, like the heat shock protein (Hsp) 90, the fusion protein is inactive. But if estrogen binds to its receptor, the Hsp complex dissociates, and the fusion protein becomes activated.*

The advantage to this system is that the ligand (β-estradiol/estrogen) is relatively cheap and easily accessible. Moreover, hematopoietic progenitor cells do not express estrogen receptors constitutively, so endogenous expression of the receptor does not interfere with the functionality of the construct of exogenous origin.

Infection of primary bone marrow progenitor cells with the ER-HoxB8 retroviral construct in the presence of estrogen produced immortalized estrogen-dependent progenitors. Those myeloid progenitor cells that express the ER-HoxB8 construct because of the retroviral transduction are the so called HoxB8 progenitors. If HoxB8 progenitors are cultured in estrogen containing media, HoxB8 protein stays active inside the cells, therefore blocking the differentiation of progenitor cells and keeping them in oligopotent state (5).

Upon estrogen withdrawal, the HoxB8 fusion protein becomes inactive. Therefore, the estrogen-dependent HoxB8 progenitors cease their proliferation, and exhibit terminal differentiation. The differentiation of HoxB8 progenitors can be guided towards myeloid cells using various cytokines. If HoxB8 progenitors are differentiated without estrogen and in the presence of G-CSF, neutrophil-like cells appear in the

cultures. Those cells that are differentiated from the HoxB8 progenitors after estrogen withdrawal in the presence of G-CSF are called HoxB8 neutrophils (Fig. 5.) (5).

Altogether, the concept of the ER-HoxB8 system can be summarized as follows and is also visualized in Fig. 5. Neutrophils are differentiated in the bone marrow through the hematopoietic stem cell (HSC)- myeloid progenitor route. In the ER-HoxB8 system, this pathway is blocked at the myeloid progenitor stage. Instead of terminal morphological and functional differentiation in the bone marrow, myeloid progenitors are collected and conditionally immortalized via the retroviral expression of the ER-HoxB8 fusion protein. These HoxB8 progenitors are cultured in vitro for long periods of time and considered immortalised as long as estrogen keeps the HoxB8 transcription factor active. After this significant detour from the classical pathway, HoxB8 progenitors can continue their differentiation towards neutrophils in vitro if estrogen is withdrawn from their environment. The in vitro differentiation this time yields neutrophil-like cells, the HoxB8 neutrophils. This simple protocol is sufficient to generate large numbers of HoxB8 neutrophils, which can be used to study neutrophil cell differentiation, signal transduction or effector functions as well.

A very important and unique aspect of the HoxB8 system is that in vitro cultured, immortalised HoxB8 progenitors can be adoptively transferred into recipient animals, therefore generating the so-called HoxB8 chimeras. Since the concentration of estrogen is lower in the body than HoxB8 progenitors require to stay immortalised, once HoxB8 progenitors are injected into live animals, they execute their differentiation into HoxB8 neutrophils. These neutrophil-like cells can then be studied extensively in vivo as well.

Others have tried alternative protocols which yielded monocytes/macrophages, dendritic cells, basophils or osteoclasts from conditionally immortalized HoxB8 progenitors using an essentially similar approach, by using different cytokines to guide the differentiation of progenitor cells in vitro (5, 154-159).

HoxB8 neutrophils have been shown to be functionally active in various in vitro experiments, like stimulus-dependent adhesion, reactive oxygen species production, degranulation, chemokine/cytokine release, chemotaxis, antimicrobial responses or apoptosis. They were also able to respond to activation through several cell surface receptors such as GPCR-coupled chemoattractant receptors, integrins and Fc-receptors (160-165). These in vitro functions are all typical for freshly isolated bone marrow or

peripheral blood neutrophils, suggesting that during differentiation from the HoxB8 progenitors, HoxB8 neutrophils develop a quite mature neutrophil-like phenotype and effector functions.

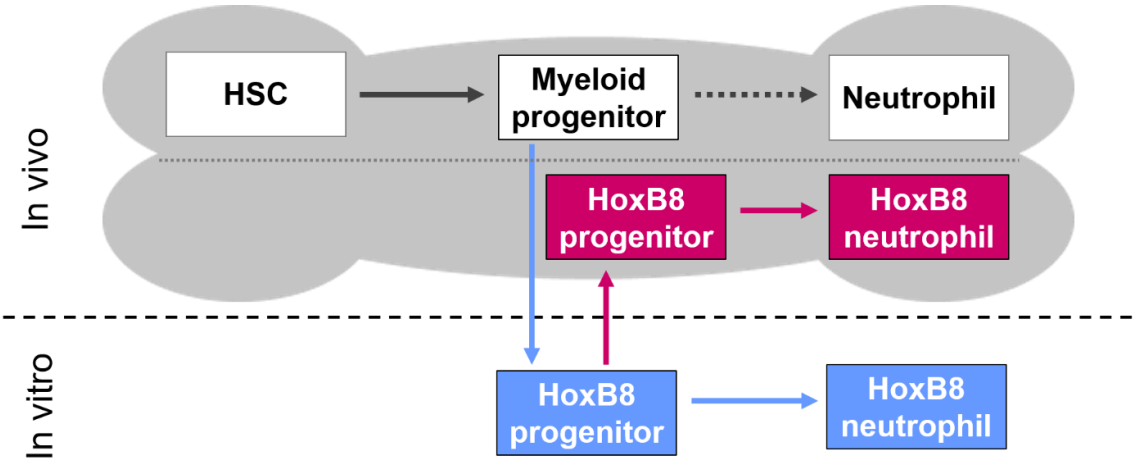

Figure 5. Concept of the ER-HoxB8 system. *In the HoxB8 cell line, the usual differentiation of neutrophils from HSC and myeloid progenitors in the bone marrow is arrested. Instead, myeloid progenitors are isolated from the bone marrow of the mice and conditionally immortalised in an estrogen-dependent manner in vitro. HoxB8 progenitors can differentiate into HoxB8 neutrophils in vitro and also in vivo if adoptively transferred into recipient mice.*

Very importantly, these findings make the ER-HoxB8 cell line eminent amongst the other cell lines mentioned in section 1.2.4. With immense numbers at reach, mostly mature phenotype and morphology coupled with functional activity comparable to primary neutrophils suggests, that HoxB8 cells can be appropriate tools in extensive neutrophil research.

However, there is very limited information about their functional responsiveness in vivo. There are only a couple of studies that focus on whether HoxB8 neutrophils can appear in vivo after injection into live mice. In most of these studies, primary neutrophils from murine bone marrow and HoxB8 neutrophils were simultaneously present in mice, hindering the possibility to analyze the functional importance of the HoxB8 progenitor-derived neutrophils only (155, 161, 166-168).

The above-mentioned information prompted us to generate immortalized HoxB8 progenitors and to examine them both in vitro and vivo. We had also developed a protocol to generate chimeras with exclusively HoxB8 progenitor-derived neutrophils in the

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circulation even for a prolonged (1-2 weeks) period. We subjected those HoxB8 chimeras to various in vivo inflammatory models in order to measure the contribution of the HoxB8 neutrophils to disease development.

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2 OBJECTIVES

During my PhD studies we have aimed to answer the following questions:

1. Are we able to generate immortalized HoxB8 progenitor cells?

2. Can we differentiate HoxB8 neutrophils from the HoxB8 progenitor cultures and characterise the effector functions of these cells in vitro?

3. Are we able to find a suitable approach to test the effector functions of HoxB8 neutrophils in vivo, both on a single-cell and whole neutrophil population-based level?

4. Characterising the effects of *Mcl1* deletion on the leukocyte populations in various tissues of *Mcl1*$^{\Delta Myelo}$ mice and reassuring the neutrophil-specificity of the mouse model.

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3 METHODS

3.1. Experimental animals

3.1.1. Animals with different genetic backgrounds and germline mutations

Wild-type C57BL/6 mice carrying the CD45.2 allele and congenic (B6.SJL-*Ptprc*^{a}) strain (referred to as BoyJ mice) carrying the CD45.1 allele on the C57BL/6 genetic background were purchased from The Jackson Laboratory.

Heterozygous mice carrying the deleted Syk allele (*Syk*^{tm1Tyb}, also referred to as *Syk*$^{+/-}$) were obtained from Victor Tybulewicz (National Institute for Medical Research, London, UK) (169). In order to maintain the mutation, mice were bread in heterozygous form on the C57BL/6 genetic background, carrying the CD45.2 allele.

Mice carrying the KRN TCR transgene (Tg(TcraR28,TcrbR28)KRNDim) (170) were obtained from the laboratory of Diane Mathis and Cristophe Benoist (Harvard Medical School, Boston, MA). These animals were maintained in heterozygous form on the C57BL/6 background. NOD (Non-obese diabetic) mice were purchased from The Jackson Laboratory, and they were mated in homozygous form. KRN-transgenic mice were then mated with NOD mice to obtain K/BxN (arthritic) and BxN (nonarthritic) F1 offspring (171, 172).

Mice carrying the *Mcl1*^{tm1Ywh} (Mcl1^{flox}) floxed allele of the Mcl-1–encoding gene (86) were obtained from You-Wen He (Duke University, Durham, NC) (86) and they were crossed to mice carrying the *Lyz2*$^{tm1(cre)Ifo}$ (Lyz2Cre; also referred to as LysM-Cre) knock-in strain expressing the Cre recombinase in the complete myeloid compartment (173) to generate *Lyz2*$^{Cre/Cre}$*Mcl1*$^{flox/flox}$ mutants (referred to as *Mcl1*$^{\Delta Myelo}$ mice). The mutations were mostly maintained by breeding *Mcl1*$^{\Delta Myelo}$ with *Lyz2*$^{Cre/Cre}$*Mcl1*$^{flox/+}$ mice, resulting in homozygous *Mcl1*$^{\Delta Myelo}$ animals and *Lyz2*$^{Cre/Cre}$*Mcl1*$^{flox/+}$ animals used as littermate controls.

Mice with a ubiquitous expression of a membrane-targeted tdTomato fluorescent protein (carrying the *Gt(ROSA)26Sor*$^{tm4(ACTB-tdTomato,-EGFP)Luo}$ allele (174) (referred to as mTmG mice) were obtained from the Jackson Laboratory.

Mice used in the experiments were genotyped by allele specific PCR using genomic DNA.

Mice were kept in individually sterile ventilated cages (Tecniplast) either in a specific pathogen-free facility or an adjacent conventional facility. Experiments were approved by the Animal Experimentation Review Board of Semmelweis University. Mice of both genders at 2-6 months of age were used for the experiments, except for the generation of HoxB8 progenitor cells and *Syk*$^{-/-}$ chimeras explained at the Methods section later.

3.1.2. Generation of chimeric animals

3.1.2.1. HoxB8 chimeras

To generate HoxB8 chimeras, recipients carrying the CD45.1 allele on the C57BL/6 genetic background, also known as BoyJ mice, were irradiated by 11 Gy (lethal irradiation) or 7 Gy (sublethal irradiation) from a ^{137}Cs source (Gamma-Service Medical D1 irradiator), followed by single (Fig. 17.A.) or multiple (Figs. 18.A. and 27.A.) intravenous injections of 3×10^7 in vitro cultured HoxB8 progenitors per recipient at the indicated time points.

3.1.2.2. Bone marrow and fetal liver chimeras

Wild type bone marrow chimeras were generated by intravenous injection of 3×10^6 unfractionated bone marrow cells from C57Bl/6 mice into BoyJ recipients.

Syk$^{-/-}$ bone marrow chimeras were generated by fetal liver transplantation using fetuses from days 15.5–18.5 of embryogenesis (E15.5–18.5), which were obtained from timed mating of heterozygous *Syk*$^{+/-}$ mice. The recipient, wild type (BoyJ) mice were lethally irradiated and then injected intravenously with unfractionated fetal liver cell suspensions (172). Fetal liver cells from a single donor were injected into 5–8 recipients. *Syk*$^{-/-}$ fetuses were identified by their characteristic petechiated appearance (171) and their genotype using allele specific PCR. Control chimeras were also generated using macroscopically normal (*Syk*$^{+/+}$ or *Syk*$^{+/-}$) sibling fetuses. To assess the chimerism, peripheral blood samples were taken 4 weeks after the transplantation and stained with antibodies (all from BD Biosciences) against CD45.2 (104), Ly6G (1A8) and B220 (RA3-

6B2). Samples were analysed using FACS Calibur flow cytometer. WT and *Syk*$^{-/-}$ chimeras were distinguished based on their peripheral B cell count, as *Syk*$^{-/-}$ animals are 95-98% deprived of their B cells due to malignancies in B cell development in connection with the loss of the Syk tyrosine kinase gene (175, 176).

3.1.2.3.Mixed chimeras

Mixed chimeras were generated via intravenous injection of a combination of donor cell types: 3×10^6 bone marrow cells and 3×10^7 HoxB8 progenitors cultured in vitro into lethally irradiated recipients (BoyJ mice). In order to distinguish recipient cells and both donor cell types in mixed chimeras, bone marrow cells from mTmG mice were used as the bone marrow cell donors.

3.2. Cell cultures

3.2.1. Generation of immortalized HoxB8 progenitor cells

SCF (Stem Cell Factor) -producing Chinese hamster ovary (CHO) cells were a kind gift from Hans Häcker (St. Jude Children's Research Hospital). These cells were cultured in RPMI 1640 (BioSera) supplemented with 10% FCS (BioSera) and 100 µg/ml penicillin/streptomycin (Sigma). Once the cells reached approximately 70-80% confluency, the SCF containing supernatant was collected, filtered through a 22 µm pore and kept at -20 ºC until use. The concentration of SCF in the supernatant was not measured directly.

The immortalized HoxB8 progenitor cells were generated as described previously (5, 163) and shown in Fig. 6. In details, HEK-293T cells were transfected with 10 µg pMSCVneo-ER-Hoxb8 retroviral and 10 µg pCL-Eco packaging vectors (5) (both were kind gifts from Hans Häcker) in 1 ml Opti-MEM medium (Lonza) in the presence of 1 µg/ml Lipofectamine 2000 (Thermo Fisher Scientific). Virus-containing supernatants were harvested 48 h after transfection and stored at -80 °C until use. Bone marrow cells of 8-10-week-old mice (WT, or chimeric mice) or fetal liver cells of embryos (embryonic day 15-16) were harvested and progenitor cells were enriched by a Histopaque-1083 (Sigma) gradient (400 g, 30 min at room temperature). Progenitor cells were collected from the gradient interface and were further cultured in RPMI 1640 supplemented with

10% FCS, 100 µg/ml penicillin/streptomycin, 10 ng/ml recombinant murine IL-3 (PeproTech), 20 ng/ml murine IL-6 (PeproTech), and 2% conditioned medium of SCF-producing CHO cell supernatant. After 72 h, the progenitor cells were transduced with the retrovirus particles by spinoculation (1500 g, 70 min, room temperature) in HoxB8 progenitor-growth media (comprising of RPMI 1640 containing 10% FCS, 100 µg/ml penicillin/streptomycin, 1 µM β-estradiol (Sigma), 2% conditioned medium of SCF-producing CHO cells and 30 µM 2-merkaptoethanol (Sigma)) with the addition of 1 µg/ml Lipofectamine 2000 (Thermo Fischer Scientific).

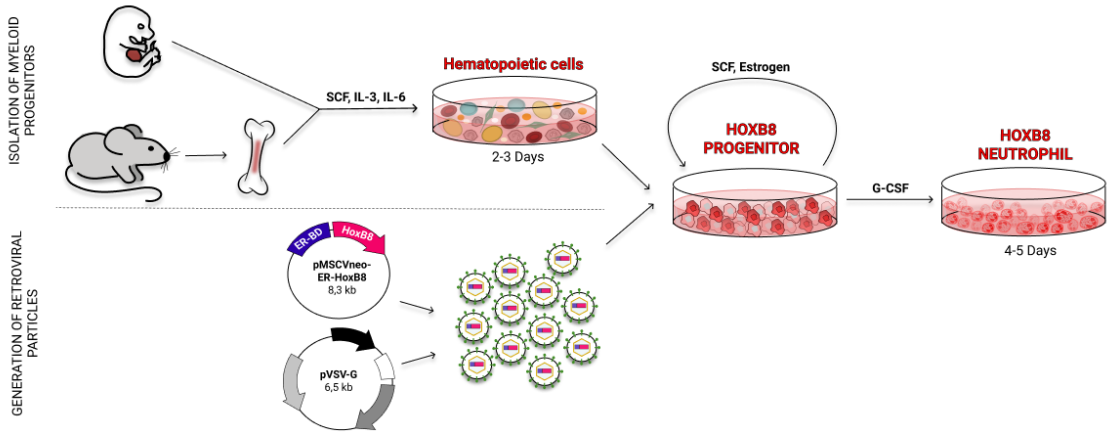

Figure 6. Generation and differentiation of immortalised HoxB8 progenitor cells. *Murine hematopoietic cells are collected either from fetal liver or bone marrow of the animals. Cell suspension is enriched for progenitor cells and cultured in the presence of SCF, IL-3 and IL-6 for 2-3 days. Meanwhile ER-HoxB8 fusion protein coding retroviral particles are generated. Progenitor cells are retrovirally transduced to express the ER-HoxB8 fusion protein leading to the emergence of conditionally immortalised HoxB8 progenitors. These cells are proliferating in SCF and estrogen containing media for prolonged periods of time. Once estrogen is withdrawn and culturing media is supplemented with G-CSF, progenitor cells differentiate into HoxB8 neutrophils in 4-5 days.*

After transduction, progenitors were cultured in HoxB8-progenitor growth media and passaged regularly to remove adherent cells and non-transduced cell debris from the culture. The first immortalized cells emerged in 10-14 days. The stable HoxB8 cell lines were established after 3-4 weeks.

HoxB8 progenitors used for the experiments were derived from 6 independent retroviral transductions, 4 of them using bone marrow and 2 of them using fetal liver cells.

No phenotypical or functional difference between HoxB8 cells from the two different sources were observed (data not shown), however for the experiments related to *Syk*$^{-/-}$ HoxB8 cells and their WT HoxB8 controls, HoxB8 progenitor cells were of bone marrow chimera origin.

3.2.2. In vitro culturing and differentiation of HoxB8 neutrophils

To elicit neutrophilic differentiation of HoxB8 progenitors, β-estradiol was removed from the culture using centrifugation (1500 rpm, 5 min at room temperature). HoxB8 progenitor cells were then counted, and the cells were further cultured in HoxB8 neutrophil-growth media (RPMI 1640 supplemented with 10% FCS, 100 μg/ml penicillin/streptomycin, 20 ng/ml mouse G-CSF (PeproTech), 2% SCF-producing CHO cell supernatant and 30 μM 2-merkaptoethanol) at appropriate concentration, typically for 5 days.

3.2.3. In vitro culture and PCR analysis of macrophages

Bone marrow cells were obtained by flushing the bone marrow, followed by red blood cell lysis with ACK buffer. Cells were washed and resuspended in macrophage-growth media (α-MEM supplemented with 10% FCS, 1% Penicillin/Streptomycin, 10 mM HEPES (pH 7.4), 1% L-glutamine and 10 ng/ml recombinant murine M-CSF). Cells were cultured for 3 days in a humidified CO_2 incubator. Cells in suspension were then collected, centrifuged and resuspended in macrophage-growth media containing 40 ng/ml recombinant murine M-CSF. Four days later, adherent cells were collected and prepared for flow cytometry or isolation of genomic DNA. For *Mcl1* genomic PCR analysis, the 5'- GGT TCC CTG TCT CCT TAC TTA CTG TAF -3' forward primer was used along with the 5'-TCG AGA AAA AGA TTT AAC ATC GCC -3' reverse primer (*Mcl1*$^{\Delta}$ allele; approximately 600 bp product length) or the 5'- CTC CTA ACC ACT GTT CCT GAC ATC C -3' reverse primer (*Mcl1*^{WT} or *Mcl1*^{flox} allele; approximately 260 and 380 bp product length, respectively). For *Itgb2* (CD18) PCR analysis, the 5'- GCC CAC ACT CAC TGC TGC TTG -3' forward primer was used along with the 5'- CCC GGC AAC TGC TGA CTT TGT -3' reverse primer (*Itgb2*^{WT} allele; approximately 480 bp product length).

3.3. Analysis of cellular phenotype

3.3.1. Preparation of cells for flow cytometry

HoxB8 cells were cultured as mentioned above and collected on the day of the experiments. Cells were washed and resuspended with PBS containing 5% FCS.

10 µl of peripheral blood samples were obtained from tail vein incision using heparinized pipette tips into PBS supplemented with 5% FCS and 1% Heparin. Samples were stained for approximately 60 min., washed (5000 rpm, 5 min, 4 ºC) and then resuspended in 500 µl of BD Biosciences' FACS Lysing Solution to lyse red blood cells.

Bone marrow and spleen samples were obtained by flushing the bone marrow or crushing the spleen through a 70 µm cell strainer, followed by red blood cell lysis with RBC Lysis Buffer (eBioscience), staining, and resuspension in PBS containing 5% FCS.

Samples were kept at 4 °C during the entire procedure. Specified volumes were used throughout, allowing a precise determination of absolute cell counts.

3.3.2. Flow cytometry and analysis

Flow cytometry was performed using a BD Biosciences FACS Calibur and analysed by FCS Express 6 Flow (De Novo Software).

During flow cytometry the leukocyte populations were distinguished based on their classic FSc (Forward scatter) (size) and SSc (Side scatter) (granularity) profiles combined with staining for the specific cell surface markers.

Antibodies used for cell staining were purchased from BD Biosciences: CD11a (clone M17/4), CD11b (M1/70), CD18 (C71/16), pan-CD45 (30-F11), CD45.2 (104), Ly6G (1A8), c-kit (CD117; 2B8), FcγRII/III (CD16/32; 2.4G2), FcγRIV (9E9), CD3 (17A2), B220 (RA3-6B2), Gr1 (RB6-8C5), 7/4 (ab53453), IgM (polyclonal, Cat. No.: 115-606-020), IgD (11-26c.2a), CD21 (7g6), CD23 (B3B4).

Red blood cell and platelet counts were measured by the Vet-Med-Labor veterinary diagnostic services (Budapest, Hungary).

Dead cells were identified using the LIVE/DEAD Fixable Far Red dead cell staining kit (Thermo Fischer Scientific). In cells with compromised membranes, the dye reacts with free amines both in the cell interior and on the cell surface, yielding intense fluorescent staining. In viable cells, the dye's reactivity is restricted to the cell-surface

amines, resulting in less intense fluorescence. The difference in intensity is typically greater than 50-fold between live and dead cells, allowing for easy discrimination.

3.3.3. Cytospin and microscopy

For cytospin analysis, HoxB8 cell were cultured, or bone marrow cells were obtained by flushing the bone marrow, followed by red blood cell lysis with eBioscience's RBC Lysis Buffer. Suspended cell counts were adjusted in PBS containing 10% FCS and cytospun onto SuperFrost slides (Thermo Scientific) for 5 minutes at room temperature using a Thermo Shandon Cytospin 3 cytocentrifuge. After drying, slides were stained with haemotoxylin-eosin or May Grünwald method as indicated at each experiment and analysed on a Nikon Ni-U Upright microscope using a 40× dry objective connected to a Nikon DS-Ri2 camera for HoxB8 cells and spleen sections and a Leica DMI6000B inverted microscope for bone marrow samples.

3.4. In vitro functional assays

3.4.1. Adherent respiratory burst

Adherent respiratory burst was measured by a cytochrome c reduction test as described previously (177) using 5×10^5/well HoxB8 neutrophils or HoxB8 progenitors as control. NUNC Maxisorp plates (BD Biosciences) were coated with either PBS plus 10% FCS (for 30-60 min.) or 20 μg/ml HSA in carbonate buffer (pH 9,6). To complete the immobilized immune complex coating, wells were blocked with PBS plus 10% FCS (60 min., room temperature) then anti-HSA was added to the surface (1:400, Sigma) for 60 minutes at room temperature. Plates were then washed with HBSS (Hank's Balanced Salt Solution) buffer. The cells were stimulated with 100 nM PMA (Phorbol 12-myristate 13-acetate) (Sigma) on the PBS-FCS coated surface or by the immobilized HAS (Human Serum Albumin)–anti-HSA immune complex (177, 178). The adherent activation of HoxB8 cells took place in HBSS buffer supplemented with 20 mM HEPES (pH 7,4) and 1 mM $MgCl_2$.

Before the measurement 100 μM ferricytochrome c was added to the cells. In case of superoxide production, the cytochrome c was reduced by the anions which resulted in a clear change in colour easily detectable by a spectrophotometer. Superoxide production

was measured in a Labsystems Multiskan Ascent multiplate reader in dual wavelength (550 and 540 nm) kinetic measurement mode for 2 hours at 37 ºC.

For the sake of presentation, unstimulated control values and values measured at 0 min. of the experiment were subtracted from those of stimulated samples.

3.4.2. In vitro migration assay

For in vitro migration experiments, Transwell inserts with a polycarbonate membrane with 5 μm pore size (Corning) and wells of a 24-well plate were precoated with fibrinogen (150 μg/ml) for 60 minutes at room temperature (177). Inserts were then washed and filled with HoxB8 cell suspensions (10^6 cells/ml), while we filled the plate wells with chemoattractant such as fMLP (N-Formylmethionyl-leucyl-phenylalanine) (1-10 μM, Sigma) or MIP-2 (Macrophage Inflammatory Protein 2) (10-100 ng/ml, PeproTech) as indicated in Fig.9.E-F. After 60 minutes incubation at 37 ºC, the plates were spun, the inserts were removed, and the number of neutrophils in the bottom of the wells was determined by an acid phosphatase assay (177). Briefly, adherent cells in the wells were lysed with acid-phosphatase buffer (pH 5,3) containing 10 μM pNPP (p-nitrophenyl phosphate) (10 minutes on ice). Then the lysates were transferred into a clear 96-well plate and incubated for 90 minutes at 37 ºC. The enzymatic reaction was stopped using 5 N NaOH buffer, and the results were detected in a spectrophotometer at 405 nm. The number of migrating cells were determined based on a calibration curve of each individual experiments.

Parallel samples were included to determine the signal intensity from the total cell number loaded into the Transwell inserts.

3.4.3. In vitro phagocytosis assay

The GFP-expressing USA300 *Staphylococcus aureus* strain was a kind gift from William Nauseef (University of Iowa). Heat-killed *Candida albicans cells* (SC5314 strain) labelled with Alexa Fluor 488 were a kind gift from Attila Gácser (University of Szeged). Briefly, *Candida albicans* SC5314 cells were inoculated into 2 ml YPD (Yeast Peptone Dextrose Broth) broth and incubated overnight at 30 °C with constant shaking. The next day 200 μl of the cell suspension was added to fresh YPD broth (2 ml) and grown overnight at 30 °C with constant shaking. Then the cells were collected via

centrifugation (3000 g, 5 min.) and washed for a total of 3 times. The pellet was resuspended in 500 µl PBS followed by heat inactivation at 100 °C for 30 minutes. The suspension was then mixed with Na_2CO_3 (1 M) and 1 mg/ml of Alexa Fluor® 488 NHS ester (Invitrogen). The labelling was done at room temperature for 60 minutes in dark. Labelled cells were then washed for a total of 3 times in PBS and the cell concentration was adjusted to 10^7/100 µl. Cell suspension was stored at 4 °C protected from light until use.

The concentration of the bacterial or fungal cells was adjusted to an OD600 of 1/cm in HBSS and opsonized by the addition of 500 µl normal murine serum to 800 µl microbial suspension for 30 min at 37 °C. The cells were then centrifuged (5 min, 3000 g, 4 °C) and washed once in HBSS.

For the analysis of in vitro phagocytosis, 10^6/ml HoxB8 cells were incubated with 10^7/ml non-opsonized or mouse serum-opsonized bacteria or fungi for 120 min at 37 °C in a linear shaker (300 rpm) and samples were taken every 60 min. Samples were then diluted 5-fold in ice-cold PBS containing 5% FCS and analysed by flow cytometry. Cells in the accurate call gate for HoxB8 progenitors and HoxB8 neutrophils were investigated for green fluorescence signal. 4 µg/ml Trypan Blue was added immediately before flow cytometric measurement to exclude cell-associated bacteria that have not been internalized.

3.5. In vivo functional assays

3.5.1. Thioglycollate-induced peritonitis

Peritonitis was induced by intraperitoneal injection of 1 ml 3% thioglycollate (Liofilchem) or PBS as control. After the indicated time, mice were sacrificed, and the peritoneum was flushed by 5 ml ice cold PBS containing 5% FCS. The lavage samples were washed (1500 rpm, 5 minutes, 4 ºC), resuspended in PBS containing 5% FCS and stained for flow cytometry analysis for approximately 60 minutes. Samples were then washed and resuspended in 500 µl PBS plus 5% FCS and analysed on a FACS Calibur flow cytometer.

3.5.2. In vivo phagocytosis

Mice were injected intraperitoneally with thioglycollate as described above (3.5.1) to induce neutrophil migration into the peritoneum. PBS was used as control treatment. 3 hours later, 50 μg/kg body weight Latrunculin A (Thermo Fisher) was injected into the peritoneum of the animals where indicated, immediately followed by 10^7 heat-killed and AF488-stained *C. albicans* suspended in 100 μl PBS (see in vitro phagocytosis protocol at 3.4.3.). 60 min later, the mice were sacrificed, the peritoneum was lavaged and staining was done as described previously. To confirm that cell associated fungi are internalized (and not only bound to the surface), 4 μg/ml Trypan Blue was added to the samples immediately before the flow cytometric analysis to quench cell-associated *Candida* particles that have not been internalized and only attached to the cells from outside.

3.5.3. Reverse passive Arthus reaction

The reverse passive Arthus reaction was triggered as described (179). Briefly, mice were anesthetized by isoflurane inhalation (Baxter Hungary) and one of the ears was injected intradermally with 20 μl rabbit polyclonal anti-ovalbumin whole serum (anti-Ova; Sigma) diluted twofold in PBS. Similarly prepared normal rabbit serum (Sigma) was injected into the other ear as control. The mice were then immediately injected intravenously with 0.8 mg Ova (ovalbumin) (Sigma), followed by the intravenous injection of 1 mg Evans blue dye 2 hours later. Evans blue is used to visualise increased infiltration and edema formation were Ova-anti-Ova immune complexes are formed. The ear thickness was measured by a spring-loaded calliper (Koeplin) both before the rabbit serum injection and before Evans Blue injection, as well. 30 min after Evans Blue injection, the mice were sacrificed and their ears were collected, cut into small pieces, and digested with Liberase II kit (Roche) on an Eppendorf Thermomixer at 1400 rpm for 1 h at 37 °C according to the manufacturer's instructions. Single-cell suspensions were then obtained by passing the digests through a 70-μm cell strainer (BD). The samples were then centrifuged (5000 g, 3 min, 4 °C), the pellet was stained and analysed by flow cytometry. Concentration of MIP-2 and LTB4 in the supernatant was determined by ELISA method (R&D Systems) (97, 179).

3.5.4. Autoantibody induced K/BxN serum-transfer arthritis

KRN transgenic animals on the C57BL/6 genetic background were mated with NOD mice yielding K/B×N (arthritic) and B×N (non-arthritic control) F1 offspring (170, 172). The arthritic animals from very early ages show inflammatory phenotype resembling human rheumatoid arthritis. The inflammation is caused by autoantibodies present in the circulation against glucose-6-phosphate isomerase (GPI) enzyme, most likely due to the presence of the KRN TCR and a certain type of MHC II (Ag^7) molecule (180).

Severe arthritis can be evoked in the healthy animals by the transfer of arthritic sera from K/B×N animals to recipients.

The presence of transgene in mice was detected using allele specific PCR, and macroscopic determination of the arthritic phenotype on the limbs. Blood was collected both from K/B×N and B×N animals through the retroorbital vein, and samples were kept refrigerated overnight. Next day, serum was extracted from above the clot, and serum samples were cleared of all cellular components via centrifugation. Control and arthritogenic sera were kept at -20 ºC until use.

Arthritis was induced by intraperitoneal injection of 300 µl K/B×N (arthritic) or B×N (control) serum, followed by daily scoring of clinical signs of arthritis and measurement of ankle thickness for 10 days as described (171, 172).

3.6. Presentation of the data and statistical analysis

Experiments were performed the indicated number of times discussed in each figure legend.

Quantitative graphs and kinetic curves show mean and SEM from all independent in vitro experiments or from all individual mice from the indicated number of in vivo experiments.

Statistical analyses were carried out by the Statistica software (StatSoft) using one-way ANOVA for evaluation of superoxide production and two-way (factorial) ANOVA for all other experiments. In case of kinetic assays, area under the curve (AUC) was used for statistical analysis. p values below 0.05 were considered statistically significant.

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4 RESULTS

4.1. Characterization of the wild type ER- HoxB8 cell line

4.1.1. Generation of the ER-HoxB8 cells

The ER-HoxB8 cell line is a myeloid progenitor cell line with conditional expression of HoxB8, leading to the formation of immortalized cells. The mechanism of generation is described in detail in section 3.2.1. and can be seen in Figure 6. Briefly, bone marrow derived progenitors are collected and cultured in IL-3 and IL-6 rich media. IL-3 is known to promote in vitro proliferation and survival of progenitor cells (181, 182), while IL-6 blocks lymphopoiesis and drives progenitor cells towards the myeloid fate (183, 184). As a result of IL-3 and IL-6 treatment, in vitro cultured bone marrow progenitors start to get committed towards the myeloid linage-specific differentiation pathway in a couple of days. At this point progenitor cells are retrovirally transduced to express an ER-HoxB8 fusion protein leading to the generation of a supposedly polyclonal myeloid progenitor cell line. Once infected, cells are further cultured in β-estrogen containing media: estrogen activates the expression of HoxB8 transcription factor, which therefore blocks the differentiation of the progenitor cells as described previously and shown in Fig. 4 (152). The intracellular overexpression of HoxB8 leads to the overall immortalization of the progenitor cell line. A stable growing, immortalized progenitor culture was established in approximately 4 weeks after retroviral transduction.

The in vitro cultured HoxB8 progenitors can be kept in culture for long periods of time, and they can expand their number very rapidly. HoxB8 progenitors show practically unlimited in vitro proliferation and self-renewal capacity in the presence of estrogen, but they are able to differentiate towards neutrophils (so-called HoxB8 neutrophils) upon estrogen withdrawal (deactivation of HoxB8) and addition of G-CSF (5). G-CSF is the key regulator in neutrophil maturation, as it is needed for a complete granulopoiesis (13). Inactivation of the HoxB8 transcription factor via estrogen withdrawal, combined with G-CSF treatment, HoxB8 neutrophils differentiate from the HoxB8 progenitors in vitro in approximately 5 days. HoxB8 neutrophils phenotypically and functionally resemble primary mouse neutrophils (5, 185).

4.1.2. Characterization of in vitro cultured HoxB8 progenitors and HoxB8 neutrophils

We have generated immortalized HoxB8 progenitors as explained on Fig. 6. (5, 163). Progenitor cells were cultured in the presence of β-estradiol to maintain HoxB8 activity, and SCF produced by CHO cells to promote progenitor cell survival and proliferation in vitro. These HoxB8 progenitors showed a typical hematopoietic progenitor-like phenotype with large, round, and uncondensed nuclei nearly completely filling the cytoplasm of the cells. Progenitor cells also showed a quite spherical shape as shown in Fig. 7. Occasionally we could find cells amongst the HoxB8 progenitors that were in the stage of mitotic division, confirming, that the HoxB8 progenitors are actively proliferating cells when the HoxB8 transcription factor is active due to the presence of estrogen in the culturing environment.

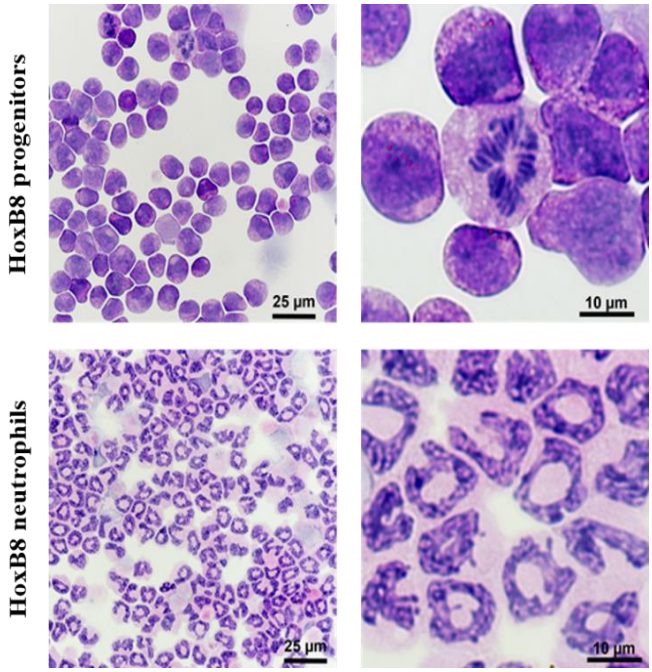

Figure 7. Microscopic images of HoxB8 progenitor and HoxB8 neutrophil cells. *Cells were stained with Haemotoxylin-Eosin dye. Images are representative of 3-5 independent experiments.*

HoxB8 progenitors were proliferating rapidly in estrogen conditioned media. Their number was easy to expand according to the experimental needs. HoxB8 progenitors tolerated changes of estrogen concentration in the culture media very well,

however they seemed to be a bit more sensitive to cell density. They preferred a maximum 70-80% cell confluency in the cultures.

In order to differentiate HoxB8 progenitors into HoxB8 neutrophils, the HoxB8 progenitors were cultured in the presence of SCF-containing supernatant and G-CSF without the addition of β-estradiol into the culture media. During the 5 days of differentiation the morphology of the HoxB8 progenitors changed dramatically: the progenitor cells that were previously cultured in suspension started to adhere to the surface of the culturing dish. The cells became smaller in size, they developed ring-shaped nuclei and their cytoplasm showed a more pinkish color under H&E-staining, specifically as a sign of granule formation (Fig. 7). All of these phenotypical changes are characteristic of murine neutrophil-like cells.

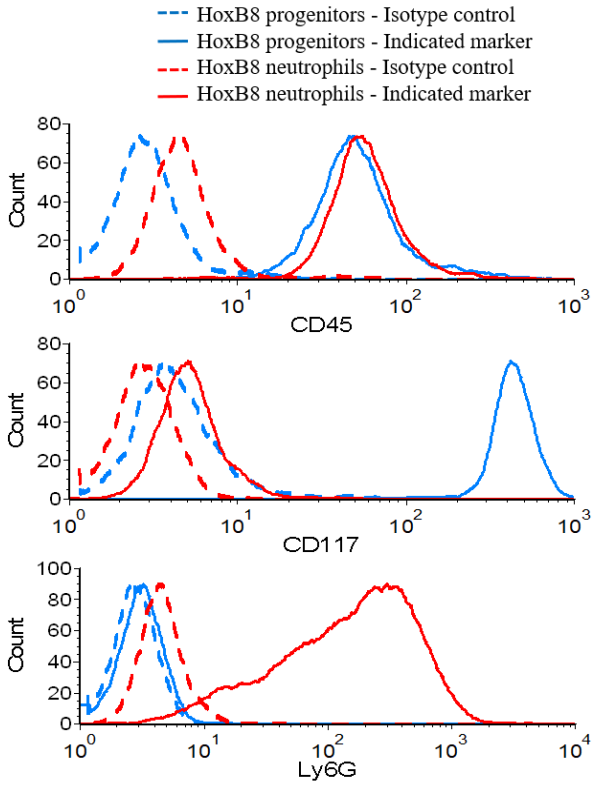

Figure 8. Histograms of CD45, CD117 and Ly6G expression of HoxB8 cells. *HoxB8 neutrophils were cultured for 5 days without estrogen. Histograms are representative of 3-5 independent experiments.*

We have performed flow cytometric analysis of HoxB8 progenitors and differentiated HoxB8 neutrophils, the latter ones after 5 days of culturing under conditions promoting neutrophilic differentiation. As shown in Fig. 8., both HoxB8 progenitors and HoxB8 neutrophils expressed the general leukocyte marker CD45 at a similar level. HoxB8 progenitors also expressed the receptor tyrosine kinase CD117 (c-Kit), the receptor for the SCF cytokine, which likely reflects their early hematopoietic cell-like phenotype.

However, CD117 expression was completely lost in HoxB8 neutrophils, as expected of a cell type that has undergone differentiation and became committed to a certain fate, losing its multipotency. The expression of the neutrophil maturation marker Ly6G showed an opposite expression pattern compared to CD117: as expected from their non-committed nature, Ly6G was absent from HoxB8 progenitors, whereas it was highly expressed on most of the HoxB8 neutrophils. It should be noted however, that the peak of Ly6G expression of in vitro-generated HoxB8 neutrophils was significantly lower than that of normal in vivo circulating neutrophils (see later on Fig. 12.), likely reflecting an incomplete differentiation in the HoxB8 neutrophil cell culture in vitro.

We have also followed the time course of neutrophil differentiation as a matter of CD117 and Ly6G expression. As shown in Fig. 9.A., the expression of CD117 dropped drastically between days 1-3 and remained low until the end of the experiment (Day 8). On the other hand, Ly6G expression started to increase on Day 3, reached its maximum on Day 5 or 6, and remained around its maximum level until Day 8 of neutrophil culture.

We have also quantified the number of living cells in our cultures and determined the number of Ly6G-negative and Ly6G-positive cell populations. As shown in Fig. 9.B., during the first days of the neutrophil culture, while the progenitor cells started to differentiate, they also kept dividing as well. Both the total number of cells and the number of Ly6G-positive cells started to decline from Day 6, and in line with our current knowledge about the lifespan of neutrophils, more and more dead cells appeared in the HoxB8 neutrophil cultures at the same time (data not shown). This kinetic curve confirmed that Day 5 is likely the most appropriate time for the analysis of HoxB8 neutrophils in vitro.

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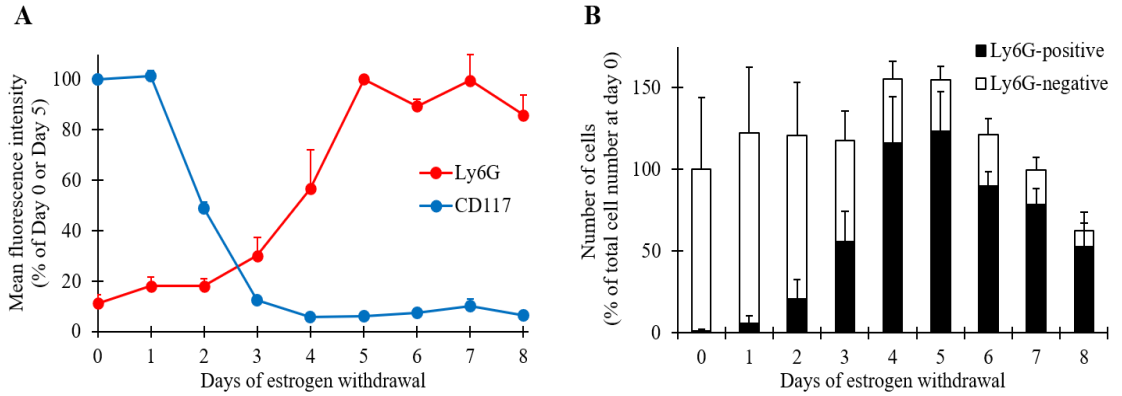

Figure 9. Differentiation pattern of HoxB8 neutrophils in vitro. *(A) Time course of CD117 and Ly6G expression level during neutrophilic differentiation of HoxB8 cells. (B) Time course of Ly6G positivity in living cells during HoxB8 cell differentiation. Quantitative data show mean and SEM from 3-5 independent experiments. Mean fluorescence intensity values (MFI) are normalized to the Day 0 (CD117) and Day 5 (Ly6G) values.*

We have also tested the expression of certain cell surface receptors known to be involved in the functional activation of neutrophils (Fig. 10.). Expression of individual α-chains of major β_2-integrins revealed that HoxB8 neutrophils and HoxB8 progenitors expressed similar levels of CD11a (integrin α_L; the α-chain of LFA-1), whereas CD11b (integrin α_M; the α-chain of Mac-1) showed a significant upregulation during neutrophilic differentiation. Likely due to the differential changes of CD11a and CD11b expression, the levels of CD18 (the β chain of all β_2-integrins) showed a slight increase on the surface of HoxB8 neutrophils compared to that of the HoxB8 progenitors. We have also tested the expression of Fcγ-receptors of HoxB8 cells. While the expression of FcγRII/III was comparable between the HoxB8 progenitors and neutrophils, FcγRIV showed robust upregulation in HoxB8 neutrophils, likely reflecting a role of Mature neutrophils in Fc-receptor-mediated immune and inflammatory reactions.

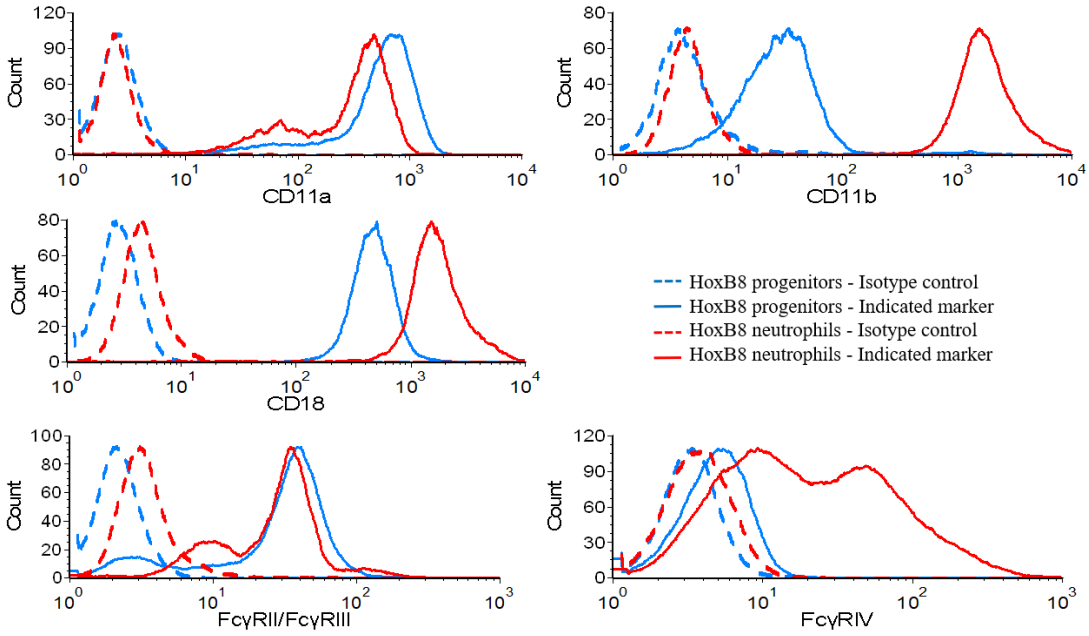

Figure 10. Histograms of various cell surface receptor staining of HoxB8 cells. *Histograms are representative of 3-5 individual experiments.*

4.1.3. In vitro functions of HoxB8 neutrophils

As a next step, we wanted to characterize the functional capacity of both HoxB8 progenitors and HoxB8 neutrophils in our hands in various in vitro experiments. Therefore, we tested in extensive experiments whether HoxB8 neutrophils can carry out classical neutrophil-like effector functions such as ROS production (Fig. 11.A -B.), migration (Fig. 11.C -D.) and phagocytosis (Fig. 11.E -F.).

As shown in Fig. 11.A., HoxB8 neutrophils showed a robust respiratory burst upon stimulation with PMA, a non-physiological stimulator of neutrophils. On the other hand, no respiratory burst response from HoxB8 progenitors could be observed ($p = 5.0\times10^{-7}$) once stimulated with PMA, compared to HoxB8 neutrophils. HoxB8 neutrophils also showed a robust respiratory burst response when plated on immobilized IgG immune complex surface, a model for Fc-receptor-dependent neutrophil activation (178), while HoxB8 progenitors stayed inactive throughout this experiment, as well (Fig. 11.B; $p = 5.5\times10^{-5}$).

We also tested the chemotactic migration of HoxB8 progenitors and HoxB8 neutrophils through a fibrinogen-coated membrane of 5 μm pore size in an in vitro Transwell system. As shown in Fig. 11.C., HoxB8 neutrophils were able to migrate

towards an fMLP source of various concentrations, whereas no substantial migration of HoxB8 progenitors could be observed ($p = 9.9\times10^{-5}$). HoxB8 neutrophils, but not HoxB8 progenitors migrated towards the MIP-2 chemokine ($p = 8.3\times10^{-6}$), as well (Fig. 11.D.).

Next, we wanted to test the phagocytosis of both bacterial (*S. aureus*) and fungal (*C. albicans*) pathogens by HoxB8 cells in vitro. The pathogens were fluorescently labelled for the sake of an easier recognition by flow cytometry. Also, at the same time we tried to analyze the importance of opsonization in case of phagocytosis as well, via using normal mouse serum-opsonized pathogens. HoxB8 progenitors and neutrophils were mixed with these non-opsonized or opsonized pathogens as described in section 3.4.3. As shown in Fig. 11.E., HoxB8 progenitors showed no substantial phagocytosis of live GFP-expressing *S. aureus* (USA 300 strain), regardless of whether the bacteria were opsonized previously or not. On the contrary, HoxB8 neutrophils showed a moderate and although statistically not significant tendency of phagocytosis of non-opsonized *S. aureus* (Fig. 11.E.; $p = 0.16$ vs. HoxB8 progenitors) while HoxB8 neutrophils showed very robust phagocytosis of serum-opsonized bacteria in the same experimental setup (Fig. 11.E.; $p = 1.3\times10^{-9}$).

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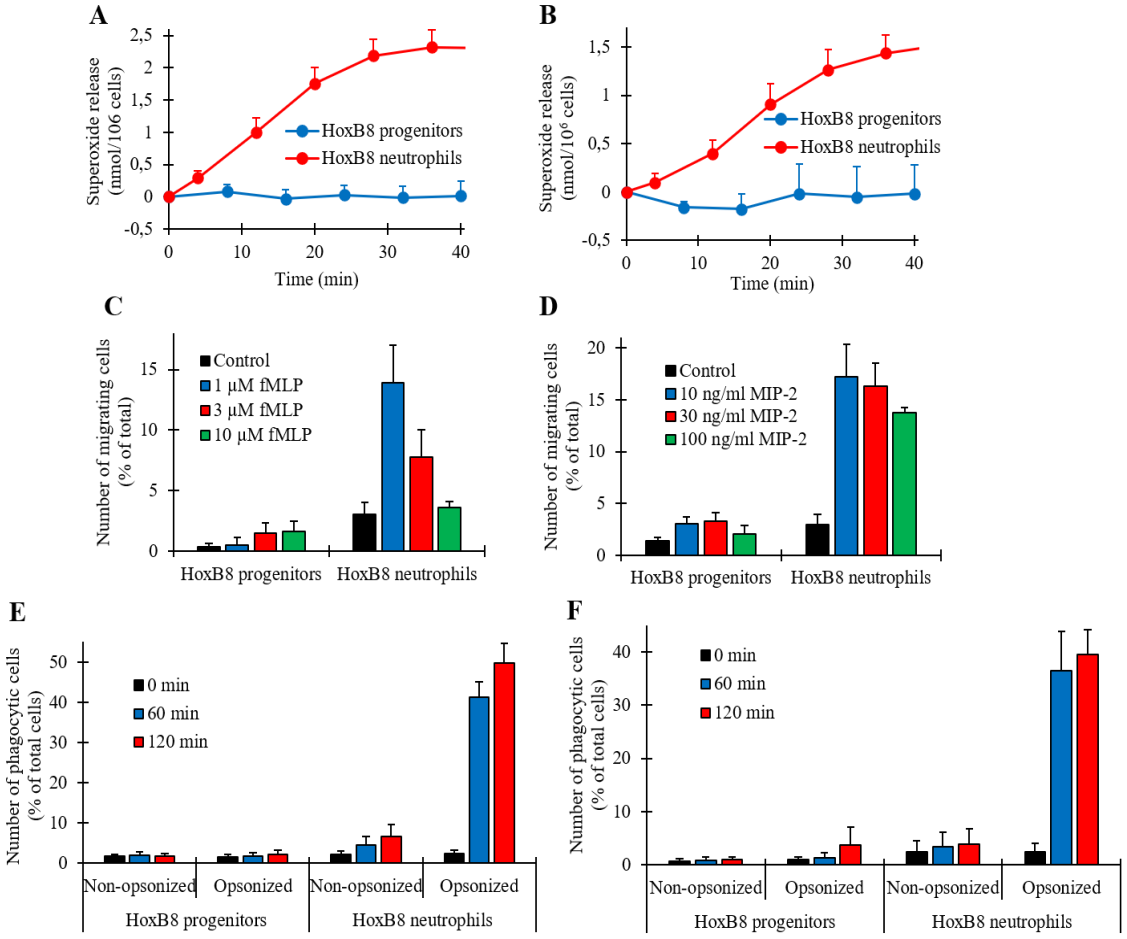

Figure 11. In vitro functions of HoxB8 neutrophils. *(A-B) Superoxide production of HoxB8 progenitors and neutrophils stimulated by PMA (A), or by immobilized IgG immune complex surface (B). Unstimulated control values were subtracted. (C-D) Migration of HoxB8 cells towards fMLP (C) or MIP-2 (D) in Transwell assay system. (E-F) Phagocytosis of non-opsonized or mouse serum-opsonized, fluorescent S. aureus (E) or C. albicans (F) by HoxB8 progenitors and HoxB8 neutrophils. Mean and SEM of results from 10 (A), 9 (B), 7 (C) and 3 (D-F) independent experiments are shown. Statistical analysis: (A-B) We used univariate test of significance (A) p = 5.0×10–7, (B) p = 5.5×10–7. (C-F) Repeated Measures Analysis of Variance method was used: (C) HoxB8 progenitor vs. HoxB8 neutrophil: p = 9.9×10–5, (D) HoxB8 progenitor vs. HoxB8 neutrophil: p = 8,3×10–6, (E) HoxB8 progenitor vs. HoxB8 neutrophil with non-opsonized bacteria: p = 0,16, HoxB8 progenitor vs. HoxB8 neutrophil with opsonized bacteria: 1.3×10–9. (F) HoxB8 progenitor vs. HoxB8 neutrophil with opsonized bacteria: 2.4×10–4.*

We have carried our very similar in vitro phagocytosis assays using fluorescently labeled heat-killed *C. albicans* yeast cells, as well. As shown in Fig. 11.F., HoxB8 progenitors failed to carry out phagocytosis when incubated with non-opsonized fungi,

neither when they were incubated with opsonized *Candida* particles. No significant phagocytosis of non-opsonized *C. albicans* by HoxB8 neutrophils was detectable either, however, HoxB8 neutrophils showed powerful phagocytosis of the serum-opsonized yeast cells ($p = 2.7\times10^{-4}$ vs. HoxB8 progenitors) in vitro.

4.1.4. HoxB8 chimeras generated with a single transplantation.

The results of the in vitro experiments indicated that HoxB8 neutrophils resemble freshly isolated mouse neutrophils as far as their functional responsiveness is concerned.

Next, we wanted to find a method that would allow the in vivo functional analysis of the HoxB8 neutrophils as well. Therefore, we decided to transfer HoxB8 progenitor cells into recipient mice. Since our HoxB8 cells expressed CD45.2 from their C57BL/6 genetic background, we chose CD45.1-expressing (also called BoyJ) recipients on the same genetic background to allow the identification of recipient and donor-derived neutrophils by flow cytometry (177). Those mice whose original leukocytes were eliminated and replaced by HoxB8 progenitor-derived HoxB8 neutrophils, as a result of irradiation and transplantation of HoxB8 progenitors, are called the HoxB8 chimeras.

We chose to transplant HoxB8 progenitors rather than in vitro differentiated HoxB8 neutrophils into the recipients. The main reason behind this approach, is that according to our experiments, we found that the maturation of HoxB8 neutrophils differentiated in vitro might not be complete. We found, that the Ly6G expression of the HoxB8 neutrophils differentiated in vitro did not reach as high levels as Ly6G expression of circulating neutrophils isolated freshly from intact mice (Fig. 12).

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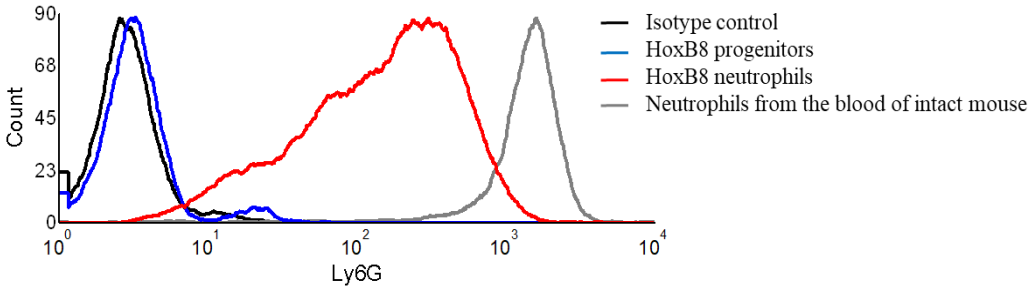

Figure 12. Ly6G expression of various cell types. *Freshly isolated neutrophils from the blood of C57Bl/6 mouse are marked with grey. In vitro cultured HoxB8 progenitors are marked with blue. In vitro differentiated HoxB8 neutrophils on day 5 of differentiation are marked with red. A single isotype control, marked with black, is representative control of all cell types used in the experiment. Histograms are representative of 5-6 individual experiments.*

Since Ly6G expression is in close correlation with the maturity of neutrophils, we concluded, that HoxB8 neutrophils differentiated in vitro may not be able to reach complete maturity. We have also acknowledged, and it can be seen in Figures 10 and 12., that the in vitro generated HoxB8 neutrophil population showed an inhomogeneous Ly6G expression, suggesting that HoxB8 progenitor cells during neutrophilic differentiation were not differentiating at the same speed. On Day 5 after estrogen withdrawal some of the cells reached their maximum differentiated stage, while others were still in pre-neutrophil-like state, according to their Ly6G expression. This incomplete maturation can probably be caused by the lack of important mediators that normally drive neutrophil differentiation, produced by various cells in the bone marrow. The currently used protocol for the in vitro differentiation of HoxB8 neutrophils might not be suitable to mimic the bone marrow niche completely, as most likely not only G-CSF but other factors might also be very important to drive myelopoiesis in vivo.

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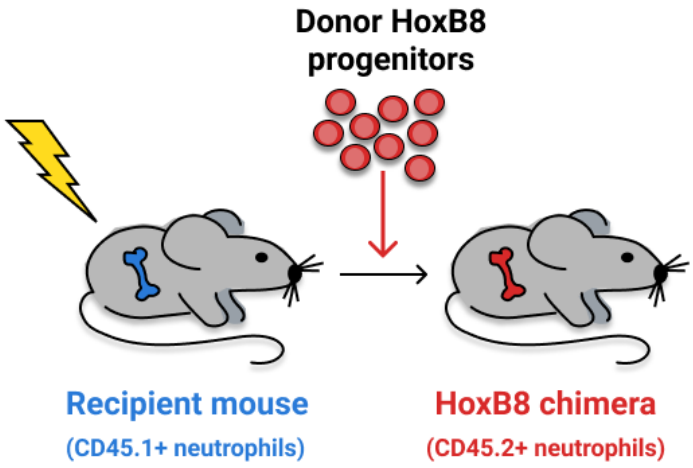

Figure 13. Generation of HoxB8 chimeras. *Recipient mice having CD45.1$^+$ neutrophils are lethally irradiated prior to the adoptive transfer of CD45.2$^+$ HoxB8 progenitor cells. Irradiation and transplantation yield HoxB8 chimeras, whose neutrophil compartment is expected to be changed completely to donor-originating cells.*

Therefore, we hoped that the in vivo environment would provide better grounds for the transplanted HoxB8 progenitors to differentiate completely into HoxB8 neutrophils. On the other hand, we chose to transplant HoxB8 progenitors into the recipients, rather than in vitro differentiated HoxB8 neutrophils, because the maintenance of the HoxB8 progenitor culture in big volumes is much easier than that of the HoxB8 neutrophils. HoxB8 progenitors are cultured in suspension, allowing for an easy passage regimen. Moreover, maintenance of the HoxB8 progenitor population is much more cost effective as well because progenitors do not need expensive cytokines (like G-CSF needed for the HoxB8 neutrophils) for their survival. The schematic design of the generation of HoxB8 chimeras is shown in Fig. 13.

At first, we wanted to find out the importance of recipient irradiation in our experimental setup. To do so, we compared the donor and recipient-derived circulating neutrophil counts between recipients that were intact (non-irradiated) and either received or did not receive HoxB8 progenitor donor cells on Day 0. As shown in Fig. 14., the circulation of both types of recipient mice contained only recipient-derived neutrophils on Day 5, irrespective of whether they received HoxB8 progenitors on Day 0. These results indicated that irradiation is essential before HoxB8 progenitor transplantation, and that recipient-derived neutrophils (and possibly other cells in the bone marrow as well)

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need to be cleared from the recipient. Otherwise, HoxB8 progenitors seemed to be unable to efficiently engraft the recipient bone marrow and differentiate into HoxB8 neutrophils, most likely because the hematopoietic niches of intact recipients are already occupied by their own hematopoietic cells.

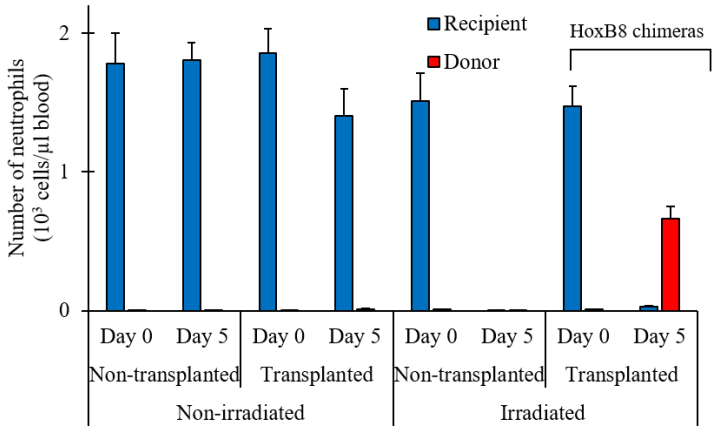

Figure 14. Comparing donor- and recipient-derived neutrophil counts in different recipient groups. *Recipient mice received or did not receive irradiation on Day 0, and also they were either subjected to HoxB8 progenitor transplantation or did not receive any donor cells on Day 0. Quantitative data show mean and SEM from 12-22 mice per group from 3 to 10 independent experiments. Except for the non-transplanted, non-irradiated group where data are from 2 mice per group from 2 independent experiments.*

In order to eliminate hematopoietic cells from the bone marrow of recipient mice, we next subjected recipient animals to lethal irradiation prior to the intravenous injection of HoxB8 progenitors. As controls, we used lethally irradiated mice which did not receive HoxB8 progenitors at all. As shown in Fig. 14., the recipient neutrophils completely disappeared from the circulation of the mice by Day 5 as a result of the irradiation. This meant that recipients that did not receive HoxB8 progenitors (irradiated, non-transplanted mice) were completely neutropenic by Day 5 after irradiation. These mice became severely ill very soon, sometimes not even reaching Day 10 of the experiment (data not shown). On the contrary, a clear donor-derived neutrophil population appeared in the lethally irradiated recipients' circulation in those mice that received HoxB8 progenitors on Day 0, after irradiation (Fig. 14.). The emerging donor-derived HoxB8 neutrophil population rescued these mice from irradiation-induced neutropenia. The existence of the

HoxB8 neutrophils in the HoxB8 chimeras could somewhat prolong the life of these mice compared to irradiated, non-transplanted control mice, as they usually survived for 12-14 days after irradiation.

For us it was very interesting to note that for some reason HoxB8 progenitors seemed to have a weaker capacity to compete for the bone marrow niche if other hematopoietic cells (other progenitors) were also present in the mice. To test this phenomenon in further detail, we generated so called mixed chimeras: lethally irradiated recipients were transplanted with untouched bone marrow cells (Fig. 15.A), HoxB8 progenitors (Fig. 15.B.) or the mixture of these two cell types (Fig. 15.C.). We have found that transplantation of bone marrow cells alone led to a gradual emergence of donor-derived neutrophils in the circulation and those counts were probably growing even after the end of the experiment (Day 10) (Fig. 15.A.). On the other hand, transplantation of HoxB8 progenitors only resulted in a brief increase of donor-derived neutrophil counts which started to decline after a peak on Day 6 (Fig. 15.B.).

Interestingly, addition of both donor cell types together (so-called mixed chimeras) resulted in the reduction of both bone marrow-derived and HoxB8 progenitor-derived neutrophil counts in the circulation on Day 5 (compare Fig. 15.A. and B. with C.), suggesting that the two donor populations were competing for limited resources and niche of the recipients.

A similar scenario could also be seen when we transplanted HoxB8 progenitors into sublethally irradiated recipients compared to mixed chimeras. As a result of sublethal irradiation, the hematopoietic cells of the recipient animals were not completely diminished. Therefore, the ideal niche for the donor cells for a successful implantation was already occupied in these mice. We suspected that donor HoxB8 progenitors had to compete with the residing recipient cells in the bone marrow, and this competition most likely reduced their overall potency to differentiate into HoxB8 neutrophils in vivo (185).

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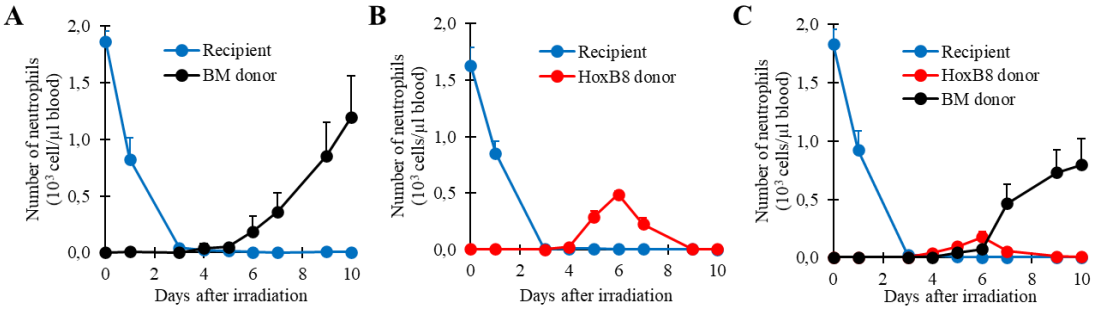

Figure 15. Neutrophil counts in various chimeric mice. *Lethally irradiated recipients were injected with BM cells only (A), HoxB8 progenitors only (B) or both cell types (C). Number of recipient and donor-derived neutrophils were followed for 10 days after irradiation. Graphs show mean and SEM from 4-5 (A), 10-13 (B) or 6-7 (C) mice per group from 3 independent experiments.*

Since lethally irradiated and transplanted animals (the so-called HoxB8 chimeras) showed the development of a very distinct, new neutrophil population, we aimed to further characterize these cells. When we checked the flow cytometric profiles of the HoxB8 chimeras both on Day 0 (immediately before irradiation/transplantation) and on Day 5, we found that the circulating neutrophils of the recipient (CD45.1 and Ly6G positive cells) were practically missing by Day 5, and they were completely replaced by neutrophils originating from the transplanted HoxB8 progenitors (CD45.2 and Ly6G positive) (Fig. 16.A.). We found very similar cellular composition in the bone marrow and the spleen of the HoxB8 chimeras 5 days after transplantation, indicating that transplanted HoxB8 progenitors can migrate into various tissues of the hematopoietic compartment where they can undergo neutrophilic differentiation. Quantitative assessment indicated that on average 97% of circulating neutrophils, and approximately 80-85% of bone marrow and splenic neutrophils were of donor origin on Day 5 of the experiments (Fig. 16.B.).

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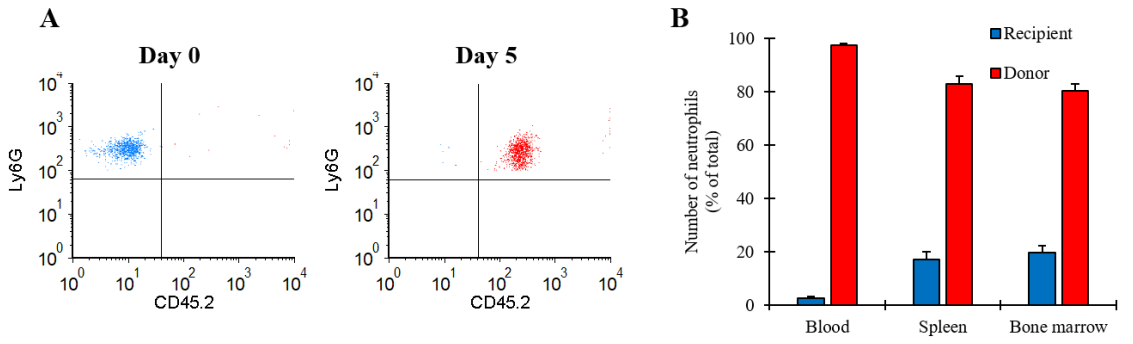

Figure 16. Recipient and donor-derived neutrophil counts in various tissues of HoxB8 chimeras. *(A) Representative flow cytometric profiles of circulating neutrophils in HoxB8 chimeras at the indicated time points after transplantation. Recipient-derived CD45.2$^-$ neutrophils are marked with blue. HoxB8 progenitor-derived CD45.2$^+$ neutrophils are marked with red colour. (B) Percentage of donor- and recipient-derived neutrophils in the different tissues of HoxB8 chimeras on Day 5 after irradiation and transplantation. Representative images are from 17-25 mice per group, quantitative data show mean and SEM from 6 mice per group from 5-10 independent experiments.*

We have also tested the time course of the differentiation of HoxB8 progenitors towards HoxB8 neutrophils in vivo, by checking the recipient- and donor-derived neutrophil counts in the peripheral blood of the chimeras for an extended time period (Fig. 17.B.). We found that the in vivo differentiation timescale of HoxB8 progenitors is very similar to what we had previously seen in vitro (Fig. 9.A.). Recipient neutrophils disappeared very rapidly after irradiation (in approximately 3-4 days). By the time we got to Day 4-5, HoxB8 neutrophils appeared in the circulation of the animals, reaching a maximum of approximately 40% of the initial circulating neutrophil count (Fig. 17.). After a peak on Day 5, neutrophil numbers began to decline, and the cells eventually completely disappeared by Days 8-10. After Day 8 we could no longer detect cells of donor origin in the circulation of HoxB8 chimeras. This meant that either all of the transplanted HoxB8 progenitors have turned into neutrophils, or because there was no exogenous SCF and estrogen administration, those HoxB8 progenitors that did not differentiate into neutrophils could not survive and proliferate in these conditions either. We believe that since the HoxB8 neutrophil population only emerged once in the circulation of the recipients after transplantation, HoxB8 progenitors were not able to colonize the hematopoietic niche of the recipient animals permanently. Therefore, differentiation of HoxB8 progenitors into neutrophils likely only occurs once, and

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transplanted HoxB8 progenitors cannot provide circulating HoxB8 neutrophils for prolonged periods.

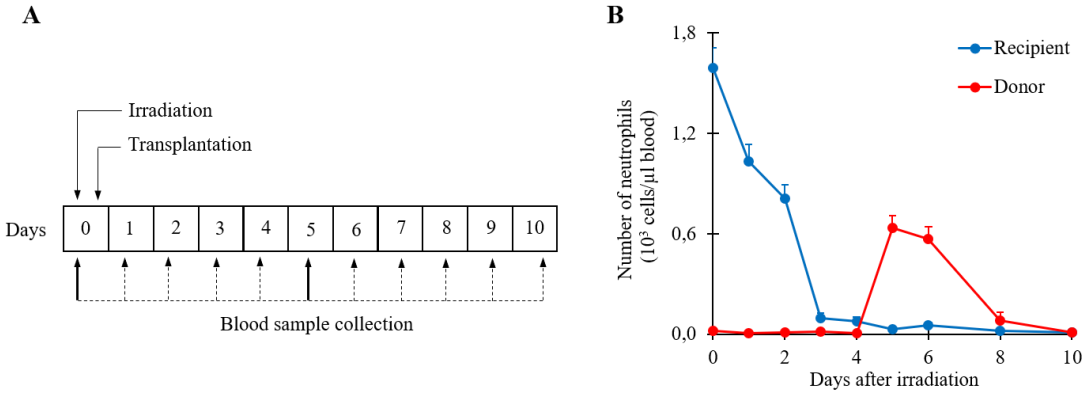

Figure 17. Circulating neutrophil counts of HoxB8 chimeras. *(A) Overview of the experimental design (B) Time course of recipient- and donor-derived circulating neutrophil counts of HoxB8 chimeras between Days 0-10 after irradiation. Quantitative data show mean and SEM from 17-25 mice per group from 5-10 independent experiments.*

4.1.5. Generation of HoxB8 chimeras with multiple, repeated transplantation of HoxB8 progenitors

According to the results of the kinetic repopulation experiments, HoxB8 neutrophils were present in the circulation of the HoxB8 chimeras only for 2-3 days (Fig. 17.B.). Therefore, we had aimed to establish a protocol which could secure the maintenance of a stable circulating HoxB8 neutrophil population for an extended period.

Therefore, we injected lethally irradiated recipients with HoxB8 progenitors repeatedly in two-day intervals (Fig. 18.A.). As shown in Fig. 18.B., transplanting HoxB8 progenitors into lethally irradiated recipients immediately after irradiation, as well as 2 and 4 days later resulted in the maintenance of a stable circulating HoxB8 neutrophil population until 10 days after irradiation. Via this multiple transplantation approach, we could generate HoxB8 chimeras that had approximately 40% of the original circulating neutrophil count for an extended period of time. Most importantly, practically no recipient-derived neutrophils could be observed in these chimeras after the disappearance of recipient neutrophils around Day 3, all the way to the end of the experiment.

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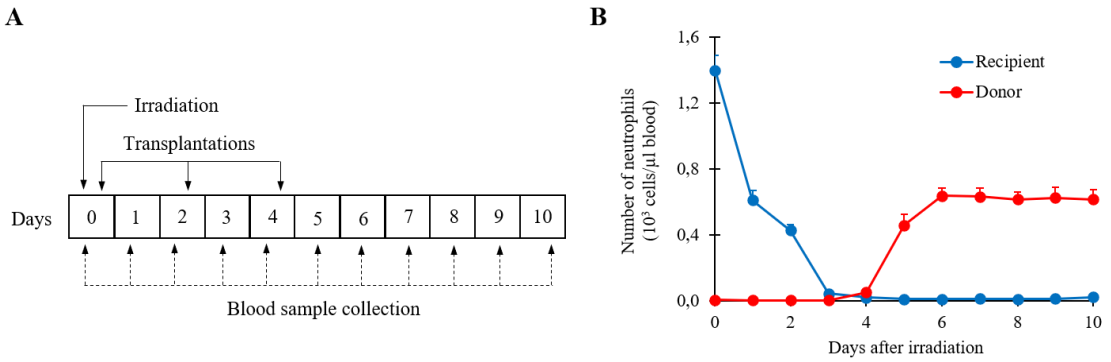

Figure 18. Longer-term maintenance of HoxB8 neutrophils in vivo. *(A) Overview of the experimental design. (B) Quantitative analysis of peripheral blood neutrophil counts after repeated HoxB8 progenitor injections into lethally irradiated recipients. Mean and SEM from 10-16 mice from 2 independent experiments are shown.*

4.1.6. Characterization of HoxB8 chimeras

As a next step, we wanted to characterize the phenotype of the HoxB8 neutrophils differentiated in vivo. To do so, flow cytometric profiles of peripheral blood samples of HoxB8 chimeras on Day 5 after HoxB8 progenitor transplantation were compared with those of intact C57BL/6 mice. Ly6G positive neutrophils within a pan-CD45 positive gate are marked with red in Figure 19.A.

As far as their size (FSc) and granularity (SSc) is concerned, the in vivo generated HoxB8 neutrophils had mostly normal size and granularity, although the neutrophil population itself seemed to be a bit more spread-out in the HoxB8 chimeras. In addition, other leukocyte populations like lymphocytes, monocytes (low SSc) and eosinophils (high SSc and high FSc profile) were less pronounced in HoxB8 chimeras than in intact mice. This finding likely reflects the fact that the lethal irradiation decimates all leukocyte populations, whereas HoxB8 progenitors primarily restore neutrophils with a limited ability to differentiate into other leukocytes (see later on Figures 21-22.).

Neutrophils from HoxB8 chimeras and intact mice expressed quite similar levels of Ly6G (Fig. 19.B.), suggesting a more complete differentiation of HoxB8 neutrophils in vivo compared to HoxB8 neutrophils differentiated in vitro as seen previously (Fig. 8 and 12.). We have also tested expression of functionally important molecules on circulating neutrophils.

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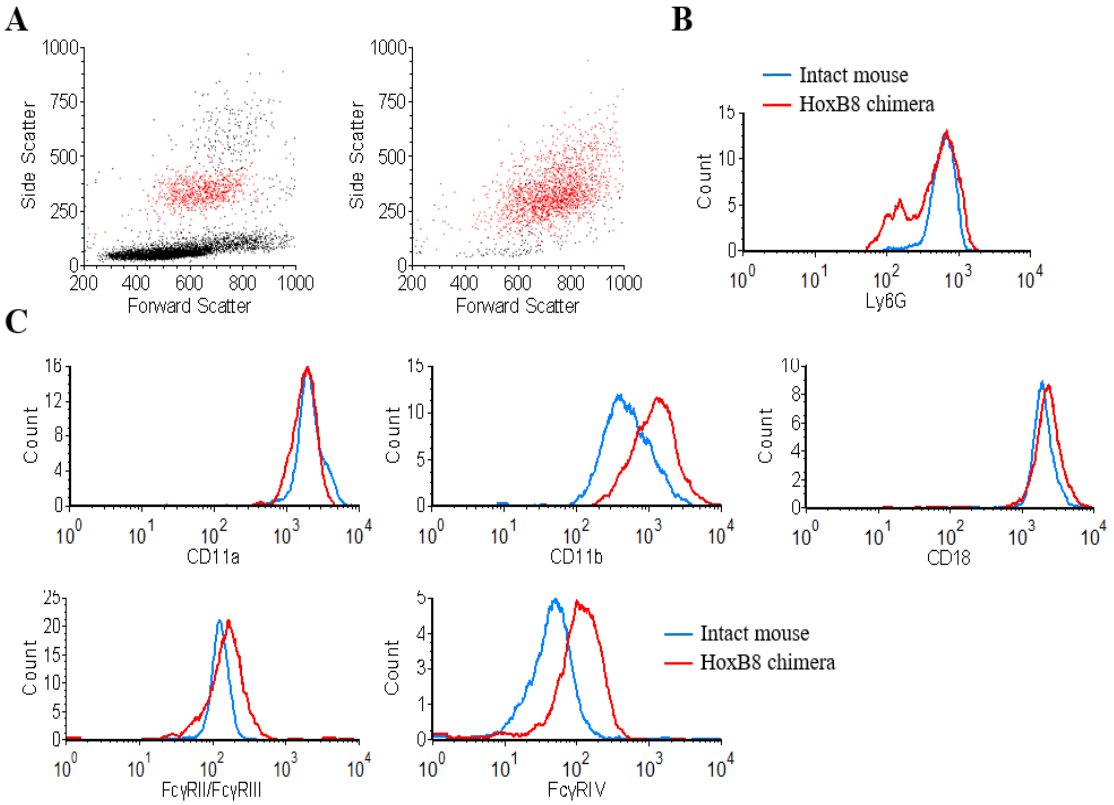

Figure 19. Surface marker expression of in vivo-derived HoxB8 neutrophils compared to circulatory neutrophils of intact mice. *Flow cytometric analysis of peripheral blood leukocytes in intact mice and HoxB8 chimeras 5 days after irradiation and transplantation. (A) Forward- and side scatter of all circulating leukocytes in a pan-CD45-positive gate. Neutrophils are marked with red. (B-C) Histograms of various cell surface receptor staining of neutrophils defined by their forward- and side-scatter characteristics (CD11a, FcγRII/III) or by Ly6G-positivity (CD11b, CD18, FcγRIV) within a CD45.2-positive gate. Representative data from 2-5 mice per group from 3 independent experiments are shown.*

As shown in Figure 19.C., the expression of various integrin chains (CD11a, CD11b, CD18) and Fcγ-receptors (FcγRII/III, FcγRIV) were very similar on neutrophils from intact mice and HoxB8 chimeras. However, moderately increased expression of some of those molecules (including CD11b, FcγRII/III and FcγRIV) could be observed in neutrophils of HoxB8 chimeras. We have later found that the increased expression of CD11b and FcγRIV in HoxB8 chimeras was likely due to the consequence of the irradiation/transplantation procedure itself, and not simply an intrinsic feature of the HoxB8 system, as 6-days-old bone marrow chimeras showed a similarly increased

expression of these two membrane proteins (Figure 20. A-B). This phenomenon was even more pronounced when we compared the CD11b and FcγRIV expression in mixed chimeras who had both BM-derived and HoxB8 progenitor-derived neutrophils in their circulation at the same time (Figure 20. C-D). A possible explanation is that the irradiation most probably evoked an inflammatory-like situation, which lead to a primed state of the hematopoietic cells (185).

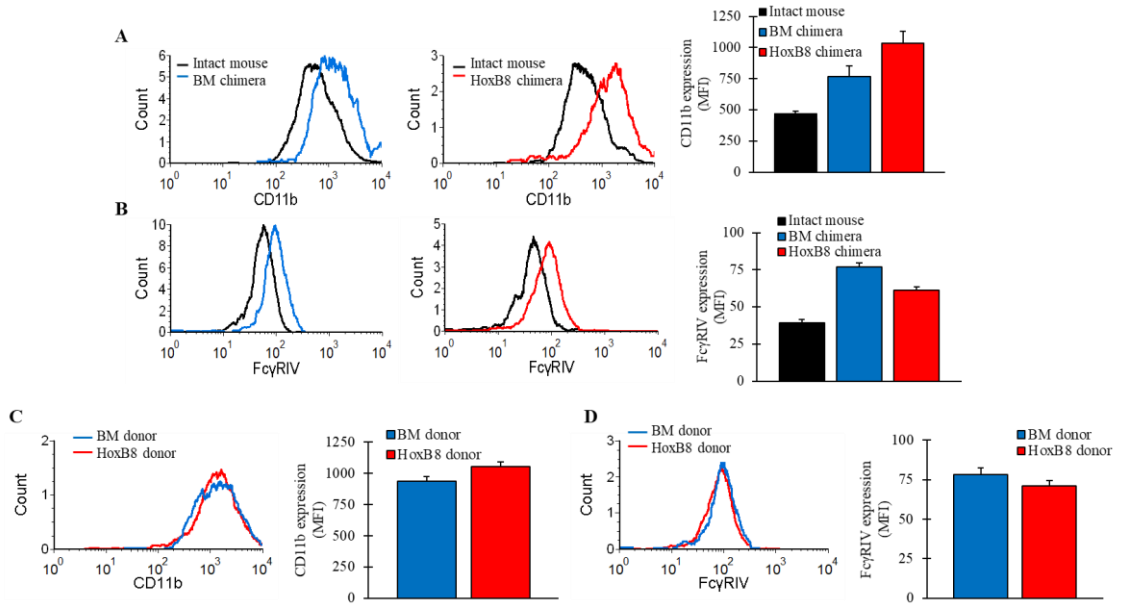

Figure 20. CD11b and FcγRIV expression on circulating neutrophils from BM, HoxB8 and mixed chimeras. *(A) analysis of CD11b (B) and FcγRIV expression on neutrophils from from BM chimeras and HoxB8 chimeras on Day 6 after irradiation, compared to neutrophils from intact mice. Summary of multiple experiments are shown in column charts. (C-D) Analysis of CD11b (C) and FcγRIV (D) expression on neutrophils from BM-HoxB8 mixed chimeras on Day 6 after irradiation. Histograms are representative of, and quantitative data show mean and SEM from, 4-8 (A-B) and 7 (C-D) mice per group from 2 independent experiments each. MFI, mean fluorescence intensity.*

As I have mentioned previously and it is visible on Fig. 19.A., likely due to the irradiation, basically all recipient-derived leukocytes are missing from the circulation of the HoxB8 chimeras. Also, we have noticed that irradiated, non-transplanted mice were severely ill within 7-8 days after transplantation, while HoxB8 chimeras were able to stay alive for a little bit longer. To investigate this phenomenon a little further and to check whether the emergence of the HoxB8 neutrophil population can be attributed to the slightly longer lifespan of the HoxB8 chimeras, we characterized the cellular composition

of the peripheral blood of non-transplanted control mice along with HoxB8 chimeras. We also checked whether other leukocyte populations emerged from the HoxB8 progenitors in the circulation of the HoxB8 chimeras.

We tested various hematological parameters in irradiated, non-transplanted mice, as well as chimeras generated using a single transplantation of bone marrow cells or single or multiple transplantations of HoxB8 progenitors at the same time. All mice received a lethal irradiation on Day 0. The number of different circulating leukocyte populations and haematological parameters were tested on Days 0, 5 and 10, except for irradiated non-transplanted control mice which could not survive until Day 10.

The lethal irradiation eliminated all recipient-derived neutrophils, monocytes, B- and T-cells in all of the animals (Fig. 21.). As expected, irradiated, non-transplanted mice had no donor-derived circulating leukocytes throughout the 10 days of the experiment. Transplantation of bone marrow cells led to the emergence of donor-derived monocytes, B-cells and a smaller number of neutrophils by Day 10, however, the T-cell population did not recover withing the assay period. Single or repeated transplantation of HoxB8 progenitors led to the emergence of donor-derived neutrophils, while no other circulating leukocytes emerged upon either single or multiple HoxB8 progenitor transplantation (Fig. 21.).

We also tested circulating red blood cell and platelet counts. We found that red blood cell counts in both types of HoxB8 chimeras were reduced to approximately 30% of their original values by Day 10, whereas they practically returned to their normal range by the same day in bone marrow chimeras (Fig. 22.A.). Very similar results could be seen in case of the platelet counts: there were basically no platelets in the circulation of the HoxB8 chimeras by the end of the experiment whereas by Day 10 the platelet numbers had already started to recover from the effects of irradiation in case of bone marrow chimeras (Fig. 22.B.).

Taken together, we could confirm that lethal irradiation basically eliminates all circulating leukocytes and platelets and decimates the red blood cell population, as well. Transplantation of bone marrow cells could revert these processes, while HoxB8 progenitor transplantation only

leads to the development of a HoxB8 neutrophil population in vivo. Nevertheless, this may be sufficient to maintain critical physiological functions, as indicated by the

declining state of irradiated, non-transplanted mice compared to a slightly longer lifespan of the HoxB8 chimeras.

Our current understanding is that according to the previous data, even with multiple, repeated HoxB8 progenitor transplantation we have roughly only 10-15 days to subject HoxB8 chimeras to in vivo experiments, and this might be the biggest limitation of our method. After 10 days, the overall health of the HoxB8 chimeras deteriorates rapidly, most likely due to the severe anaemia and platelet defect, which may result in the death of the animals.

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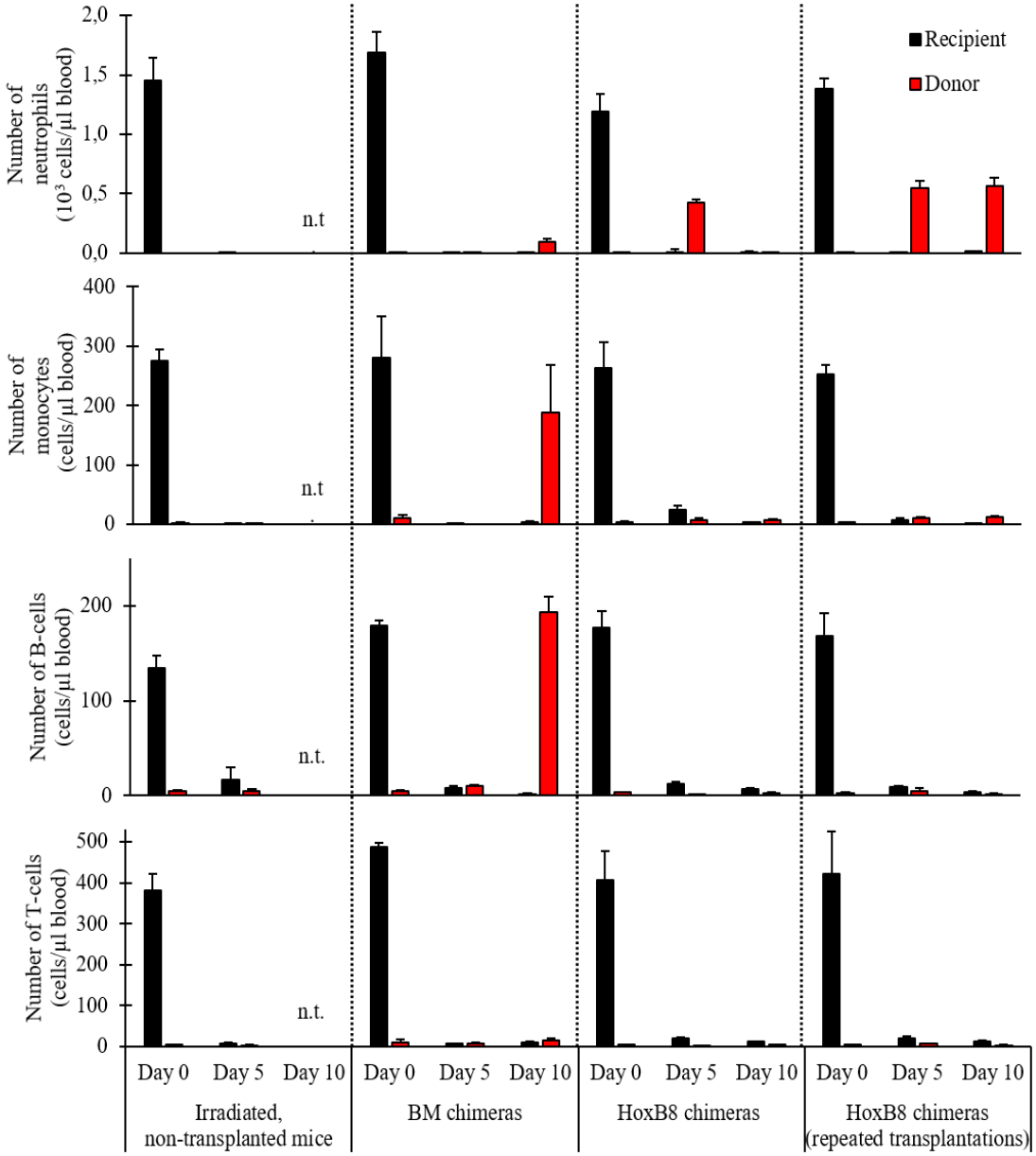

Figure 21. Cell counts of leukocytes in various recipient groups. *Time course of donor- and recipient-derived circulating neutrophil, monocyte, B-cell, and T-cell counts of irradiated, non-transplanted mice, bone marrow (BM) chimeras and HoxB8 chimeras generated via a single or repeated injection of HoxB8 progenitors respectively. Quantitative data show mean and SEM from 3-18 or 3-11 mice per group from 2-4 independent experiments. n.t., not tested.*

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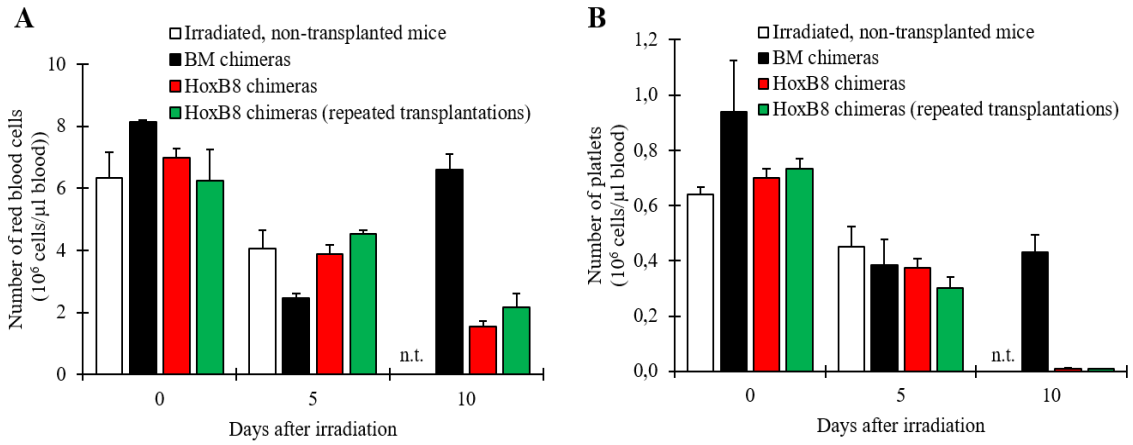

Figure 22. Red blood cell and platelet counts in various recipient groups. *Time course of red blood cell (A) and platelet (B) counts of irradiated, non-transplanted mice, bone marrow (BM) chimeras and HoxB8 chimeras generated via a single or repeated injection of HoxB8 progenitors. Quantitative data show mean and SEM from 3-18 or 3-11 mice per group from 2-4 independent experiments. n.t., not tested.*

4.1.7. In vivo functions of HoxB8 neutrophils

4.1.7.1. Accumulation of HoxB8 neutrophils in induced peritonitis

Since we were able to generate HoxB8 chimeras which have a substantial number of circulating HoxB8 neutrophils that are very similar to intact mouse neutrophils in their cell surface protein expression (Fig. 19.), we next began testing the functional characteristics of these cells in vivo. First, we wanted to check whether these HoxB8 neutrophils can leave the circulation and migrate towards the extracellular space, if needed. Therefore, we subjected HoxB8 chimeras to a thioglycollate-induced sterile peritonitis on Day 5 after transplantation. During the following experiments, HoxB8 chimeras generated via a single transplantation were used either 5 or 6 days after irradiation and transplantation of HoxB8 progenitors as shown in Fig. 17.A.

We found that thioglycollate induced a robust infiltration of neutrophils into the inflamed peritoneum during the 4-hour assay period (Fig. 23. A-C.).

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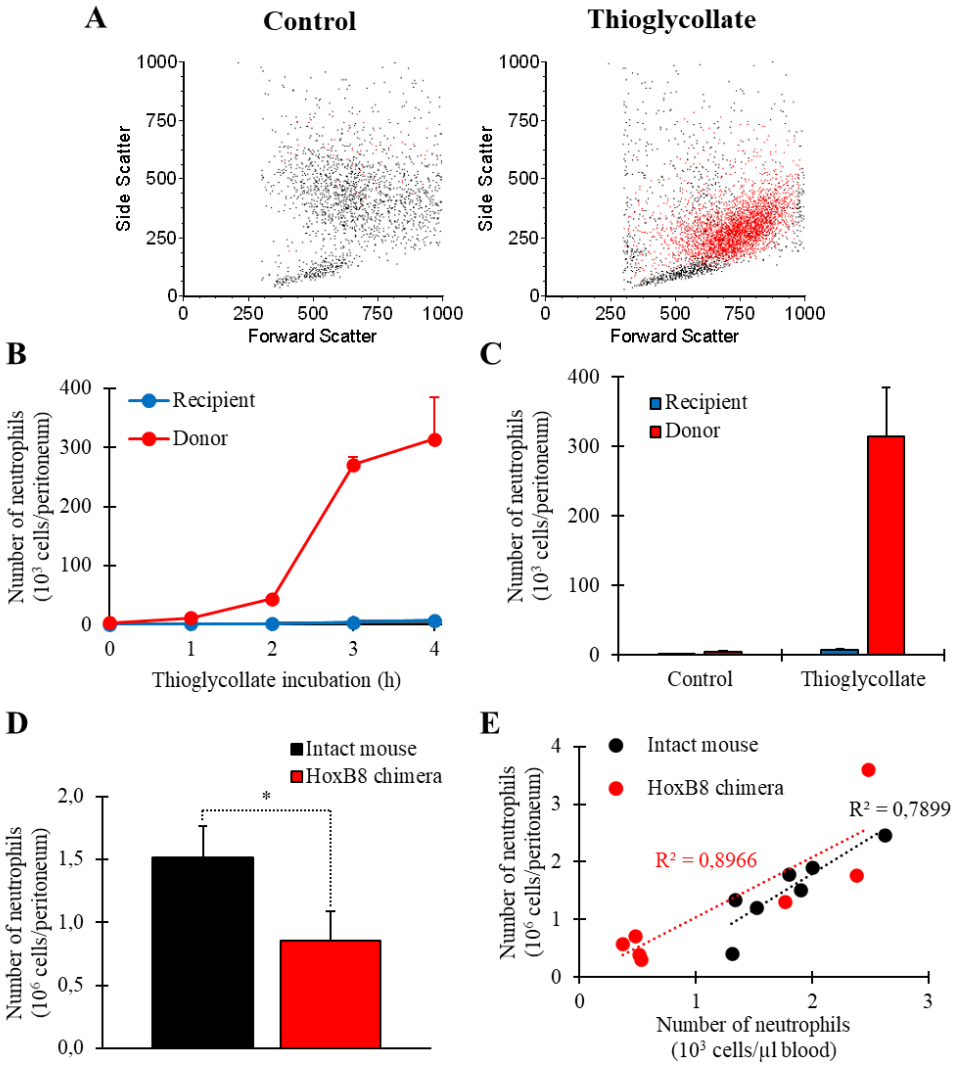

Figure 23. Peritoneal accumulation and phagocytic activity of in vivo derived HoxB8 neutrophils. *(A) Forward- and side-scatter profiles of peritoneal cells in HoxB8 chimeras upon control or thioglycollate treatment. Red coloured dots represent the Ly6G expressing HoxB8 neutrophil population. (B-C) Quantitative analysis of donor- and recipient-derived peritoneal neutrophils in HoxB8 chimeras 0-4 h (B) or 4 hours (C) after peritonitis induction. (D) Quantitative analysis and comparison of peritoneal neutrophil counts in intact mice and HoxB8 chimeras 4 hours after thioglycollate-dependent peritonitis induction. *: p=0,077 (E) Correlation between circulating and peritoneal neutrophil counts in intact mice and HoxB8 chimeras. Each dot represents an individual intact mouse or HoxB8 chimera. Dot plots are representative of, and quantitative data show mean and SEM from 18 (A, C), 4-9 (B) or 6-7 (D-E) mice per group from 4-6 independent experiments.*

Kinetic analysis revealed that the majority of infiltration happened during the last two hours of the assay (Fig. 23.B.). These infiltrating neutrophils were almost entirely of donor origin (Fig. 23.C.), in line with the practically complete absence of recipient-

derived circulating neutrophils in these chimeras on Days 5 and 6 (Fig. 17.B.). We further compared the efficacy of thioglycollate-induced accumulation of the HoxB8 neutrophils to neutrophils of intact mice. We found that the number of neutrophils in the peritoneum of HoxB8 chimeras during thioglycollate-induced peritonitis was closely 40% of that in intact mice (Fig. 23.D.). This percentage was comparable to the number of circulating neutrophils in HoxB8 chimeras relative to intact animals (Fig. 17.B.). Indeed, comparing the neutrophil counts in the circulation and in the peritoneum of all mice individually revealed that HoxB8 neutrophils appear to have a quantitatively similar capability of accumulating in the inflamed peritoneum as intact mouse neutrophils (Fig. 23.E.).

4.1.7.2. In vivo phagocytosis by HoxB8 neutrophils

Since HoxB8 neutrophils were able to migrate into the inflamed peritoneum, we wanted to test whether they are also able to show any phagocytic capacity once they encounter pathogens outside the circulation. Therefore, thioglycollate-induced sterile peritonitis was triggered as above to initiate extravasation of HoxB8 neutrophils. 3 hours after thioglycollate injection, heat-killed fluorescently labeled *C. albicans* suspension was injected into the peritoneum of HoxB8 chimeras. After an additional hour, the peritoneum was lavaged and the phagocytosis of *C. albicans* by HoxB8 neutrophils was determined by flow cytometry. A parallel cohort of mice received latrunculin A, a known phagocytosis blocker (an inhibitor of actin polymerization), immediately before the injection of the heat-killed yeast cells.

As shown in Fig. 24., one hour after pathogen administration, more than 30% of HoxB8 neutrophils phagocytosed the fluorescent *C. albicans* particles inside the peritoneum. This response was completely blocked by latrunculin A, which confirms the role of the cytoskeletal machinery in the phagocytic response mounted by the HoxB8 neutrophils ($p = 2.1\times10^{-7}$). Immediately before the flow cytometric measurement, samples were treated with trypan blue dye in order to quench the fluorescence of candida particles that might be bound to the neutrophils from outside. Therefore, HoxB8 neutrophils showed green fluorescence only if they engulfed the *C. albicans* particles.

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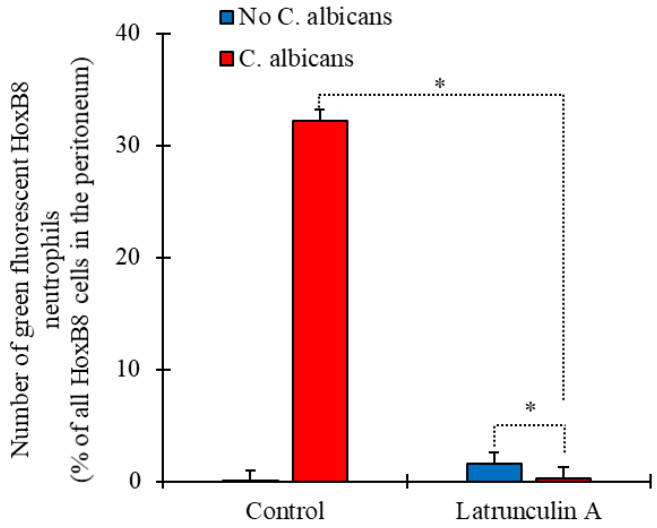

Figure 24. Phagocytic capacity of HoxB8 neutrophils in vivo. *Quantification of phagocytosis of fluorescently labeled heat-killed C. albicans by peritoneal HoxB8 neutrophils with or without 50 µg/kg latrunculin A treatment. Quantitative data show mean and SEM from 6-12 mice per group from 8 independent experiments. *: p < 0,05.*

4.1.7.3. Reverse passive Arthus reaction

The above experiments confirmed that HoxB8 neutrophils are capable of various functional responses in vivo, such as migration and phagocytosis of pathogens comparable to circulating neutrophils (Figs. 23 and 24.). However, all of the above-mentioned experiments tested the functional capacity of the HoxB8 neutrophils on a single-cell level only. Therefore, we hoped to subject HoxB8 chimeras to experiment, where the entire donor-derived HoxB8 neutrophil compartment, as a whole, is able to mediate neutrophil-dependent functional responses. We chose two different experimental models to answer this question.

The first one of these models was the reverse passive Arthus reaction, which is known to be an acute, neutrophil-dependent inflammatory mouse model (186). HoxB8 chimeras were intraperitoneally injected with ovalbumin (Ova) on Day 5 after irradiation/transplantation, along with local intradermal injection of rabbit polyclonal anti-Ova antibodies into one of the ears. As control, normal rabbit serum was injected into the other ear of the same mice. The same experiments were also performed on irradiated, non-transplanted mice and, in some cases, intact C57BL/6 mice to serve as controls. The inflammatory reaction was tested by measuring the accumulation of the

Evans Blue dye in the ears which reflects the inflammation-induced loss of vascular integrity and edema formation. Ear thickness measurement, as well as determination of the tissue accumulation of neutrophils and production of various inflammatory mediators, were also assessed.

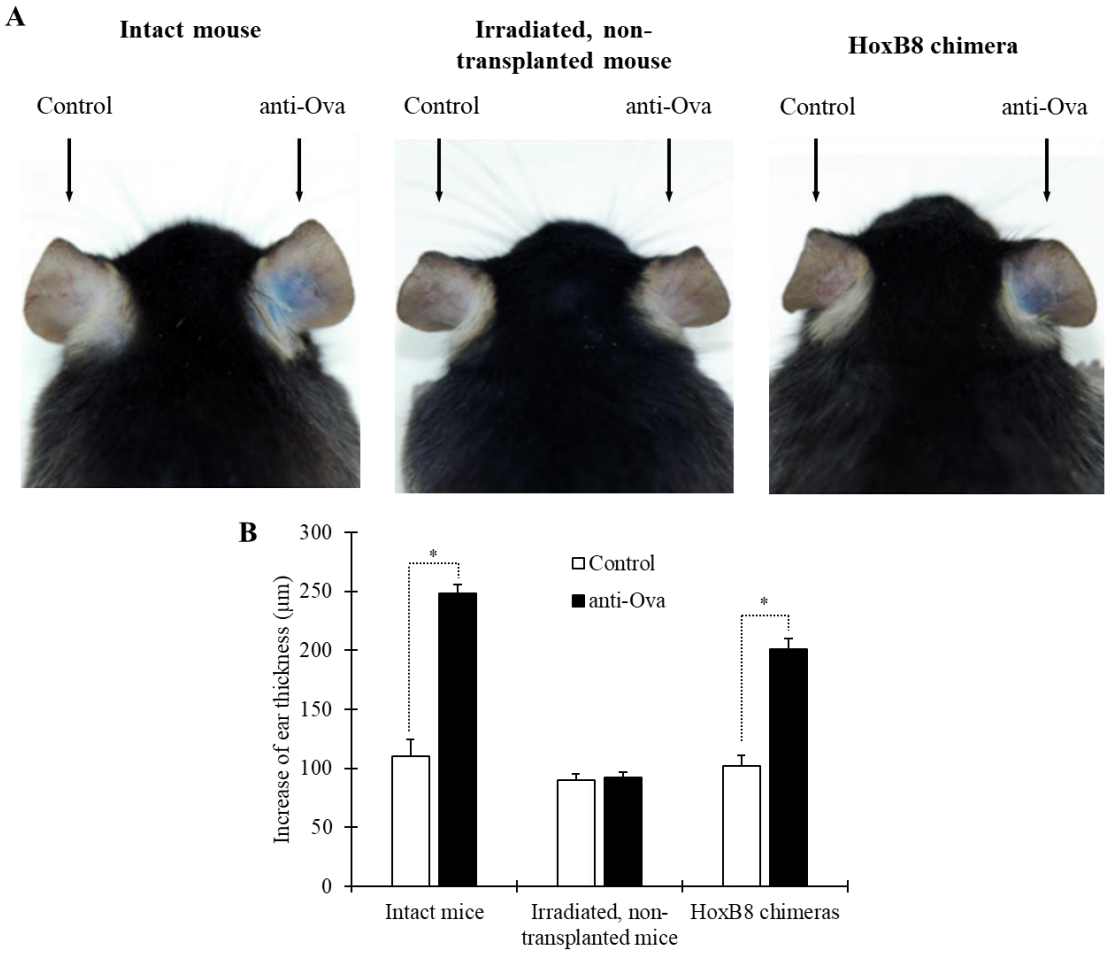

Figure 25. Reverse passive Arthus reaction in HoxB8 chimeras. *Reaction was triggered in intact mice, irradiated, non-transplanted mice, or HoxB8 chimeras. Inflammation and edema formation was assessed by following the extravasation of intravenously injected Evans Blue dye (A) or measurement of ear thickness (B). Images are representative of, and quantitative data show mean and SEM from 6-10 mice per group from 3 independent experiments. *: $p < 0,05$.*

Anti-Ova but not control treatment resulted in edema formation indicated by the blue color of the ear treated with anti-OVA (tissue accumulation of Evans Blue) and the increase of ear thickness in intact C57BL/6 mice (Fig. 25.). Similarly, blue coloring and ear thickening of anti-Ova treated ears could be seen in HoxB8 chimeras but not in

irradiated non-transplanted mice (Fig. 25. A-B.) ($p = 5.0\times10^{-9}$ for comparison between the last two groups).

To check if the edema formation was accompanied by the infiltration of neutrophils, we measured the number of neutrophils from ear tissue lysates using flow cytometry, comparing HoxB8 chimeras with irradiated, non-transplanted control mice. As shown in Fig. 26.A., a substantial neutrophil infiltration was observed in the anti-Ova-treated ears of HoxB8 chimeras but not the control treated ears or in any of the ears of irradiated, non-transplanted control animals ($p = 3.1\times10^{-4}$). Also, anti-Ova treatment induced the accumulation of the pro-inflammatory cytokine IL-1β ($p = 1.1\times10^{-5}$) and the MIP-2 chemokine ($p = 0.043$) in HoxB8 chimeras but not in irradiated non-transplanted mice (Fig. 26. B-C.). These results also suggested, that HoxB8 neutrophils are able to produce various cytokines in vivo, which is another important effector function of mature circulatory neutrophils.

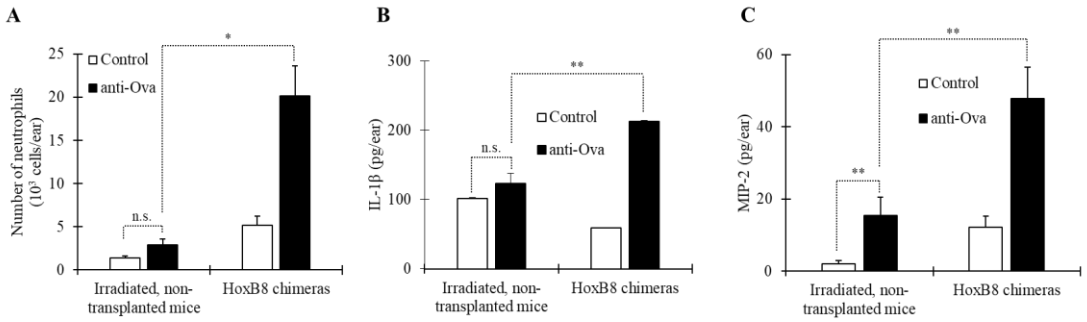

Figure 26. Reverse passive Arthus reaction induced in HoxB8 chimeras. *Analysis of the accumulation of neutrophils in anti-OVA or control treated ears of HoxB8 chimeras and irradiated, non-transplanted mice by flow cytometry (A). Production of IL-1β (B) or MIP-2 (C) by accumulated neutrophils in anti-Ova or control treated ear samples. Quantitative data show mean and SEM from 6-10 mice per group from 3 independent experiments. n.s.: not significant, *: p <0,05, **: p < 0,05.*

Taken together, we could confirm that the HoxB8 neutrophil population in the HoxB8 chimeras can take important part in the development of complex inflammatory reactions, just like how the circulating neutrophils usually perform in vivo. The fact that no such reactions could be observed in irradiated non-transplanted control mice under the same circumstances further indicates a critical role for HoxB8 neutrophils in the development of the inflammatory response.

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4.1.7.4. Autoantibody-induced arthritis in HoxB8 chimeras

As I have explained previously, subjecting lethally irradiated recipient mice to multiple transplantation of HoxB8 progenitor cells makes it possible to maintain a quite stable HoxB8 neutrophil population in the circulation for prolonged periods of time (depending on the number of transplantations) (Fig. 18.). Therefore, our next aim was to test whether we can also induce longer-term inflammatory processes that are dependent on the function of the complete circulating HoxB8 neutrophil compartment. To do so, we turned to the K/B×N serum-transfer arthritis, a strongly neutrophil-dependent autoantibody-induced in vivo model of autoimmune arthritis as a second type of whole neutrophil population-based mouse model (35, 36).

The K/B×N serum-transfer arthritis experiment usually takes about 14 days (36, 97, 171). Therefore, we have transplanted lethally irradiated CD45.1-expressing recipients with HoxB8 progenitors immediately after the irradiation (Day 0), as well as every two days until Day 10 (6 times altogether). We have also reduced the length of the overall experiment to 10 days (instead of 14) due to the declining health status of the HoxB8 chimeras as we reached Day 15 after lethal irradiation. Animals were injected with arthritogenic (K/B×N) or non-arthritogenic control (B×N) serum on Day 5, when donor-derived HoxB8 neutrophils were clearly present in the circulation (Fig. 27.B.). The experimental setup is explained on Fig. 27.A.

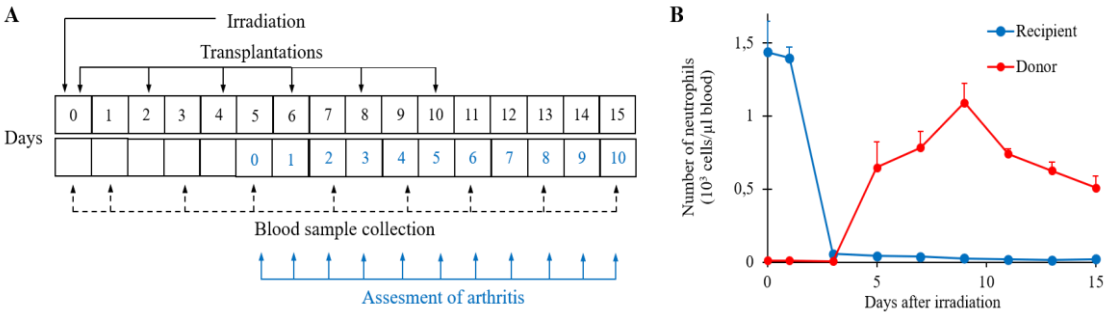

Figure 27. Autoantibody-induced arthritis in HoxB8 chimeras. *(A) Overview of the experimental design. (B) Quantitative analysis of peripheral blood neutrophil cell counts in HoxB8 chimeras. Quantitative data show mean and SEM from 3-6 control and 4-8 arthritic serum-treated individual mice per group from 3 independent experiments.*

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From Day 0 all the way to the end of the experiment (Day 15) circulating neutrophil numbers of HoxB8 chimeras were assessed (Fig. 27.B.) and the disease course (Fig. 27. A-C.) was followed for 10 days after the serum injection (between Days 5 and 15 from irradiation and first HoxB8 progenitor injection). We have also included intact C57BL/6 mice and irradiated, non-transplanted recipients to serve as controls.

In HoxB8 chimeras treated according to Fig. 27.A., we have found extensive circulating HoxB8 neutrophil numbers, just like in experiments shown previously on Figs. 17.B. and 18.B. Recipient neutrophils were practically completely missing from the circulation by Day 3 and remained so until the end of the experiment. Donor-derived HoxB8 neutrophils appeared in the circulation by Day 5 and their numbers remained at around 40-50% of the initial neutrophil counts until the end of the experiment. There was basically no difference in the circulating neutrophil counts observed between arthritogenic and control serum treated HoxB8 chimeras (data not shown). As expected, irradiated, non-transplanted control mice had no circulating neutrophils during the experiment, and in most cases these animals had a poor health status towards the end of the experiment, and had to be euthanized.

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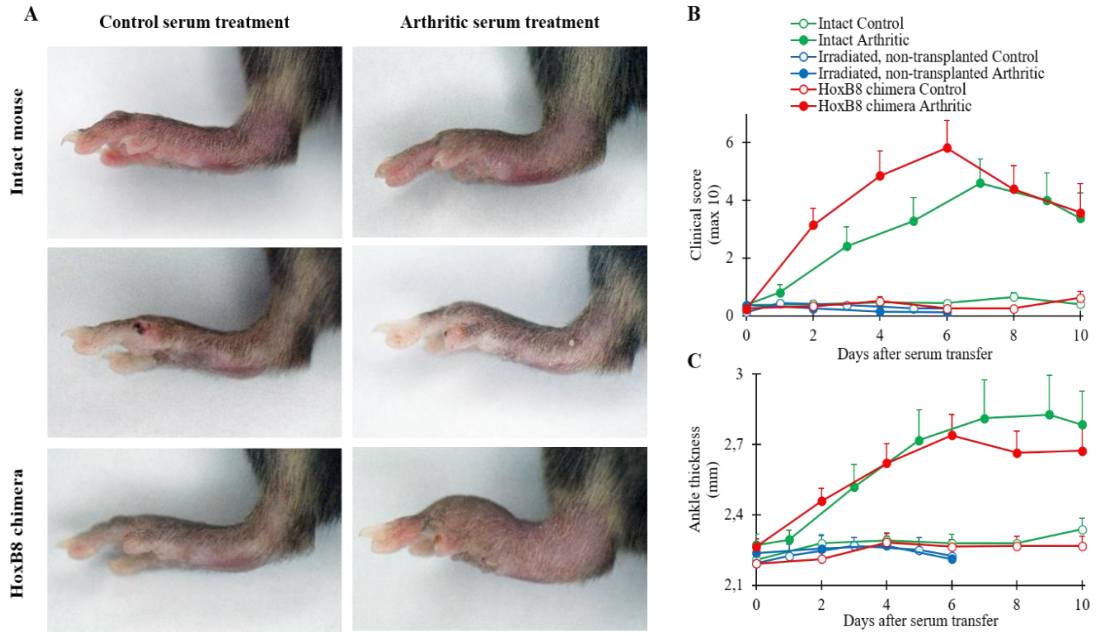

Figure 28. Autoantibody-induced arthritis in HoxB8 chimeras. *K/B×N serum transfer arthritis in intact mice, irradiated, non-transplanted mice and HoxB8 chimeras. Analysis of the development of the arthritic phenotype by photographing on Day 7 (A), clinical scoring of the hind limbs (B) or ankle thickness measurement (C). Images are representative of, and quantitative data show mean and SEM from 3-6 control and 4-8 arthritic serum-treated individual mice per group from 3 independent experiments.*

The disease course was assessed immediately before, and then each day after the serum treatment. This meant that the limb of the animals was checked for any signs of inflammation (swelling, thickening, redness, deformity, etc.) and clinical scores between 0-10 were given to represent the severity of inflammation. Ankle thickness was measured using a caliper, and photos were taken usually on Day 7-8 after serum injection (Days 12-13 after the irradiation/first transplantation). As shown in the photographs (Fig. 28.A.), arthritogenic but not control serum injection triggered robust arthritis in intact C57BL/6 mice, whereas no signs of arthritis could be observed in irradiated, non-transplanted mice. Most importantly, injection of arthritogenic serum also triggered robust arthritis in the HoxB8 chimeras, while the control serum treated HoxB8 chimeras remained healthy.

Clinical scoring (Fig. 28.B.) and ankle thickness measurements (Fig. 28.C.) revealed that the disease course reached its maximum around Day 7 after disease induction in arthritogenic serum-treated intact mice. According to clinical scoring and

ankle thickness, irradiated, non-transplanted mice were completely protected upon arthritic serum treatment, supposedly indicating a very important role of circulating leukocytes in the disease development. On the contrary, severe arthritis development could be observed in HoxB8 chimeras, which was similar to, or in the case of ankle thickening, even slightly more pronounced than in intact mice (Fig. 28. A-C.). Statistical analysis revealed that arthritis development in HoxB8 chimeras compared to irradiated, non-transplanted mice was significantly higher ($p = 0.016$ and 0.021 for clinical score and ankle thickness during Days 0-6 from serum transfer, respectively) but it did not differ significantly from that of the intact animals ($p = 0.39$ and 0.57, respectively).

Our results indicate that if a stable HoxB8 neutrophil population is present in the circulation of the chimeras for several days as a result of repeated transplantation of the HoxB8 progenitors, these HoxB8 chimeras can be the subjects of long-term in vivo inflammatory model experiments. This further suggests the cell line's usefulness in neutrophil research, as the in vivo differentiated HoxB8 neutrophils are not only functionally active individually, but the whole HoxB8 neutrophil compartment can contribute to inflammatory disease development, just like their normal circulating counterparts.

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4.2. Characterization of Syk KO HoxB8 neutrophils

All of the previously described in vitro and in vivo results regarding the functional activity of the HoxB8 cells pointed towards the same conclusion, that the HoxB8 neutrophils are very similar to circulating neutrophils both in their phenotype and effector functions. These results on their own would indicate that the ER-HoxB8 myeloid progenitor and neutrophil cell line could be a useful tool in the future for studying neutrophil biology.

Even though we had very promising outcomes from the previously mentioned experiments, we wanted to further verify the accuracy of the HoxB8 system. Therefore, we turned to our previously reported observations regarding the role of Syk (spleen tyrosine kinase) in neutrophil function. It is known that Syk is an essential component of integrin signaling in neutrophils (177), and it was also reported, that immune complexes trigger neutrophil activation in Syk-dependent manner as well (187). Syk has numerous functions in neutrophil activity (176), from mediating adhesion and extravasation (177, 188, 189), directing pathogen recognition (190) all the way to coordinating complex inflammatory neutrophil-specific responses. Our group has previously reported, *Syk*-deficient mice ($Syk^{-/-}$) are completely protected in autoantibody-induced arthritis (98, 171) or in the autoimmune skin inflammation model of epidermolysis bullosa acquisita (EBA) as well (187).

Considering that previous observations highlighted the role of *Syk* in neutrophils' various functions, we wanted to see if *Syk* deletion in HoxB8 progenitors could result in the same functional defect of HoxB8 neutrophils generated from $Syk^{-/-}$ HoxB8 progenitors in vitro and in vivo, as well. Since germline Syk deletion is known to cause perinatal lethality (169, 191), we had two options the collect progenitor cells for the HoxB8 cell line generation (according to Fig. 6.). One was to use fetal liver cells of a $Syk^{-/-}$ mouse embryo and generate HoxB8 cells directly from the progenitors residing in the fetal liver. Alternatively, we could generate $Syk^{-/-}$ BM chimeras via transplantation of fetal liver cells of a $Syk^{-/-}$ embryo into lethally irradiated recipients, and 4 weeks later collect bone marrow of these chimeras to generate HoxB8 progenitors, from supposedly $Syk^{-/-}$ bone marrow cells. We tried both methods and found that there was no phenotypical or functional difference between $Syk^{-/-}$ HoxB8 progenitors originating from fetal liver or bone marrow progenitor cells (data not shown).

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4.2.1. *Syk*$^{-/-}$ HoxB8 cells in vitro

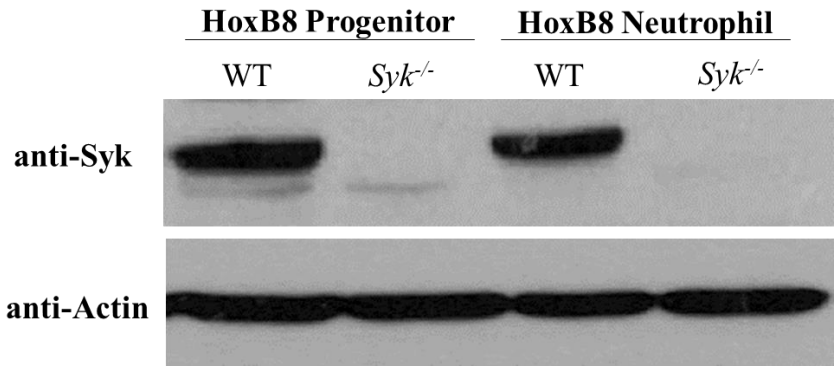

Figure 29. Western-blot of in vitro cultured HoxB8 progenitor and HoxB8 neutrophil cell lysates. *The presence of Syk was determined by monoclonal anti-Syk antibody and actin served as loading control.*

As it can be seen in Figure 29., western-blotting confirmed that Syk was expressed in WT HoxB8 progenitors and WT HoxB8 neutrophils alike. However, Syk was missing from the *Syk*$^{-/-}$ HoxB8 progenitors and neutrophils differentiated from them in vitro. We observed, that *Syk*$^{-/-}$ HoxB8 progenitor cells were able to proliferate just as quickly as WT HoxB8 progenitors, and once we followed the usual differentiating protocol (Fig. 6.), estrogen withdrawal and G-CSF treatment yielded HoxB8 neutrophils of practically completely similar overall morphology both in the WT and *Syk*$^{-/-}$ cultures. We found that both WT and *Syk*$^{-/-}$ HoxB8 progenitors express CD45 and CD117 (c-kit) markers at very similar levels (Fig. 30. A-B). As a sign of mouse neutrophilic differentiation HoxB8 neutrophils generated from WT and *Syk*$^{-/-}$ progenitors expressed similar levels of Ly6G (Fig. 30.C.). Cell surface receptors like CD11a, CD11b, CD18, FcγRII/III and FcγRIV attributed to various neutrophil functions showed identically low expression in WT and *Syk*$^{-/-}$ HoxB8 progenitors, as well, while all of these markers were upregulated in WT and *Syk*$^{-/-}$ HoxB8 neutrophils differentiated in vitro at the same manner (Fig. 30. D-H).

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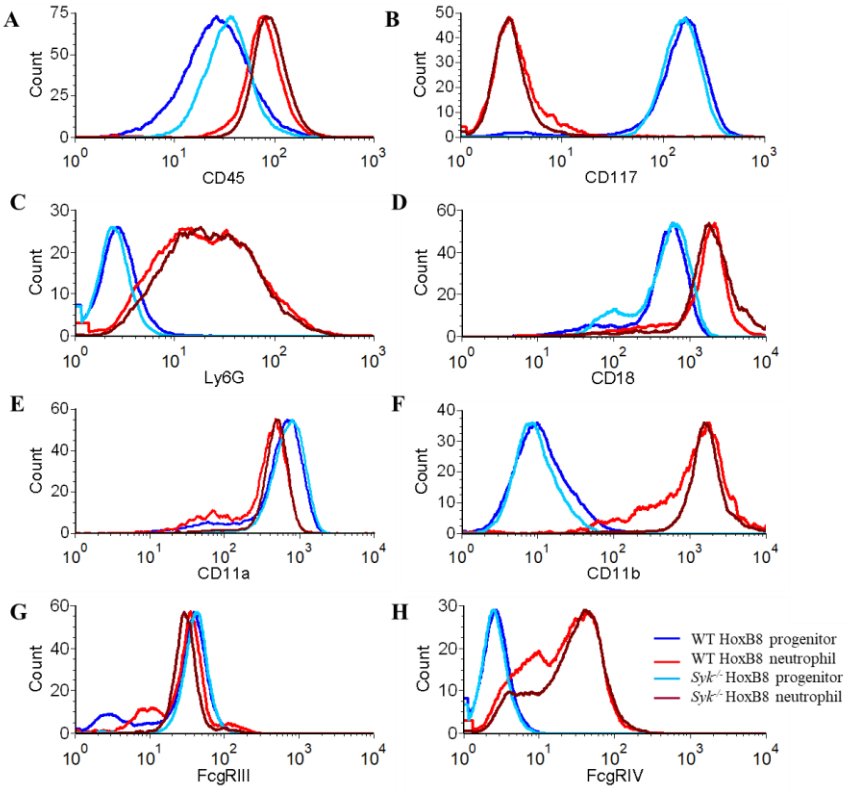

Figure 30. Histograms of various cell surface receptor staining of WT and Syk$^{-/-}$ HoxB8 cells cultured in vitro. *Cell surface receptor expression was assessed by flow cytometry. Histograms are representative of 3-5 individual experiments.*

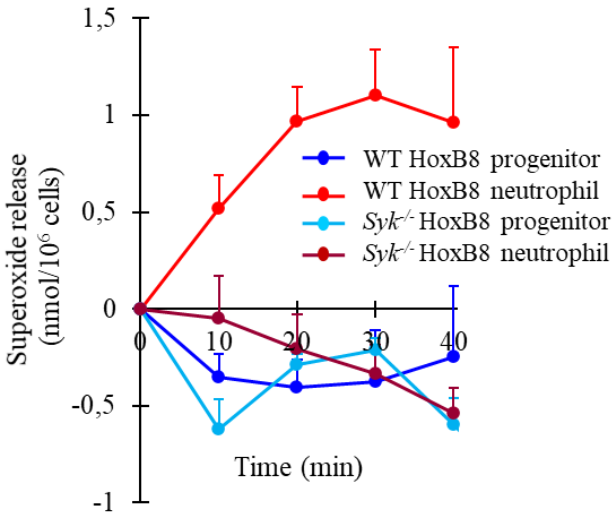

Figure 31. ROS production of WT and Syk$^{-/-}$ HoxB8 progenitors and neutrophils in vitro stimulated by immobilized IgG immune complex surface. *Quantitative data show mean and SEM of results from 4 independent experiments.*

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As far as the in vitro functions of these cells are concerned, we have found that *Syk*$^{-/-}$ HoxB8 neutrophils were not able to produce reactive oxygen species upon immobilized immune complex stimulation, while WT HoxB8 neutrophils in the same experiment were just as functionally active as previously shown on Fig. 9.B. Not surprisingly, both WT and *Syk*$^{-/-}$ HoxB8 progenitor cells stayed inactive throughout the experiment and were unable to produce ROS (Fig. 31).

4.2.2. *Syk*$^{-/-}$ HoxB8 neutrophils in vivo

Since we observed practically the same potential of WT and *Syk*$^{-/-}$ HoxB8 progenitors to differentiate into HoxB8 neutrophils in vitro (Fig. 30.), we hoped to follow the same protocol as before (Figs. 11, 17.A. and 18.A.) to generate *Syk*$^{-/-}$ HoxB8 chimeras via single or repeated transplantation of *Syk*$^{-/-}$ HoxB8 progenitors into lethally irradiated recipients.

What we observed was basically the same phenomenon as I described in detail in case of WT HoxB8 neutrophil differentiation in vivo. Briefly, following lethal irradiation by Day 3 all of the recipient-derived neutrophils disappeared from the circulation of the HoxB8 chimeras. Meanwhile *Syk*$^{-/-}$ HoxB8 progenitors started to differentiate, leading to the emergence of the *Syk*$^{-/-}$ HoxB8 neutrophil population 5 days after irradiation and transplantation. If we carried out transplantation of HoxB8 progenitors only once, the *Syk*$^{-/-}$ HoxB8 neutrophil population disappeared from the circulation of the chimeras after a peak in cell number at Day 6 (data not shown.) However, using the previously successful protocol (Fig. 18.A.) of multiple *Syk*$^{-/-}$ HoxB8 progenitor injections (every 2 days for 6 times altogether), we were able to maintain the *Syk*$^{-/-}$ HoxB8 neutrophil cell counts in the circulation of the chimeras at quite stable level for a total of 10 days (Fig. 32.).

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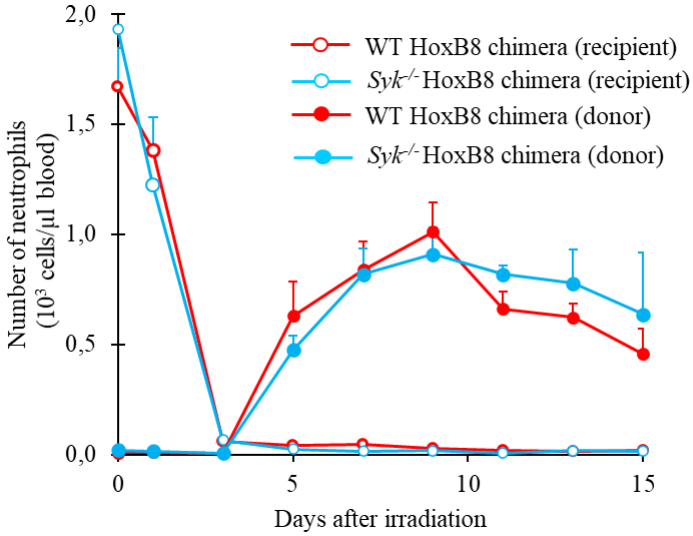

Figure 32. Peripheral blood neutrophil cell counts in WT and Syk$^{-/-}$ HoxB8 chimeras. *Quantitative data show mean and SEM from 10-16 mice from 3 independent experiments.*

As it is presented on Figure 32., there was basically no difference between the circulating neutrophil counts in the parallel generated WT and *Syk*$^{-/-}$ HoxB8 chimeras.

If we compare various cell surface receptor expression in WT and *Syk*$^{-/-}$ HoxB8 neutrophils present in the circulation of the chimeras on Day 5, we can say that they are very similar cells. Expression of the usual neutrophil markers like CD45, Ly6G, α- and β-chains of β2-integrins and FcγRII/III and FcγRIV showed to be completely overlapping in case of WT and *Syk*$^{-/-}$ HoxB8 neutrophils in flow cytometric measurements (Fig. 33. A- G.).

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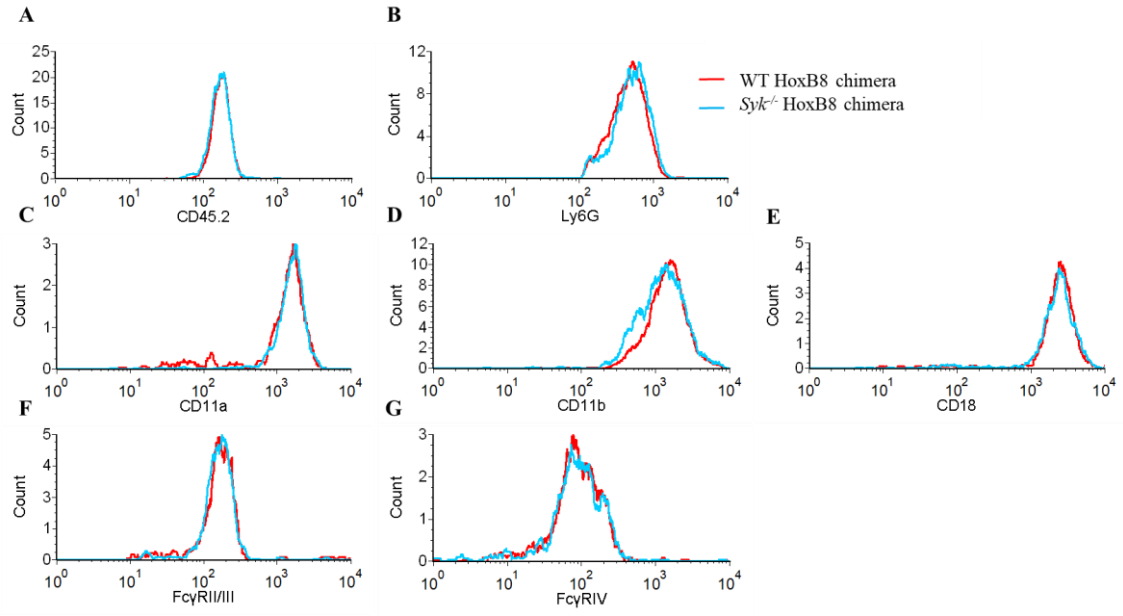

Figure 33. Surface marker expression of in vivo-derived HoxB8 neutrophils. *Histograms of various cell surface receptor staining of WT and Syk$^{-/-}$ HoxB8 neutrophils defined by their forward- and side-scatter characteristics and Ly6G-positivity within a CD45.2-positive gate. Representative data from 2-5 mice per group from 3 independent experiments are shown.*

4.2.3. Autoantibody-induced arthritis in *Syk*$^{-/-}$ HoxB8 chimeras

Since we hoped to use the *Syk*$^{-/-}$ HoxB8 chimeras to verify the accuracy and efficiency of the HoxB8 system in neutrophil research, we thought to subject *Syk*$^{-/-}$ HoxB8 chimeras to a complex, neutrophil-specific, inflammatory mouse model. Therefore, just like with WT HoxB8 chimeras, we turned to the K/B×N serum-transfer arthritis, a heavily neutrophil-dependent autoantibody-induced in vivo model of autoimmune arthritis (35, 36).

As shown is Fig. 27.A., we generated *Syk*$^{-/-}$ HoxB8 chimeras using multiple transplantation of HoxB8 progenitors into lethally irradiated recipients and WT HoxB8 chimeras to serve as a reference in our experiment. We injected control and arthritic sera into mice of both chimera groups and assessed the development of the disease by measurement of ankle thickness, clinical scoring of phenotypical changes, and we took photos of the animals on Day 7 and 8 after serum injection (Day 12, 13 after irradiation), as well.

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As seen in the photographs (Fig. 34.A.), arthritogenic but not control serum injection triggered robust arthritis in HoxB8 chimeras. Meanwhile, most importantly, no signs of arthritis could be observed in *Syk*$^{-/-}$ HoxB8 chimeras upon arthritic serum administration.

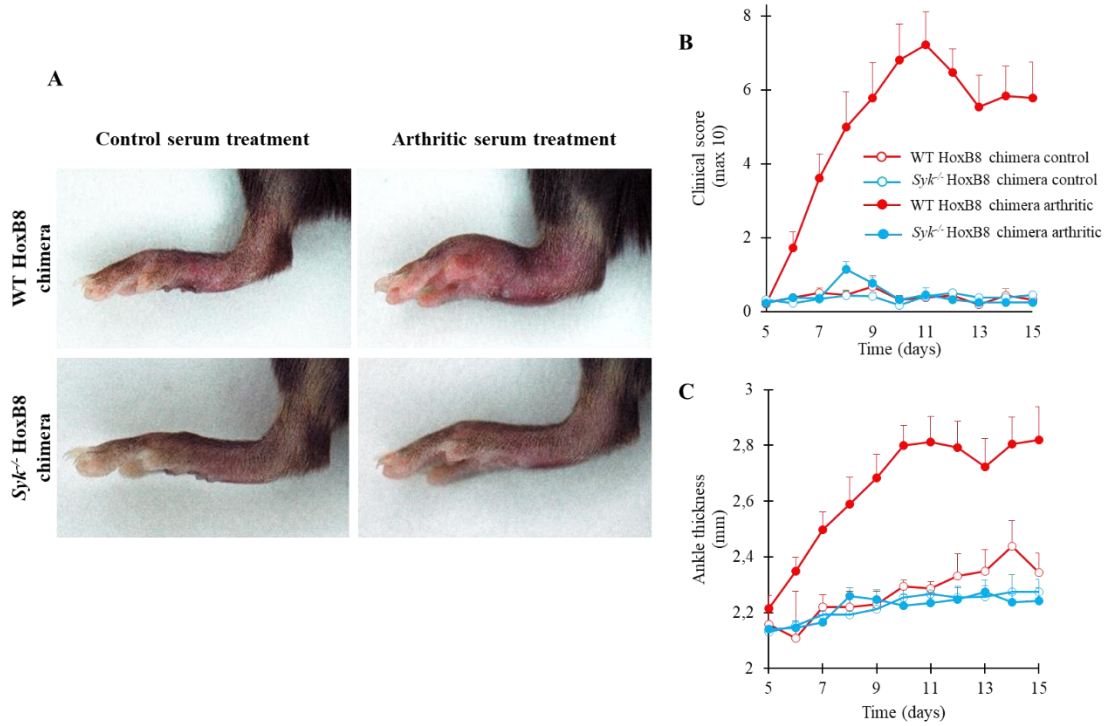

Figure 34. Autoantibody-induced arthritis in WT and Syk$^{-/-}$ HoxB8 chimeras. *Analysis of arthritis development by photographing on Day 7 (A), clinical scoring of the hind limbs (B) or ankle thickness measurement (C). Images are representative of, and quantitative data show mean and SEM from 3-4 control and 5-6 arthritic serum-treated individual mice per group from 3 independent experiments.*

Clinical scoring (Fig. 34.B.) and ankle thickness measurements (Fig. 34.C.) revealed that the disease course reached its maximum around Day 6 after disease induction in arthritogenic serum-treated mice. Severe arthritis development could be observed in WT HoxB8 chimeras, which was similar to what we had seen previously (Fig. 28. A-C.). However, just as it was observed by Jakus et al. using *Syk*$^{-/-}$ fetal liver chimeras (171), *Syk*$^{-/-}$ HoxB8 chimeras were completely protected from autoantibody-induced serum transfer arthritis upon arthritic serum treatment. Control serum did not induce any disease formation neither in WT nor in *Syk*$^{-/-}$ HoxB8 chimeras.

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Statistical analysis revealed that arthritis development in WT HoxB8 chimeras compared to *Syk*$^{-/-}$ HoxB8 chimeras was significantly higher ($p = 0.0014$ and 0.011 for clinical score and ankle thickness during Days 0-10 from serum transfer, respectively) but there was no significant difference between the control serum treated groups in any of the datasets ($p = 0.77$ and 0.49, respectively).

Altogether, our results with the *Syk*$^{-/-}$ HoxB8 neutrophils generated both in vitro and in vivo were very similar to what we had previously reported using Syk-deficient fetal live chimeras. Therefore, these results again confirmed that HoxB8 neutrophils differentiated from the immortalized HoxB8 progenitors are just as functionally active as any bone marrow-derived neutrophils in the circulation, further proving that the HoxB8 system could be one of the best tools currently available to conduct research on neutrophils both in vitro and in vivo.

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4.3. Characterization of *Mcl1$^{\Delta Myelo}$* mice

Even though there are many in vitro techniques and in vivo models which have been very useful to characterize the role and effector mechanisms of neutrophils in the last decades, unfortunately there are still numerous questions left unanswered regarding the important characteristics of these cells. Therefore, it is of utmost importance to constantly seek, develop, and characterize new tools that could bring us closer to the depth of neutrophil biology.

We believe that the HoxB8 system could provide a great framework for many of the upcoming neutrophil-related research, but to further enlarge the toolbox for neutrophil studies, we have developed and described a very promising new tool as well. During my PhD studies I had the opportunity to work together with Janka Zsófia Csepregi to characterize the *Mcl1$^{\Delta Myelo}$* neutropenic mouse model, described in detail in section 1.2.1.2.7. The elaborate characteristics of this mouse model can be found in our publication (36) and also in the PhD thesis of Janka Zsófia Csepregi. But briefly, *Mcl1$^{\Delta Myelo}$* mice have a significantly (98%) reduced neutrophil cell count (Fig. 2.A.) without a drastic effect on fertility and survival rate of these animals (36). This strongly neutropenic phenotype of the *Mcl1$^{\Delta Myelo}$* mice was also noticeable in inflammatory conditions, as thioglycollate injection failed to evoke neutrophil accumulation in the peritoneum of the animals (Fig. 2.B.) (36). We have found that *Mcl1$^{\Delta Myelo}$* mice can survive and breed in homozygous form in SPF (specific pathogen free) conditions for long periods of time (36). We have also found that *Mcl1$^{\Delta Myelo}$* mice were completely protected in two separate types of neutrophil-mediated autoantibody-induced inflammation models (Fig. 2.C.) (36). In addition, we could confirm that *Mcl1$^{\Delta Myelo}$* mice seemed to be highly susceptible to infectious challenge by bacterial or fungal pathogens, such as *S. aureus* (Fig. 2.D.) or *C. albicans*, most likely because of the defect in the neutrophil-mediated elimination of the pathogens (36).

A very important feature of a neutropenia mouse model is its neutrophil specificity. Since Lysozyme-M is known to be expressed in every myeloid cell type, we were very interested in characterising the effect of Mcl-1 deletion in our mouse model in various tissues. Janka Zsófia Csepregi analysed the circulation of the *Mcl1$^{\Delta Myelo}$* mice in this regard, while I focused on the cellular composition and possible changes of the leukocyte cell counts in the bone marrow and spleen of *Mcl1$^{\Delta Myelo}$* mice.

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4.3.1. Leukocyte populations in the bone marrow of *Mcl1$^{\Delta Myelo}$* mice

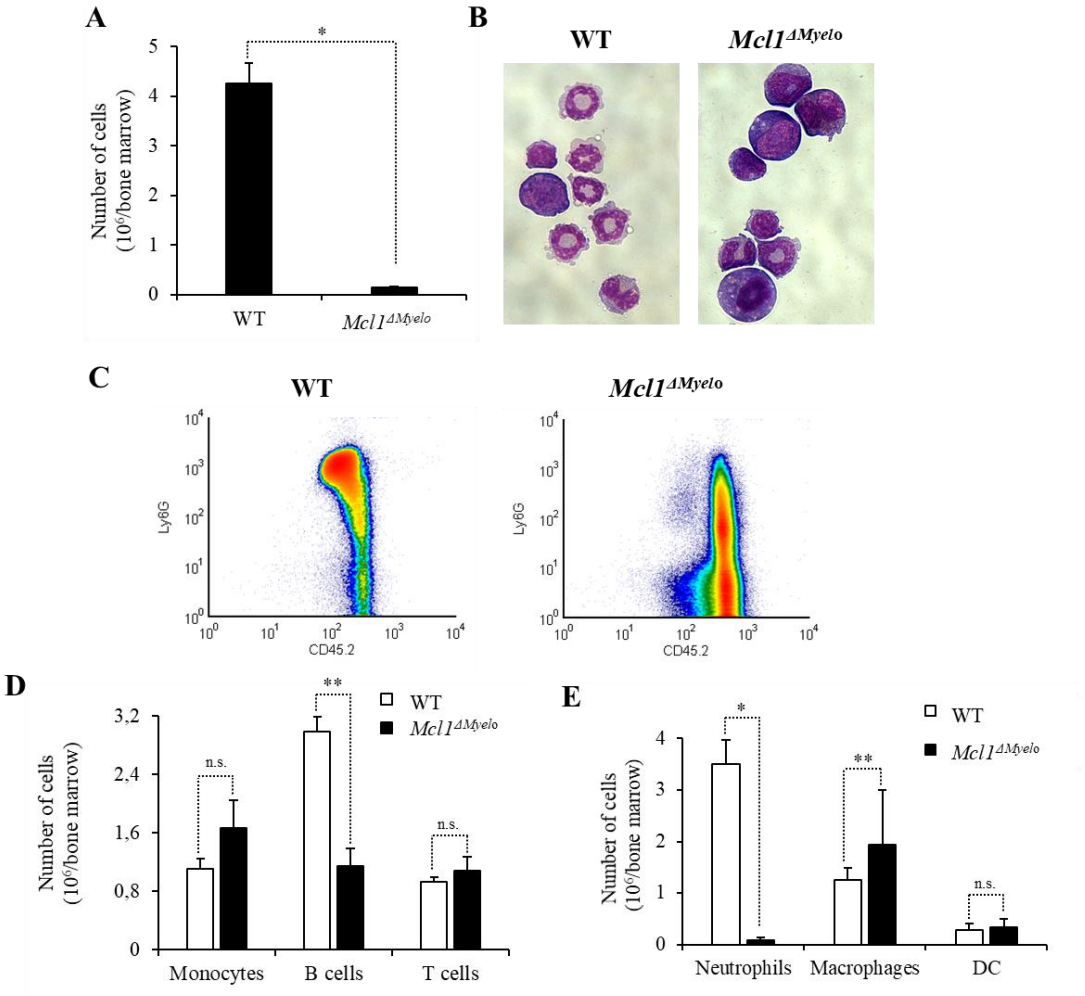

Figure 35. Leukocyte populations in the bone marrow of Mcl1$^{\Delta Myelo}$ mice. *Tissue neutrophils and other leukocytes were analysed in wild type (WT) and Mcl1$^{\Delta Myelo}$ mice from bone marrow samples. (A) Mature neutrophils were identified as CD11b^{+}Ly6G^{+} cells. (B) Cytospin image of bone marrow samples. (C) Density plots of WT and Mcl1$^{\Delta Myelo}$ mouse bone marrow cells, characterized by Ly6G and CD45 (CD45.2) staining using flow cytometry. (D) Absolute number of other leukocytes in the bone marrow. (E) Macrophages and dendritic cells (DC) from the bone marrow of WT and Mcl1$^{\Delta Myelo}$ mice. Macrophages were identified as CD45^{+}F4/80^{+} cells and DC were identified as CD45^{+}iA/B^{+}CD11c^{+} cells by flow cytometry. Bar graphs show the absolute number of leukocyte populations. Data show mean and SEM from 5-6 mice per group from 2-3 independent experiments. n.s.: not significant, *: p < 0,05, **: p < 0,05.*

Since we have found that the circulating neutrophil count in the *Mcl1$^{\Delta Myelo}$* mutants was reduced by 98.1% relative to wild type animals without substantially affecting other

circulating leukocyte numbers (36), we were interested in checking the effect of the *Mcl1*$^{\Delta Myelo}$ mutation on tissue leukocyte numbers.

Using flow cytometry, we found that the number of Ly6G-positive neutrophils in the bone marrow was strongly reduced in the *Mcl1*$^{\Delta Myelo}$ animals (96% reduction; $p=1.1\times10^{-5}$) (Fig. 35.A). This phenomenon was further confirmed by cytospin preparation and Giemsa-May-Grünwald dye staining of bone marrow cells. We could see a strong reduction in the number of cells with neutrophil-like donut-shaped nuclear morphology (Fig. 35.B).

When we further analyzed the Ly6G expression in the bone marrow, we found that although the Ly6G^{high} neutrophil population was practically absent in *Mcl1*$^{\Delta Myelo}$ mice, the immature neutrophil populations with Ly6G$^{med/dim}$ expression were not reduced, suggesting that the *Mcl1*$^{\Delta Myelo}$ mutation does not eradicate the myeloid progenitors all together, but leaves behind an early neutrophil-lineage-like compartment (Fig. 35.C.)

Next, we analysed the number of other leukocytes, as well, in the bone marrow and found that no reduction of monocytes or T cells could be observed in *Mcl1*$^{\Delta Myelo}$ mice (Fig. 35.D.; p=0.20 and 0.48, respectively). However, there was a clear reduction in bone marrow B cell counts ($p=4.0\times10^{-4}$) (Fig. 35.D), even though circulating B cell numbers were not affected (36). Other results later suggested that the reduced bone marrow B cell counts are likely due to a disturbed bone marrow B cell niche (rather than an intrinsic B cell defect) and that this bone marrow phenotype is well compensated in the periphery (36).

Further analysis of bone marrow did not reveal any difference in macrophage and dendritic cell counts between wild type and *Mcl1*$^{\Delta Myelo}$ mice either (Fig. 35.E.).

4.3.2. Leukocyte populations in the spleen of *Mcl1*$^{\Delta Myelo}$ mice

We continued the characterization of the *Mcl1*^{Myelo} neutropenia mouse model with the analysis of leukocyte populations in the spleen of the animals. As shown in Fig. 36.A., splenic neutrophil numbers were strongly reduced in *Mcl1*$^{\Delta Myelo}$ animals (93% reduction; $p=1.5\times10^{-6}$). However, the number of splenic T or B cells was not affected (p=0.77 and 0.092, respectively) (Fig. 36.B.).

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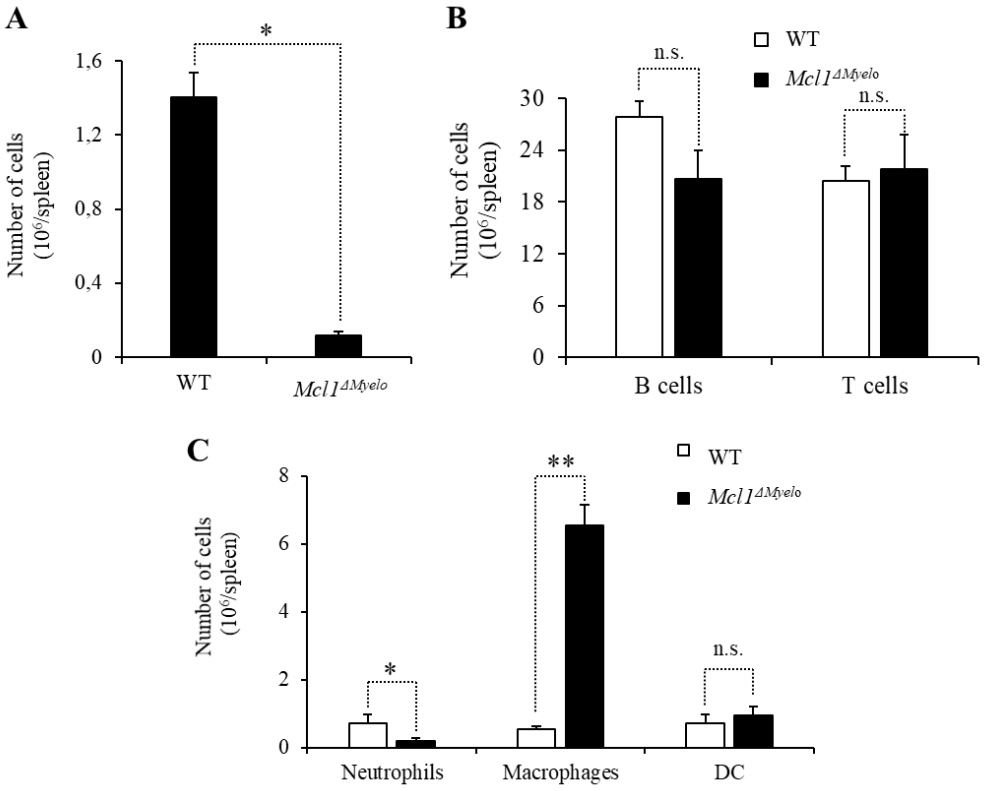

Figure 36. Leukocyte populations in the spleen of Mcl1$^{\Delta Myelo}$ mice. *Tissue neutrophils and other leukocytes were analysed in wild type (WT) and Mcl1$^{\Delta Myelo}$ mice from spleen samples. Absolute number of mature neutrophils (A), B- and T lymphocytes (B), macrophages and dendritic cells (C) in the spleen of the animals can be seen on the bar graphs. Data show mean and SEM from 5-6 mice per group from 3 independent experiments. n.s.: not significant, *: p < 0,05, **: p < 0,05*

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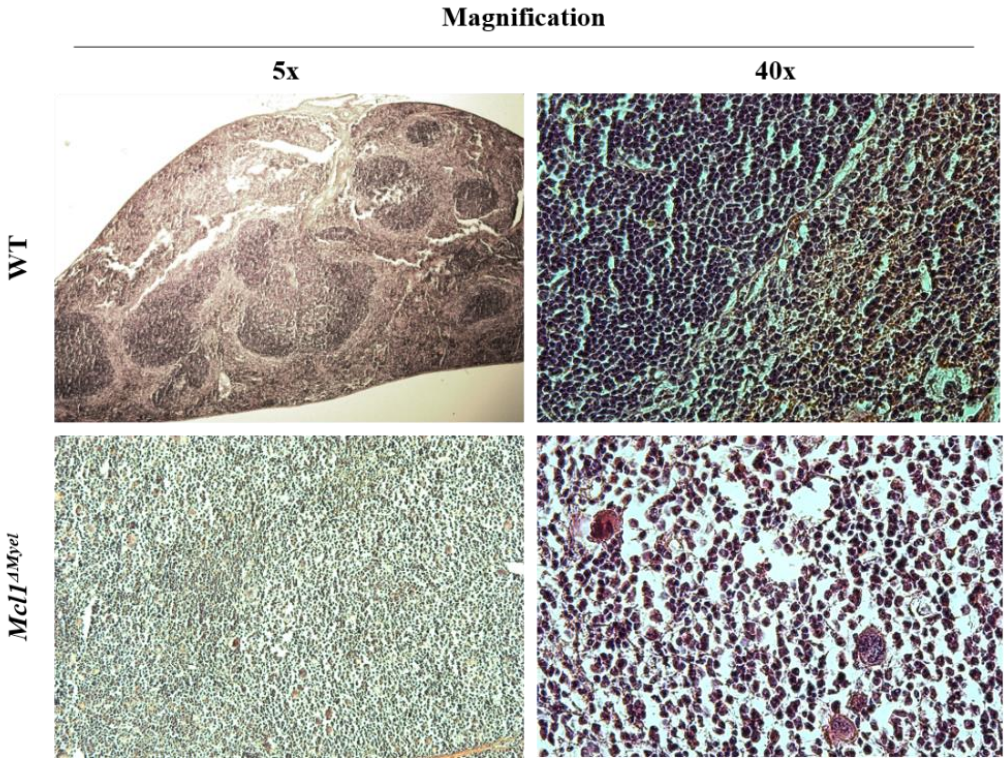

Figure 37. Microscopic images of the spleen of Mcl1$^{\Delta Myelo}$ mice. *Section of spleen was cut and stained with haemotoxylin-eosin dye. Structure of the spleen was analysed using 5x or 40x magnification on a Nikon Ni-U Upright microscope. Photos are representative of 2-3 mice per group from 1 experiment.*

We could not find any reduction in the number of splenic macrophages or dendritic cells either. On the contrary however, we detected a significant increase in the number of splenic macrophages in *Mcl1$^{\Delta Myelo}$* animals (Fig. 36.C.). We had also found that the increased macrophage numbers in the spleen correlated with the increased size of the organ in the *Mcl1$^{\Delta Myelo}$* mice (i. e. the difference disappeared after normalization for the weight of the spleen). Therefore, we believe that the increased macrophage number is related to splenomegaly of the mice (36) (Fig. 37), reflecting the fact that macrophages represent one of the predominant cell types in this organ. Splenomegaly of the mice was quite pronounced in the *Mcl1$^{\Delta Myelo}$* animals as shown on Fig. 37, and microscopic analysis also revealed that the spleen of these mice was not only bigger in size, but its classical structure was also disturbed. The clear margin between the red and white pulp of the spleen was completely missing in *Mcl1$^{\Delta Myelo}$* mice, compared to the WT control animals (Fig. 37.). However, since the overall health of the animals was not affected by the splenomegaly, we did not seek further explanation of the phenomenon.

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4.3.3. Analysis of the differentiation of macrophages from *Mcl1*$^{\Delta Myelo}$ mice in vitro

Since we found that *Mcl1* deletion caused somewhat confusing results regarding the macrophage numbers in the spleen of the animals, we have also tested the in vitro differentiation of macrophages from *Mcl1*$^{\Delta Myelo}$ bone marrow cells to test if the gene mutation had any side effects on the macrophage biology apart from the arrest in neutrophil differentiation.

We did not observe any difference between the number of bone marrow-derived macrophages generated from wild type or *Mcl1*$^{\Delta Myelo}$ bone marrow cells (Fig. 38.A.), and the morphology and F4/80 expression profile was also similar between those genotypes (data not shown).

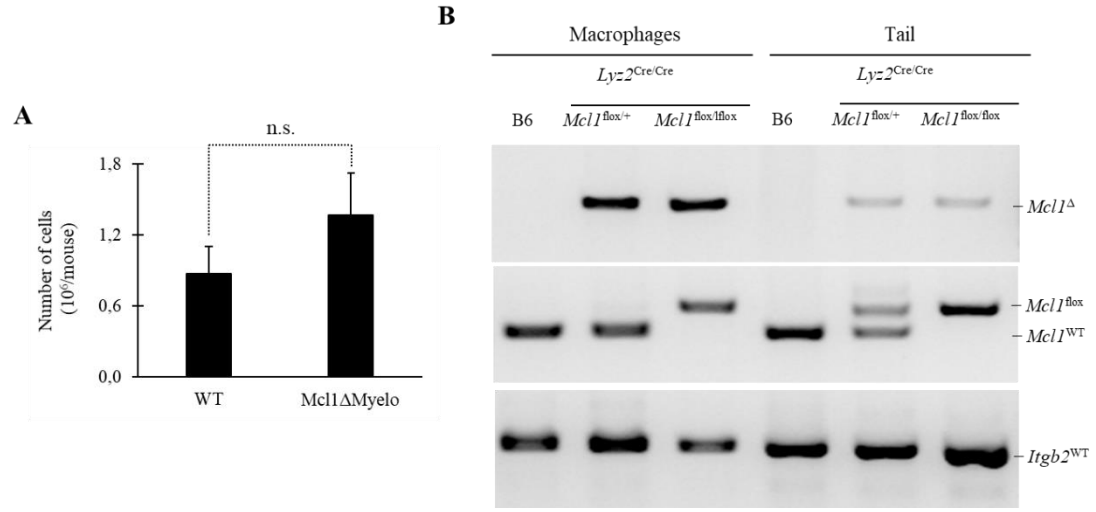

Figure 38. Analysis of bone marrow-derived macrophages. *(A) Macrophage cell counts of Mcl1*$^{\Delta Myelo}$ *bone marrow chimeras. (B) PCR-based analysis of Cre mediated deletion of the Mcl1 gene of in vitro cultured macrophages and tail samples. Mcl1*$^{\Delta}$ *indicates Cre-mediated deletion of the Mcl1*^{flox} *allele. The Mcl1*^{flox} *and Mcl1*^{WT} *PCR protocol used the same primer set with different product lengths. The Itgb2*^{WT} *PCR was used as an irrelevant control. n.s.: not significant.*

On the other hand, PCR analysis of genomic DNA confirmed deletion of the *Mcl1*^{flox} allele in bone marrow-derived macrophage cultures while only a slight deletion (likely due to the presence of tissue macrophages or osteoclasts) was seen in tail biopsy samples (Fig. 38.B.). These results indicated that the normal number and differentiation capacity of macrophages were retained even though Mcl-1 was effectively deleted from these cells as well. Because of the saturating nature of PCR reactions, the deletion of the

Mcl1^{flox} allele is best seen in the *Lyz2*$^{Cre/Cre}$*Mcl1*$^{flox/+}$ samples. Here, the almost complete lack of the *Mcl1*^{flox} PCR product indicates strong deletion of the floxed allele in macrophages, as compared to the normal presence of the *Mcl1*^{flox} allele in the genome of the mice (36). Furthermore, the presence of the *Mcl1*^{flox} PCR product in the *Mcl1*$^{\Delta Myelo}$ macrophage samples most probably reflects the fact, that *Mcl1*^{flox} allele still is present in these samples in a low amount in the absence of competing *Mcl1*^{WT} sequences, rather than the lack of deletion of the *Mcl1*^{flox} allele, which is represented by the strong *Mcl1*$^{\Delta}$ product present in those specific samples (36).

The overall normal appearance of macrophages likely reflects the fact, that Mcl-1 is only required for the survival of neutrophils but not of the cells of the monocyte/macrophage lineage, likely because those cells also express antiapoptotic proteins other than Mcl-1 (86). These results indicate that *Mcl1* deletion does not affect the overall morphology, proliferation or differentiation of macrophages, further confirming that the *Mcl1*$^{\Delta Myelo}$ mice might be a very promising tool for the analysis of the role of neutrophils in in vivo models of both normal and pathological conditions in experimental mice (36).

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5 DISCUSSION

Neutrophils have long been known to be crucially important in antimicrobial host defence mechanisms during bacterial and fungal infections. But just like the whole immune system itself, they are carrying out their functions on the edge of being extremely effective or overactivated. The latter is the reason why neutrophils are also involved in the development of various diseases like autoimmune inflammation or cancer, when the fine tuning of the immune activation is damaged (1, 3, 4, 36).

However relevant it may be in many illnesses, understanding the functional biology of neutrophils has never been an easy task. The short-lived, terminally differentiated nature of the neutrophils strongly limit our options to genetically manipulate these cells. Proper genetic analysis of neutrophils has long been only possible by analyzing neutrophils from transgenic mice carrying germline mutations, but the cost of generating and maintaining most of these mouse models are very significant. Not to mention, if in vitro analysis of the cells is needed, the inaccessibility of great number of neutrophils and their lack of suitability for tissue culturing has been the biggest challenge of the field.

During my studies I have worked on two different approaches that could serve as useful tools in neutrophil research, which could help us deepen our knowledge on the molecular machinery behind the effector mechanisms of neutrophils in various diseases.

5.1. The ER-HoxB8 progenitor cell line

5.1.1. Generation and differentiation of the HoxB8 cells in vitro

A more recent approach (5) brought about the generation of a conditionally immortalized murine myeloid progenitor cell line, the so-called HoxB8 progenitors. HoxB8 progenitor cells are cultured in vitro, and they can be differentiated into mature myeloid cells in basically unlimited numbers via a simple protocol.

We generated such HoxB8 progenitors by retroviral integration of an ER-HoxB8 fusion protein into the genome of isolated murine progenitor cells (Fig. 6.), that yielded an immortalized HoxB8 progenitor cell line growing and proliferating in estrogen containing media. We could culture these HoxB8 progenitor cells for prolonged periods without seeing changes in their phenotype or proliferating capacity. We have found that

it is advisable to follow the usual cell culturing guidelines with these close to primary cells. Meaning that as soon as a stable cell line is established after retroviral transduction (approximately 3-4 weeks), cells should be frozen very delicately in several aliquots. These aliquots can then be cultured and used for experiments up to 1,5-2 months with a 2 times/week passage rate after thawing without detecting much variability between samples during this time period.

Even though this kind of immortalization is extremely effective, the retroviral transduction likely results in a polyclonal population of the HoxB8 progenitor cells, due to several independent retroviral integrations into the genome of the progenitor cells. We suspect that this polyclonal nature of the cultures is most likely changing during the freeze-thaw cycles and long-time culturing of the HoxB8 progenitors, and it is highly likely that the most competent cells overgrow the less efficient ones over time. We have made several attempts to support this thesis of ours via detecting the number and the exact genomic position of the retroviral integration sites in various HoxB8 progenitor cell cultures and also in the single cell clones of these progenitor populations, but our results are preliminary and so far we could not confirm the polyclonal nature of the original cell culture.

When we withdrew estrogen from the culturing media of the HoxB8 progenitors, therefore switching off the exogenous HoxB8 transcription factor, progenitors started to differentiate. One of the very important properties of the HoxB8 progenitor cell line is that progenitors keep their multipotent phenotype even after retroviral transduction. It means that after the inactivation of the HoxB8 transcription factor via estrogen withdrawal, the differentiation of the progenitor cells can be guided towards various myeloid cell fates using different cytokines. Using ultimately the same protocol HoxB8 macrophages (5, 154, 155, 192, 193), HoxB8 dendritic cells (154, 155, 158, 159), HoxB8 osteoclasts (157) and also various HoxB8 neutrophil, eosinophil and basophil granulocytes (5, 155, 156, 161-164, 167, 168) can be differentiated from the progenitor cells. This was also possible in our hands as well, as we could generate HoxB8 macrophages and HoxB8 osteoclasts from the HoxB8 progenitors that we usually use to differentiate neutrophil-like cells (unpublished observation).

To guide their differentiation towards neutrophil-like cells, along with estrogen withdrawal we supplemented the culturing media with G-CSF. In 5 days, HoxB8

progenitors turned into cells with a completely different phenotype. As shown in Figs. 7, 9, 8 and 10, the differentiation of HoxB8 progenitors resulted in the emergence of neutrophil-like cells, the HoxB8 neutrophils, which had the classical segmented, ring-shaped nuclei, they lost hematopoietic stem cell-specific CD117 (c-kit) marker expression. We and others (164) have also confirmed that HoxB8 neutrophils highly expressed many neutrophil-specific cell surface markers like CD45, Ly6G, CD11a, CD11b, CD18 integrin chains, FcγRII/III and FcγRIV as a result of their maturation. The lifespan of HoxB8 neutrophils even reflected that of normal circulating neutrophils as they reached their close-to mature stage 5 days after initiation of differentiation and stayed alive for approximately 8 days.

5.1.2. In vitro functions of HoxB8 neutrophils

Apart from the cells' phenotypical characterisation, we tested various functional responses of the HoxB8 neutrophils in vitro. We could confirm the previously published observation (164-166) and it is shown in Fig. 11.A-B., that HoxB8 neutrophils were able to produce reactive oxygen species upon a less specific stimulation (PMA), as well as through an immobilized immune complex-dependent manner which require activation through cell surface FcγRs.

HoxB8 neutrophils consistently showed the capacity of chemotaxis (161-163, 165). We have also demonstrated that they could migrate for example towards fMLP and the MIP-2 chemokine (Fig.11. C-D.), indicating that HoxB8 neutrophils likely have various cytokine and chemokine receptors on their surface, which usually drive neutrophil movement from the circulation towards peripheral tissue during invasion by pathogens.

It was previously published, that HoxB8 neutrophils show phagocytic activity towards the yeast cell particle zymosan (161, 165). We have also confirmed this activity using fluorescently labelled *Candida albicans* particles (Fig. 11. F), and we were able to demonstrate, that HoxB8 neutrophils could also show extensive phagocytosis of bacterial (Fig. 11.E) particles in vitro as well. To further expand our knowledge on the phagocytic activity of HoxB8 neutrophils, we tested whether this effector function of the HoxB8 cells depends on the pre-opsonization of the pathogenic particles. We found that the in vitro phagocytic action significantly increased when pathogens of various kind were opsonized by serum proteins in advance. This further demonstrates that HoxB8 neutrophils likely

have various functionally active Fc- and complement receptors, and signalling machinery, which drive pathogen elimination very similarly to circulating neutrophils.

HoxB8 progenitors stayed inactive in all the above-mentioned functional experiments in vitro, indicating that the above processes were indeed related to neutrophilic differentiation of the HoxB8 progenitors.

Taken together, our in vitro experiments indicated that HoxB8 neutrophils differentiated from HoxB8 progenitors in our hands are able to perform numerous functional responses typical of freshly isolated human or mouse neutrophils. This is in line with prior reports on the in vitro functions of these cells (163-165).

5.1.3. Generation of HoxB8 chimeras and differentiation of HoxB8 neutrophils in vivo

We next decided to focus our work on the in vivo characterization of HoxB8 neutrophils. Since we found that the in vitro cultures likely provided suboptimal conditions for proper neutrophilic differentiation, as it can be seen on the Ly6G expression of in vitro-derived HoxB8 neutrophils (Figs. 8 and 12), we were concerned that the in vitro experiments might not be completely accurate to analyze the functional activity of HoxB8 neutrophils. But most importantly, we were very interested in whether the HoxB8 neutrophils can be studied in vivo at all, considering the fact that, according to our knowledge, there is no cell line currently available, which could differentiate into mature, active neutrophils both in vitro and in vivo.

Using the HoxB8 cells in vivo is a quite rare approach according to the literature. Some have tried to fluorescently label and inject in vitro differentiated HoxB8 neutrophils into intact mouse (161, 168) and they could follow their journey in the body until they undergone cell death. But this method is not suitable to characterize these cells extensively. Others transplanted HoxB8 progenitors in combination with whole bone marrow cell suspension into lethally irradiated recipient mice (155, 166, 167), and they found that HoxB8 neutrophils were able to differentiate in vivo from the injected HoxB8 progenitors. However, no one ventured to study the effector functions of these HoxB8 cells in vivo. This suggested to us, that the Hoxb8 cells might provide a good tool to study neutrophils in vivo.

To this end, we came up with the idea to replace the neutrophil compartment of mice into HoxB8 neutrophils, via injecting HoxB8 progenitors only, into lethally irradiated recipient mice. These animals are called the HoxB8 chimeras. Very importantly, using this approach the neutrophil compartment of HoxB8 chimeras consisted practically exclusively of HoxB8 progenitor-derived cells by day 5 after the irradiation and (first) transplantation (Figs. 14, 16, 17, 18.B., 27.B., 32). These HoxB8 neutrophils could be present in the circulation up to 2-3 days. We generated HoxB8 chimeras using both single (Fig. 13.) or repeated (Figs. 18.A. and 27.A.) injections of HoxB8 progenitors into lethally irradiated recipient mice, as well. We chose the multiple transplantation approach when we wished to test the functional responses of HoxB8 neutrophils in vivo for a longer period of time.

A key point in our experimental setup was to use lethally irradiated mice as recipients, since we found that HoxB8 progenitors were unable to engraft non-irradiated recipients (Fig. 14.). Our explanation on this was that HoxB8 progenitors mostly likely need to occupy certain parts of the hematopoietic niches for their proper differentiation and these niches are preoccupied by recipient-derived cells in non-irradiated recipients. The preoccupation of the right niche might prevent the survival/differentiation of the donor HoxB8 progenitors in the bone marrow. Our mixed bone marrow chimeric experiments further confirmed this hypothesis, as we could see competition between normal bone marrow-derived hematopoietic cells and HoxB8 progenitors (Fig. 15.C.) when they were co-injected into the recipients. So, our approach to generate HoxB8 chimeras yielded much higher number of circulating HoxB8 neutrophils compared to studies where HoxB8 progenitors and bone marrow cells were injected in combination (155, 166, 167). Moreover, we were able to generate animals that have HoxB8-derived neutrophils only in their circulation for a prolonged period, making it highly possible to study the effector functions of these cells in vivo, without having to worry about other bone marrow-derived cells masking the functions of HoxB8 neutrophils.

Flow cytometric analyses revealed that HoxB8 neutrophils were able to replace the recipient neutrophil population in the circulation of HoxB8 chimeras as well as in other hematopoietic tissues (Figs. 16, 17.), and their overall number reached approximately 40% of the original neutrophil counts. The HoxB8 neutrophil cell counts

could be maintained in the circulation for prolonged periods via the repeated transplantation of HoxB8 progenitors (Fig. 18, 27.B., 32.).

One of our key findings was that the consistently lower Ly6G expression in in vitro generated HoxB8 neutrophils 5 days after estrogen removal (Fig. 12.), was completely missing in donor-derived neutrophils of HoxB8 chimeras on Day 5 after transplantation (Fig. 19.), and in vivo differentiated HoxB8 neutrophils seemed to reach complete maturation.

HoxB8 neutrophil cell surface receptor expression in vivo was very similar to HoxB8 neutrophils differentiated in vitro (compare Fig. 10 with Fig. 19.). However, certain functional receptors (CD11b, FcγRII/III, FcγRIV) showed upregulation in HoxB8 neutrophils of the HoxB8 chimeras compared to neutrophils of intact animals (Fig. 19 and 20.). This finding, however, is likely related to a proinflammatory post-irradiation environment (194), also observed when normal bone marrow cells were injected into lethally irradiated recipients (Fig. 20.) (185). This slightly primed state of circulating HoxB8 neutrophils may also contribute to the normal or even enhanced inflammatory reaction of HoxB8 chimeras in the K/B×N serum-transfer arthritis model (Fig. 28.) even though the overall neutrophil count in the HoxB8 chimeras was only approximately half of that in intact mice (Fig. 27.B.).

Extended analysis of diverse hematopoietic lineages confirmed our original assumption that HoxB8 progenitors were only able to efficiently restore the neutrophils but not other leukocyte lineages in vivo (Figs. 21 and 22.). This feature is an important limitation of our approach since the lack of various leukocyte cell types could make the interpretation of complex inflammatory experiments with HoxB8 chimeras somewhat difficult later. To overcome this issue, we tried to generate HoxB8 chimeras using non-irradiated *Mcl1*$^{\Delta Myelo}$ neutropenic mice as recipients. However, HoxB8 progenitors were unable to differentiate into HoxB8 neutrophils in these animals, most likely due to the previously mentioned aspects of an occupied bone marrow niche by recipient cells (data not shown). On the other hand, having HoxB8 neutrophils only in the HoxB8 chimeras can be a great asset, as well, because using these chimeras we could carry out experiments in a very neutrophil-specific manner.

During our studies we have found that not all of the HoxB8 progenitor populations, generated separately at different time points in the last 2-4 years, are able to

differentiate into HoxB8 neutrophils in vivo. Some of the HoxB8 progenitor cultures did not differentiate in vivo at all, while others yielded very high numbers of neutrophils. Moreover, at very rare occasions, the in vivo differentiation of the HoxB8 progenitor cells resulted in monocyte-like or eosinophil-like cell populations. This diverse differentiation profile of various separate HoxB8 progenitor cultures might come from the random nature of the retroviral integration during the generation of the immortalized cells. However interesting may this be, we did not design or execute further experiments to uncover the possible mechanisms behind this phenomenon. Rather we continued our work using only those HoxB8 progenitor populations that demonstrably yielded neutrophil-like cells in vivo.

Another limitation of the HoxB8 chimera system is that keeping HoxB8 chimeras alive and using them in long-term experiments requires multiple transplantations. We believe that it would be difficult to continue this procedure for a substantially longer time than what we had shown on Figs. 27, 28, 32 and 34, without negative effects on the overall health of the mice. The wasting phenotype of irradiated, non-transplanted mice indicates that without sufficient number of red blood cells and platelets in the circulation, the mice cannot survive for significantly longer than 7-8 days (Fig. 22.). The fact that HoxB8 chimeras stayed alive for longer than that suggests, that even though lethal irradiation basically killed every cell in the circulation, the donor-derived HoxB8 neutrophils were able to save chimeras from premature death, for at least approximately one more week.

5.1.4. In vivo functions of HoxB8 neutrophils

A very unique part of our study compared to previously published observations on HoxB8 neutrophils is that the generation of HoxB8 chimeras allowed us to perform various single-cell-based (migration- Fig.23 and phagocytosis- Fig.24.) and whole population-based short-term (reverse passive Arthus reaction; Figs. 25 and 26.) and long-term (K/B×N serum-transfer arthritis; Figs. 28 and 34.) in vivo experiments. All of these functional assays further indicated that HoxB8 neutrophils differentiated in vivo are able to carry out multiple functional responses characteristic of circulating neutrophils. HoxB8 neutrophils can migrate into inflamed tissues (e.g.: peritoneum, ear, joints) and perform either immunological functions like phagocytosis, or due to their strong activation, they further promote the development of the inflammatory phenotype itself.

Comparing the functional activity of WT and *Syk*$^{-/-}$ HoxB8 neutrophils in a complex inflammatory response emphasized the potential of the HoxB8 cell line in neutrophil research. One of the biggest advantages of the HoxB8 system is that HoxB8 progenitors can be generated basically from any mouse bone marrow or fetal liver sample irrespective of their genotype. Even those genotypes could be studied, via recreating them in the HoxB8 cell format, which are known to be difficult breeders or suffer from early lethality. Not to mention the possibility of genetic manipulation to create a neutrophil-specific gene deletion in general, using the CRISPR-Cas9 system on the in vitro cultured HoxB8 progenitor cells instead of the generation of germ line mutations in mouse embryos. The genetic manipulation of the HoxB8 progenitors in vitro seems to be quite challenging as they are close to primary cells, but others (157, 193) and we have also been successful to manipulate the genome of the HoxB8 progenitors using lentiviral vectors targeting multiple genes (unpublished observations of Anita Orosz and Attila Mócsai).

Taken together, our experiments indicate that we are able to generate HoxB8 neutrophils of various genotypes both in vitro and in vivo. It is technically possible to establish and maintain circulating neutrophil populations derived from previously in vitro cultured donor HoxB8 progenitors in HoxB8 chimeras. Moreover, HoxB8 neutrophils seem to be able to perform majority of neutrophil-specific effector functions (Fig. 1.) both in vitro and in vivo. Therefore, the ER-HoxB8 cell line could be a very promising and useful approach for neutrophil function-related studies. Furthermore, generation of HoxB8 chimeras would very reliably allow the rapid testing of the effects of genetic modifications of HoxB8 progenitors under in vivo condition, as well.

5.2. Characterisation of *Mcl1*$^{\Delta Myelo}$ neutropenic mouse strain

During my studies, I have also worked on another approach that could serve as a useful tool in studying neutrophil cell biology. One commonly used approach to study the role of a cell type is to deplete the cell type of interest in vivo in order to characterize the phenotypical and functional outcome.

Neutrophil deletion can be achieved by various genetic modifications resulting in neutropenic mouse strains. Even though there are several neutropenic mouse models available, most of them have very serious limitations, like only partial deletion of the neutrophil population, extended genetic disturbance in various leukocyte lineages,

difficult population maintenance and low breeding efficiency, etc. Therefore, there was no highly accurate neutropenia mouse model available before the generation of the *Mcl1*$^{\Delta Myelo}$ mice (36).

Our results indicate that *Mcl1*$^{\Delta Myelo}$ mice lacking *Mcl1* in the myeloid lineage are severely neutropenic but survive and breed in homozygous form (36). We have found that drastic reduction in neutrophil numbers in *Mcl1*$^{\Delta Myelo}$ mice is not exclusive to the circulation but can also be detected in other hematopoietic tissues like the bone marrow or spleen of the animals (Figs. 35.A and 36.A.). It was also revealed that *Mcl1* deletion in neutrophils cause neutropenia in the animals most likely because it leads to incomplete maturation of neutrophils in *Mcl1*$^{\Delta Myelo}$ mice, as premature neutrophils could be observed in the bone marrow of these animals (Fig. 35. B-C.) (36).

Detailed analysis of bone marrow and splenic tissues revealed that in comparison to other neutropenia mouse models (e.g.: G-CSF receptor and G-CSF knock out mice (72, 73, 75) or Cxcr2 knock out mice (76)), the deletion of *Mcl1* in *Mcl1*$^{\Delta Myelo}$ mice did not affect the cell numbers of other leukocyte populations (Figs. 35. D-E. and 36. B-C.). The slight reduction in B-cell numbers in the bone marrow is likely due to a disturbed bone marrow B-cell niche which becomes well compensated once B-cells get to the periphery (36). It should also be noted that an increased number of splenic macrophages (Fig. 34.C.) was observed in *Mcl1*$^{\Delta Myelo}$ mice which likely reflects the splenomegaly in those animals (Fig. 35) However, it does not affect neither the in vitro nor the in vivo differentiating capacity of macrophages derived from the bone marrow of *Mcl1*$^{\Delta Myelo}$ mice (Fig. 36).

Taken together, our results indicate that the *Mcl1*$^{\Delta Myelo}$ neutropenia mouse strain is highly suitable for the analysis of the role of neutrophils in normal and pathological processes in experimental mice.

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6 CONCLUSIONS

According to the objectives presented previously, I am summarizing my conclusions in 5 points:

1. We were able to generate HoxB8 progenitors of various genotypes both from progenitor cells derived of the bone marrow or fetal liver of mice. We could not detect any phenotypical differences between HoxB8 progenitors of different origin. HoxB8 progenitor cells could be kept in culturing media supplemented with SCF and estrogen, for long periods of time. During culturing, HoxB8 progenitors were able to divide and proliferate in a rapid manner, without significant apoptosis along the way.

2. With the right culturing protocol (estrogen withdrawal and G-CSF addition to the culture), HoxB8 progenitors differentiated into HoxB8 neutrophils in my hands. We could generate basically unlimited numbers of HoxB8 neutrophils in vitro. HoxB8 neutrophils differentiated in vitro resembled neutrophils originating from WT mice both phenotypically and functionally.

3. We were able to generate HoxB8 chimeras for the in vivo characterization of the HoxB8 cells. HoxB8 progenitors differentiated into mature neutrophil-like cells (the HoxB8 neutrophils) with a strong, convincing neutrophilic nature. The number of HoxB8 neutrophils could be kept steady for up to 10 days with repeated transplantation of HoxB8 progenitors into lethally irradiated recipients.

4. HoxB8 neutrophils could develop all tested functional properties of circulating neutrophils both at a single-cell and whole neutrophil population-based level. Their functional activity was comparable to neutrophils of intact animals, and they even contributed to the development of complex inflammatory conditions, as well.

5. The deletion of *Mcl1* does not affect the cell number of the majority of the leukocyte populations in the bone marrow and the spleen of $Mcl1^{\Delta Myelo}$ mice, except for a drastic reduction in neutrophil counts.

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7 SUMMARY

Even though neutrophils play an important role in immunity and inflammation, their extensive analysis is greatly hindered by their short lifespan and terminally differentiated nature. According to prior studies, a conditionally immortalized myeloid progenitor cell line could be established via the overexpression of the HoxB8 transcription factor. These so called HoxB8 progenitors can be cultured in estrogen-containing media, followed by differentiation towards neutrophils upon estrogen withdrawal and G-CSF treatment. We confirmed that in vitro generated HoxB8 neutrophils showed a neutrophil-like phenotype and were able to perform various neutrophil-specific functions in vitro. However, little is known about their capacity to perform in vivo neutrophil-like functions, so I focused my attention to address this issue by an in vivo transplantation approach. Intravenous injection of HoxB8 progenitors into lethally irradiated recipients yielded a well-defined population of donor-derived HoxB8 neutrophils in the circulation of HoxB8 chimeras. Repeated injection of HoxB8 progenitors allowed us to maintain stable circulating HoxB8 neutrophil counts for several days. In vivo differentiated HoxB8 neutrophils were phenotypically similar to neutrophils in the circulation of intact mice. HoxB8 neutrophils could migrate to the inflamed peritoneum and could carry out phagocytosis of heat-killed Candida particles. Complex inflammatory responses like the reverse passive Arthus reaction or K/B×N serum transfer arthritis could also be induced in HoxB8 chimeras. The functional responsiveness of HoxB8 neutrophils were further confirmed by subjecting *Syk*$^{-/-}$ HoxB8 chimeras to neutrophil-specific inflammatory experiments as well, where *Syk*$^{-/-}$ HoxB8 chimeras were completely protected compared to their wild type counterparts. Taken together, our results indicate that the HoxB8 system could provide a great model for the in vivo analysis of neutrophil function.

Another approach to investigate the biological importance of neutrophils in various diseases could be to use the *Mcl1*$^{\Delta Myelo}$ neutropenia mouse model that I helped to characterise as well. It might be one of the best neutropenia mouse models as we could confirm an impressive 98% reduction of neutrophils in the circulation, and substantial neutrophil deficiency in the bone marrow and spleen of the animals, while the number of other leukocyte populations were not substantially affected.

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8 ÖSSZEFOGLALÓ

Régóta ismert tény, hogy a neutrofil granulociták a normál immunválasz és a kóros gyulladás kialakulásának egyaránt fontos szereplői. Bár számos megközelítés létezik, vizsgálatuk mégis korlátozott a sejtek rövid élete és terminálisan differenciált volta miatt. Az egyik lehetséges megoldást a neutrofilek vizsgálatához az úgynevezett HoxB8 immortalizált mieloid sejtvonal alkalmazása jelentheti. Ezek a HoxB8 progenitor sejtek ösztrogén jelenlétében tenyészthetőek, ösztrogén elvonás hatására pedig neutrofil-szerű sejtekké differenciálódnak. Munkánk során igazoltuk, hogy a HoxB8 progenitorokból in vitro körülmények között differenciáltatott HoxB8 neutrofilek fenotípusukban és funkcióikat tekintve is nagyon hasonlóak a keringő neutrofilekhez. A HoxB8 neutrofilek in vivo vizsgálatához kidolgoztuk az ún. HoxB8 kiméra állatok létrehozásának módszerét, amelynek lényege, hogy letálisan besugarazott recipiens egerekbe injektáltunk in vitro tenyésztett HoxB8 progenitorokat. A donor sejtekből HoxB8 neutrofilek differenciálódtak in vivo. A HoxB8 neutrofilek száma a HoxB8 progenitor sejtek többszöri injektálásával hosszabb távon is fenntarthatónak bizonyult. A recipiensek keringésében megjelenő HoxB8 neutrofilek fenotípusukban teljesen hasonlítottak a keringő neutrofilekhez. A HoxB8 neutrofilek képesek voltak gyulladás hatására kilépni a keringésből és a szövetek közé vándorolni. Továbbá fagocitálták a periférián található *Candida albicans* kórokozót is. A HoxB8 neutrofilek kiemelten fontosnak mutatkoztak olyan, a teljes HoxB8 neutrofil populációt érintő bonyolult gyulladásos folyamatok kialakításában is, mint a K/B×N szérum transzfer arthritisz modell vagy a reverz passzív Arthus reakció. A K/B×N szérum transzfer artritisz modellben ugyanakkor a Syk hiányos HoxB8 neutrofileket tartalmazó $Syk^{-/-}$ HoxB8 kimérák teljesen védettnek bizonyultak. Ezen eredmények tovább erősítik azt az elképzelést, hogy a HoxB8 neutrofilek kiváló eszközei lehetnek a neutrofilek működésében fontos mechanizmusok feltárásának.

Munkám során részt vettem az $Mcl1^{\Delta Myelo}$ neutropéniás egértörzs jellemzésében is, amely a HoxB8 sejtekhez hasonlóan nagyon hasznos módszer lehet a neutrofilek vizsgálatához. A $Mcl1^{\Delta Myelo}$ egerek keringésében és más nyirokszervében is 98%-os neutrofilszám csökkenést tapasztaltunk. Ugyanakkor a neutrofil-specifikus géntörlés, nem okozott jelentős változást más fehérvérsejt populációk méretében és működésében.

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II. Csepregi JZ*, **Orosz A***, Zajta E, Kása O, Németh T, Simon E, Fodor S, Csonka K, Barátki BL, Kövesdi D, He YW, Gácser A, Mócsai A. (2018) Myeloid-specific deletion of Mcl-1 yields severely neutropenic mice that survive and breed in homozygous form. *J Immunol,* 201: 3793-3803. (**shared first authors*)

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I. Ayoglu B., Szarka E, Huber K, **Orosz A,** Babos F, Magyar A, Hudecz F, Rojkovich B, Gáti T, Nagy Gy, Schwenk J, Sármay G, Prech J, Nilsson P, Papp K (2014) Bead arrays for antibody and complement profiling reveal joint contribution of antibody isotypes to C3 deposition. *PLos ONE,*9(5): e96403

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11 DIVISION OF RESULTS BETWEEN CO-AUTHORS

1. Orosz et al. *J Immunol*, 206 (2): 432-445

There are no shared results between co-authors. The initial HoxB8 cell line and the plasmids used for the generation of the immortalised HoxB8 progenitors are from the laboratory of Barbara Walzog. I designed, performed and analysized all of the experiments shown. The project was supervised by Attila Mócsai.

2. Csepregi et al. *J Immunol* 2018, 201: 3793-3803

The majority of the experiments were conducted by Janka Zsófia Csepregi. I was responsible for the analysis of bone marrow and spleen samples to characterise various leukocyte cell populations in the mouse model. I performed sample preparation and flow cytometric measurements of the bone marrow and spleen samples of the mice and performed citospin experiments. Spleen histology samples, staining and microscopic analysis was performed by myself.

I carried out in vitro macrophage experiments and designed PCR probes for the extended analysis of gene deletion in the $Mcl1^{\Delta Myelo}$ mice. The project was supervised by Attila Mócsai.

Figures in the 'Results' section of this thesis do not occur in any other dissertations.

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