THE IMPACT OF CD44 AND OSTEOPONTIN ON HEMATOPOIESIS

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Zusammenfassung (Abstract)

Abstract

Hematopoiesis is the process, which enables the replenishment of blood cellular components in mammals throughout their lifespan. Hematopoietic stem cells (HSC), which reside within the bone marrow (BM), are the primary progenitor unit for the production of myeloid and lymphoid blood cell populations within the living organism. Balance disturbances between self-renewal and differentiation in HSCs, which may occur in case of mutations, can turn healthy hematopoiesis into hematological diseases. Mutations in a huge variety of genes in HSCs have been analyzed by scientists over decades, in order to find treatment angles for severe blood disorders. Within this PhD thesis, the impact of two genes (CD44 and osteopontin) on hematopoiesis has been investigated. CD44 encoding a cell-surface glycoprotein involved in cell-cell interactions, is expressed by HSCs while osteopontin (OPN) is abundantly present in the HSC niche as an extracellular matrix protein. Within the pre-experimental work of this thesis, CD44 and OPN double deficient mice ($CD44^{-/-}$:$OPN^{-/-}$) have been successfully bred by mating of $CD44^{-/-}$ and $OPN^{-/-}$ single deficient mice. The resulting offspring being $CD44^{-/-}$:$OPN^{-/-}$, $CD44^{+/-}$:$OPN^{+/-}$ and $OPN^{-/-}$ have been examined in their peripheral blood and BM composition in comparison to wild-type mice ($CD44^{+/+}$:$OPN^{+/+}$), which possess fully functional CD44 and OPN genes, under steady-state and stress-state experimental conditions. Steady-state conditions define analyses of blood and BM composition of mice under unaffected living conditions during their whole lifespan (3-21 months), whereas stress-state conditions are represented by the impact of a serial stem cell transplantation, G-CSF and Poly(I:C) stress. Blood and BM status of mice have been investigated with the BC-5000-Vet blood analyzer (Mindray) and by Fluorescent-activated cell-sorting (FACS; BD FACS Canto II). Steady-state results reveal elevated thrombocyte and leukocyte cell counts in the peripheral blood of $CD44^{-/-}$:$OPN^{-/-}$ and $OPN^{-/-}$ mice during their lifespan, especially in the older cohorts, while the erythrocyte blood values are only elevated in young $OPN^{-/-}$ and old $CD44^{-/-}$:$OPN^{-/-}$ mice. $CD44^{-/-}$:$OPN^{-/-}$ mice display higher B- and T cell levels in their peripheral blood than wild-types in the majority of the observed age cohorts. Furthermore, old $CD44^{-/-}$:$OPN^{-/-}$ mice exhibit reduced granulocyte- and Lin^{-} $cKit^{+}$ $Sca1^{+}$ (LKS) numbers within the BM, whereas the size of the HSC pool in young and old $CD44^{-/-}$:$OPN^{-/-}$ mice is unaltered

referring to controls. OPN-deficiency enhances HSC proliferation in BM of young mice and shows a tendency of enhanced MPP proliferation in BM of old mice. In a serial stem cell transplantation young $CD44^{-/-}$:$OPN^{-/-}$-BM-recipient cells fail to adequately reconstitute thrombocyte, leukocyte and erythroid replenishment in the peripheral blood resulting in hematopoietic failure at 16 weeks after 3rd round of transplantation shown by the premature death of 3 out of 10 mice while all 10 out of 10 wild-type-BM-recipient mice (controls) have survived. G-CSF treatment lowers blood T cell levels in wild-type, $CD44^{-/-}$:$OPN^{-/-}$ and $OPN^{-/-}$ mice, reduces blood monocyte levels in $OPN^{-/-}$ mice and elevates blood granulocyte levels in wild-type and $CD44^{-/-}$:$OPN^{-/-}$ mice at 6 days after treatment. Thrombocyte levels in the peripheral blood of wild-type and $OPN^{-/-}$ mice have been reduced under G-CSF stress, while those of $CD44^{-/-}$:$OPN^{-/-}$ mice have not been affected. Therefore, $CD44^{-/-}$:$OPN^{-/-}$ mice might have an impaired thrombocyte function. Furthermore, G-CSF treatment does not alter LKS, hematopoietic progenitor cell (HPC), HSC and MPP numbers in BM of $CD44^{-/-}$:$OPN^{-/-}$ mice. Leukocytes in the peripheral blood of $CD44^{-/-}$:$OPN^{-/-}$ mice react with different intensity to the viral-infection simulation of Poly(I:C) compared to wild-type leukocytes, implicating an impaired immune response capability of $CD44^{-/-}$:$OPN^{-/-}$ mice. Poly(I:C) increases HSC proliferation in BM of $OPN^{-/-}$ mice and MPP proliferation in BM of $CD44^{-/-}$:$OPN^{-/-}$ mice in comparison to untreated controls, underlining an impaired immune response of the referring mice. Summarizingly, the double-deficiency of CD44 and OPN in HSCs has a profound impact on blood and BM cell populations of the referring mice under steady- and stress-state conditions.

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Authentizitätserklärung **III**

Ich versichere, diese Arbeit selbstständig und nur unter Verwendung der angegebenen Quellen und Hilfsmittel angefertigt zu haben. Sollten andere Personen hier gezeigte Ergebnisse beigetragen haben, so wird ausdrücklich darauf hingewiesen.

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List of Abbreviations:

abbrevation	meaning
AML	acute myeloid leukaemia
BM	bone marrow
bp	base pairs
C57Bl/6	inbred mouse strain (black six)
CAM	cell adhesion molecule
CD44	50kB sized highly conserved gene
CD44s	standard CD44 Isoform
CD44v	variant isoform of CD44
CFU_c	Colony-forming unit culture
CFU_s	spleen colony-forming unit
CIC	Cancer-initiating cell
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
CXCL12	Chemokine protein (synonymous for SDF1)
$CD44^{-/-}$:$OPN^{-/-}$	double knockout mouse lacking the CD44 and Osteopontin gene
ECM	extracellular matrix
Endosteum	connective tissue lining the inner surface of the bone
FACS	fluorescence-activated cell sorting
GMP	Granulocyte-macrophage progenitor
HET	Haus Für Experimentelle Therapie (Uniklinikum Bonn)
HPCs	hematopoietic progenitor cells
HSC	hematopoietic stem cell
HSCs	hematopoietic stem cells
HSPC	Hematopoietic stem and progenitor cells
integrins	transmembran receptors, that faciliate cell-extracellular matrix adhesion
ip	intraperitoneal
kB	kilo bases
kDA	kilo Dalton
LIC	leukaemia-initiating cells
LKS	Lineage negative, c-kit and Sca1 positive cells
LT-HSCs	long-term hematopoietic stem cells

MEP	Megakaryocyte-erythroid progenitor
MMPs	matrix metalloproteinases
MPP	multipotent progenitors
m-RNA	messenger RNA
OPN	osteopontin
osteoblasts	cells with a single nucleus, which synthesize bone
PBLs	peripheral blood lymphocytes
PCR	polymerase chain reaction
Poly I:C	polyinosinic:polycytidylic acid
RBC	red blood cells
SDF-1	stromal cell-derived factor-1
sialic acid	derivative of neuraminic acid
SIBLINGs	Small Integrin-Binding LIgand N-linked Glycoproteins
ST-HSC	short-term hematopoietic stem cell
v	variant

1. Introduction

1.1 Hematopoiesis

The lifespan of mature blood cells is relatively short (from a few hours to over 100 days), therefore they have to be replenished continuously during the lifetime of an organism (Orkin and Zon, 2008; Rieger and Schroeder, 2012; Seita and Weissman, 2010). The process of blood cell renewal is called hematopoiesis (Jagannathan-Bogdan and Zon, 2013). During embryonic development and throughout adulthood of the referring vertebrate, hematopoiesis ensures production and replenishment of blood cells (Jagannathan-Bogdan and Zon, 2013). Being a highly dynamic developmental process, hematopoiesis is shaped by hematopoietic stem cells (HSCs), which are able to perform self-renewal and differentiation into all mammalian blood cell types (Hu and Shilatifard, 2016; Seita and Weissman, 2010). HSCs reside in small numbers in the bone marrow (BM) in adult mammals. They are the origin of blood cell supply (Orkin, 2000).

Blood has several functions, which are essential for the metabolism of an organism: transport (of gases, nutrients, waste products and hormones), regulation of cell surrounding fluid, reduction of injury induced fluid loss and regulation of body temperature and immunity defenses (Weiss and Jelkmann, 1989). Blood is composed of the pale yellow plasma (called serum when the fibrinogen is removed) and three different kinds of corpuscles, which are suspended within it: red corpuscles (erythrocytes), white corpuscles (leukocytes) and platelets (thrombocytes) (Weiss and Jelkmann, 1989). Blood function is maintained by blood cell populations, which perform their duties acting highly specific (Weiss and Jelkmann, 1989).

Red blood cells (erythrocytes) are responsible for the oxygen and carbon dioxide transport between tissues and lung (Rieger and Schroeder, 2012).

Erythropoiesis is the process, which sustains the red blood cell (RBC) life cycle (Daugas et al., 2001). In the red bone marrow, erythroid progenitors differentiate from proerythroblasts to polychromatophylic erythroblasts and orthochromatic erythroblasts till they finally reach their fully developed mature erythrocyte state (Daugas et al., 2001).

White blood cells (leukocytes) are cells, which attack foreign antigens and help to protect the organism from pathogens in order to prevent infections (Fathima and Khanum, 2017). Leukocytes are divided into five subgroups: neutrophil-, eosinophil- and basophil granulocytes, monocytes and lymphocytes (B cells, T cells and natural killer (NK) cells) (Fathima and Khanum, 2017). The process, in which white blood cells are produced and regulated, is called leukopoiesis (Adimy et al., 2006).

1.1.1 Hematopoiesis in mice

In mice, embryonic hematopoiesis starts from the mesoderm, progresses to the extraembryonic yolk sac to produce primitive erythrocytes and some myeloid cells around embryonic day 7.5 (E7.5) (Medvinsky et al., 2011). After this first hematopoietic wave, HSCs are generated in the aorta–gonad–mesonephros (AGM) region around E9.5, which contribute to the development of all hematopoietic lineages (de Bruijn et al., 2000).

The complex developmental process of forming the HSC pool within the embryo takes place in several tissues: the yolk sac (Lux et al., 2008; Mikkola and Orkin, 2006), the AGM region, the placenta and the fetal liver (Mikkola and Orkin, 2006). Shortly before birth, HSCs migrate to the bone marrow (BM), where they reside throughout life (Baron et al., 2012; Hu and Shilatifard, 2016). HSC, which are capable to reconstitute multi lineage haematopoiesis for at least 6 months are termed long-term repopulating hematopoietic stem cells (LT-HSCs) (Pang et al. 2005). These cells are very rare as they represent less than 0.1% of nucleated marrow cells (Pang et al., 2005).

1.1.2 The role of HSCs in hematopoiesis

HSCs reside in the BM of every mammalian organism and are the origin of blood cell replenishment in the peripheral blood (Jagannathan-Bogdan and Zon, 2013).

Hematopoiesis is a hierarchical process, in which HSCs give rise to progenitors at first and secondly differentiate into multiple or single pathway committed precursors, which give rise to all blood cell subsets (Orkin, 2000).

Hematopoietic differentiation from HSCs into myeloid and lymphoid blood cell lines is shown in Figure 1. (Hu and Shilartifard, 2016).

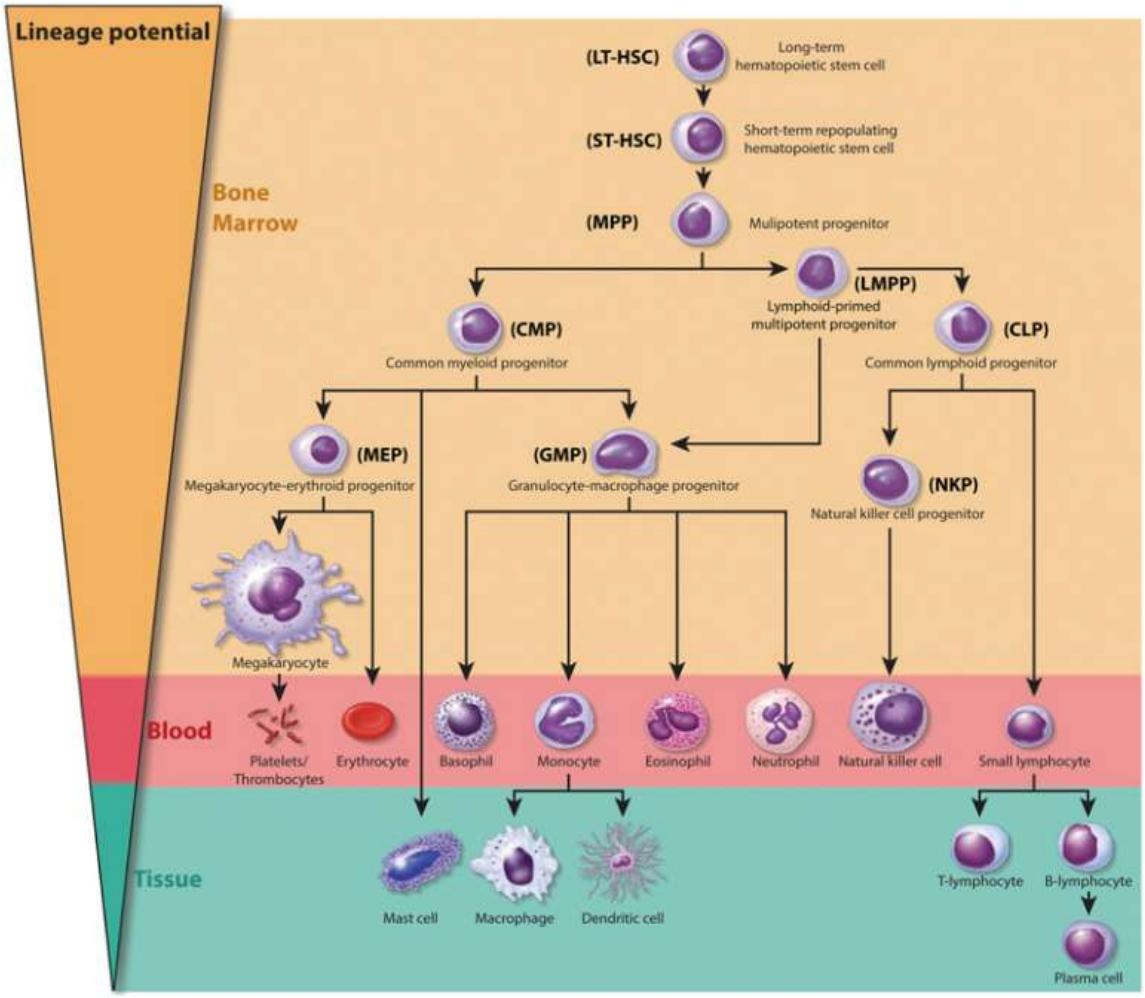


Figure 1. Schematic overview of normal hematopoietic hierarchy in adult mice (by Hu and Shilartifard, 2016). HSCs sit at the top of the hierarchy and have both the capacity of self-renewal and the multipotent potential to give rise to all mature hematopoietic cell lineages (Hu and Shilartifard, 2016). After receiving a differentiation signal, HSCs first lose self-renewing capacity and then progressively lose lineage potential, as they are restricted to a certain lineage (Hu and Shilartifard, 2016). (LT-HSC) Long-term HSC; (ST-HSC) short-term repopulating HSC; (MPP) multipotent progenitor; (CMP) common myeloid progenitor; (CLP) common lymphoid progenitor; (LMPP) lymphoid-primed multipotent progenitor; (MEP) megakaryocyte/erythroid progenitor; (GMP) granulocyte-macrophage progenitor (Hu and Shilartifard, 2016).

Multiple factors of extrinsic and intrinsic origin (e.g. niche-associated factors, signal transduction pathways, transcription factors, and chromatin modifiers) shape the dynamic equilibrium between self-renewal and the multilineage differentiation potential

of HSCs (Hu and Shilatifard, 2016). The highly specialized microenvironment in adult BM is defined as stem cell niche (Brizzi et al., 2012). The currently identified niche cells consist of non-hematopoietic cell types (Nakamura-Ishizu and Suda, 2014). Non-hematopoietic cells include immature osteoblasts (Zhang et al., 2003; Arai et al., 2004), endothelial cells (Ding et al., 2012; Butler et al., 2010), perivascular cells (Ding et al., 2012; Sugiyama et al., 2006), mesenchymal stem cells (MSC) (Méndez-Ferrer et al., 2010), sympathetic nerve cells (Katayama et al., 2006), adipocytes (Naveiras et al., 2009), and non-myelinating Schwann cells (Yamazaki et al., 2011) (Nakamura-Ishizu and Suda, 2014). In addition, mature hematopoietic cells like macrophages/monocytes (Chow et al., 2011), regulatory T cells (Fujisaki et al., 2011) and osteoclasts (Kollet et al., 2006), also regulate HSCs or progenitor cells in an indirect way via modulation of non-hematopoietic niche cells (Nakamura-Ishizu and Suda, 2014).

1.1.3 Stem cell niches

The majority of HSCs reside in a specific microenvironment within the bone marrow, which is called the stem-cell “niches” (Suárez-Álvarez et al., 2012). Within the osteoblastic niche HSCs perform self-renewal (a quiescence state), whereas within the vascular niche HSCs are activated for proliferation and/or injury repair, maintaining a dynamic balance between self-renewal and differentiation (Suárez-Álvarez et al., 2012).

The finding that spleen colony-forming unit cells (CFU_s) near the bone surface are proliferating at a faster rate than those more distant from the bone, but that hematopoietic colonies being grown in agar culture (CFU_c) have a fast proliferation rate, irrespective of their position in the distribution (Lord et al., 1995), supports the assumption that the BM microenvironment is able to regulate hematopoietic stem and progenitor cells (HSPCs) state and function (Boulais and Frenette, 2015).

Cytokines like angiopoietin-1(Ang-1), CXCL12, thrombopoietin (TPO) and extracellular matrix proteins, such as osteopontin and tenascin-C, as well as adhesion molecules such as N-cadherin are produced by niche cells and actively regulate HSCs (Ding et al.,

2012; Sugiyama et al., 2006; Arai et al., 2004; Yoshihara et al., 2007; Nilsson et al., 2005; Hosokawa et al., 2010) (Nakamura-Ishizu and Suda., 2014).

Furthermore, hyaluronan is an important component of the hematopoietic stem cell (HSC) niche (Nilsson et al., 2003; Legras et al., 1997, Goncharova et al., 2012). It participates in HSC lodgment in the endosteal region, functioning in HSC proliferation and differentiation (Cao et al., 2016).

The various interactions between the stem cell niche, ECM, integrins and HSCs, which have a profound impact on haematopoiesis in mammals, are still poorly understood in their complexity (Brizzi et al., 2012). Therefore more studies referring to this topic are needed in order to find more possible treatment ankles for haematological diseases. The identification of molecular cues regulating HSC fate will improve the knowledge on the regulation of hematopoiesis in health and disease (Boulais and Frenette, 2015).

1.2 Genes and hematopoiesis

1.2.1.1 The CD44 gene and protein family

CD44 is a cell-surface glycoprotein, which is expressed by a variety of hematopoietic and non- hematopoietic cells. It has first been described as a lymphocyte homing receptor (Gallatin et al., 1983) and is coded by a 50 kB sized highly conserved gene, which is located on chromosome 11 in humans and on chromosome 2 in mice (Naor et al., 1997). CD44 is coding a heterogeneous protein group, which is represented by cell adhesion molecules (CAM) (Cao et al., 2016). Protein size varies due to alternative splicing between 80-200 kDA (Naor et al., 1997). More than 20 isoforms of CD44 proteins are known (Goodison et al., 1999), of which 10 are generated by alternatively splicing (variants off CD44) (Ponta et al., 2003) (Figure 2(a)).

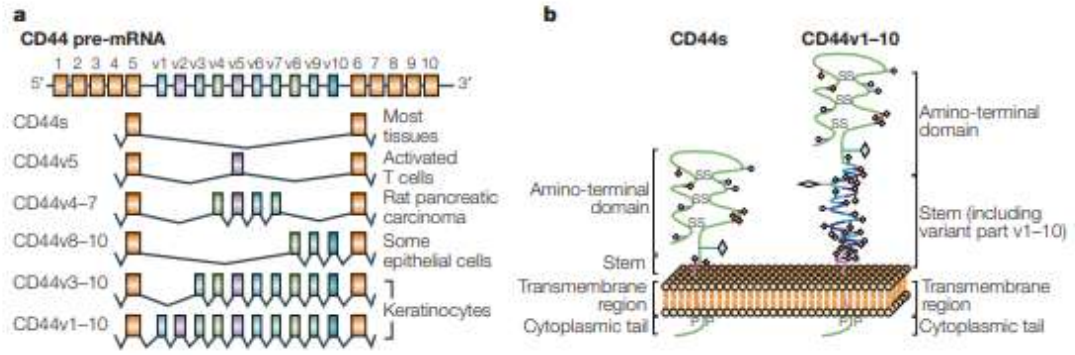


Figure 2. Isoforms of CD44 and protein structure (on the basis of Ponta et al., 2003). **(a)** CD44 pre-mRNA is encoded by 20 exons, 10 of which can be regulated by alternative splicing (variant or `v` exons) (Ponta et al., 2003). The smallest CD44 isoform, which is known as CD44 standard (CD44s), is ubiquitously expressed in vertebrates in developing and adult organisms (reviewed in (Naor et al., 1997)), whereas the larger variant isoforms are expressed in only a few epithelial tissues, mainly in proliferating cells, and in several cancers (Ponta et al., 2003). **(b)** The protein structure of CD44s is compared with that of the largest variant isoform CD44v1-10, which shows that the sequences encoded by the variant exons are in the stem region (Ponta et al., 2003).

CD44 plays an important role in organogenesis, neuronal axon guidance, immunological- and hematopoietic processes (Ponta et al., 2003). The isoform CD44s (standard CD44) is the most abundant one, being expressed by most mammalian cells (Wheatley et al., 1993). The protein structure of CD44s is shown in figure 2(b). CD44s participates in hyaluronan uptake and degradation (Culty et al., 1992), angiogenesis (Griffioen et al., 1997), wound healing (Jain et al., 1996), tissue formation and patterning (Wheatley et al., 1993). CD44 is the principal cell surface receptor for hyaluronan (Aruffo et al., 1990) as well as a receptor for collagens, matrix metalloproteinases (MMPs) and osteopontin (Ponta et al. 2003).

Furthermore, CD44 proteins are important regulators of cell growth, differentiation, cell survival and motility (Nam et al., 2015). In long-term bone marrow (BM) cultures of mice, the blocking of CD44s inhibits lymphopoiesis (Miyake et al., 1990). CD44 is involved in T-precursor trafficking to the thymus and lymph nodes (O'Neill, 1989) and participates in B-cell activation (Rachmilewitz and Tykocinski 1998).

Dysfunction of CD44 proteins or altered expression play a role in a huge variety of pathological conditions (Ponta et al., 2003). Researchers have shown that there is a

linkage between the expression of CD44 variant isoforms (CD44v) and tumor progression, including high grade Non-Hodgkin's lymphoma (hgNHL) (Khaldoyanidi et al., 1996). Khaldoyanidi et al., 1996 found some CD44 variants (mostly exons v5, v6, v7 and, less frequently, exon ~10) in elevated levels in peripheral blood lymphocytes (PBLs) of patients with acute and chronic myeloid leukaemia (AML: 16%, CML: 25%), Hodgkin's disease (HD: 17%), multiple myeloma (MM: 22%), polycythemia vera (PV: 33%), acute lymphoid leukaemia (ALL: 23%) and, most frequently, in PBLs of patients with non-Hodgkin's lymphoma (NHL: 54%). Variations of the CD44 protein have been found in breast and prostate cancer (Basakran, 2015; Louderbough and Schroeder, 2011). Interestingly, the role of CD44 in breast cancer has been found to be dualistic, showing growth and metastasis suppression on the one hand and tumor promotion on the other hand (Louderbough and Schroeder, 2011).

CD44 is highly expressed in healthy HSCs as well as in malicious HSCs (leukemic stem cells) and might therefore be used as a potential protein target in treatment of leukemia (Krause et al., 2006; Krause et al., 2013). CD44s/CD44v are CIC/LIC (cancer-initiating; leukaemia-initiating cell) markers (Ratajczak, 2005; Zöller, 2011). Antibody-based inhibition of CD44 does not only have an impact on leukemic stem cells, but also leads to an impairment of healthy HSCs function (Williams, 2013; Miyake et al., 1990). In a study from 2016, researchers found a CD44 deletion in mice having only a minor effect on embryonic and adult hematopoiesis, but also stated, that this deletion led to an increase in HSCs in the referring mice compared to wild-type controls (Cao et al., 2016).

Summarizing, it is very probable that different variants of CD44, which undergo differential splicing and post-translational-modifications and are involved in distinct biological processes, are the reason for findings of CD44 acting as cancer-inducing in some study cases and cancer-suppressing in others.

The widespread expression of the CD44 cell-surface glycoprotein-gene in combination with its post-translational modifications, which lead to several variants of this gene on the protein-level, offer a huge variety of possible weak points, in which CD44 function can be altered what may lead to the development of cancer in the impacted tissue.

1.2.1.2 CD44 in hematopoiesis

CD44 influences hematopoietic maintenance in humans and is involved in hematopoietic stem and progenitor cell migration (Cao et al., 2016). The CD44 standard isoform (CD44s) is expressed in various cell types, being most abundantly present in cells of the hematopoietic system (Zöller, 2015). The expression of CD44 variant isoforms (CD44v) is more restricted (Zöller, 2015). Both CD44 variants (CD44s and CD44v) were first identified as stem cell markers and found to be involved in the initiation of cancer- and leukemic cells (Zöller, 2015). CD44 strongly influences the homing ability of adult HSCs (Cao et al., 2016). It is additionally involved in lymphocyte migration (O'Neill, 1989; Jalkanen et al. 1986; Jalkanen et al. 1987; Cao et al., 2016) and activation (Cao et al., 2016). CD44s as well as CD44v are involved in the maintenance of stem cell features (Zöller, 2015). They participate actively in generating and embedding in a niche, homing into the niche, maintenance of quiescence, and relative apoptosis resistance (Zöller, 2015).

In 2004, Avigdor et al. found that hyaluronan (HA) and stromal cell-derived factor-1 (SDF-1) are expressed on human BM sinusoidal endothelium and endosteum. The authors suggest a key role for CD44 and HA in SDF-1–dependent transendothelial migration of HSCs/HPCs and their final anchorage within specific niches of the BM (Avigdor et al., 2004).

CD44 interacts with the surrounding niche while being located in membrane subdomains, which are sensitive for collecting signal transduction molecules, proteases, and cytoskeletal components (Zöller, 2015). CD44 regulates HSC and their BM microenvironment by influencing matrix assembly, cytokine/chemokine capture and/or release, cytoskeletal linker protein binding (eg. ankyrin, ezrin, radixin and moesin) including signal transduction, and HSC adhesion, homing, migration, quiescence, resistance to oxidative stress as well as mobilization (Zöller, 2015).

Interactions of CD44 with the BM niche environment are highly complex processes and depend on its post-translational modifications. CD44s is expressed in fetal

hematopoietic organs (Ren et al. 2003; Huang and Auerbach, 1993), but its function in this context is not fully elucidated (Cao et al., 2016).

1.2.2.1 Osteopontin

Osteopontin (OPN) (also known as early T cell activation gene-1 (Ashkar et al., 2000) and Spp-1 gene) is a chemokine-like, non-collagenous, sialic-acid-rich, calcified extracellular matrix (ECM)-associated protein, which is part of the Small Integrin-Binding Ligand N-linked Glycoproteins (SIBLING) family (Bellahcène et al., 2008; Rangaswami et al., 2006). OPN is expressed and secreted by osteoclasts (Chellaiah et al., 2003; Dodds et al., 1995; Maeda et al., 1994). It stimulates bone resorption and osteoclast motility, increasing the number and depth of resorption pits produced by osteoclasts (Chellaiah et al., 2000). Osteoclasts from $OPN^{-/-}$ mice are hypomotile and less active than wild-type (WT) osteoclasts in bone resorption (Chellaiah et al., 2000). The schematic structure of the osteopontin (OPN) protein is shown in Figure 3. (Denhardt et al., 2001). Spp-1 has been characterized independently as mouse2ar (Craig et al., 1989), mouse Eta-l (Patarca et al., 1989), mouse osteopontin (Miyazaki et al., 1990), rat osteopontin (Oldberg et al., 1986), rat 44K bone phosphoprotein (Prince and Butler, 1987; Butler, 1989), rat pp69 (Chackalaparampil et al., 1985; Nemir et al. 1989), porcine Spp-1 (Wrana et al. 1989), humanbone sialoprotein I (Fisher et al. 1987; Kiefer et al. 1989; Young et al., 1990) and mammalian transformation-associated phosphoprotein (Senger et al. 1988; Senger et al., 1989)(Craig and Denhardt, 1991).

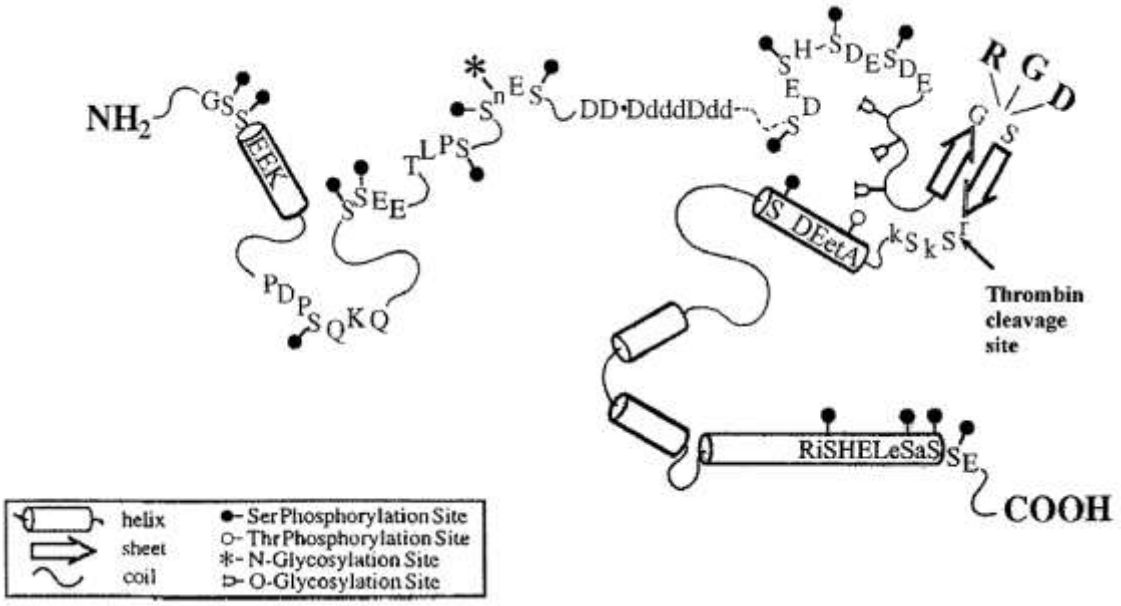


Figure 3. Schematic structure of the osteopontin (OPN) protein (by Denhardt et al., 2001). Structural motifs are shown on the cartoon (Denhardt et al., 2001). OPN from seven species (rat, mouse, cow, pig, human, rabbit and chicken) has been sequenced: The capital letters indicate amino acids that were identical in the seven species, whereas the lower case letters indicate conserved amino acids (Denhardt et al., 2001). The signal sequence that directs secretion of the protein is not illustrated (Denhardt et al., 2001). The central aspartic acid-rich segment is believed to be important in binding to bone mineral (Denhardt et al., 2001). Immediately C-terminal to the arg-gly-asp (RGD) sequence is the conserved thrombin cleavage site (adapted by (Denhardt et al., 2001) from (Sodek et al., 2000) with permission of the authors and publisher).

OPN is expressed in kidney, body fluids, and is abundant in bone and other mineralized tissues (Furger et al., 2001). OPN contains a protease-hypersensitive site, which is located between the integrin- and CD44-binding domains. OPN is secreted by osteoblasts and hematopoietic cells (Liersch et al., 2012) and has been found to be produced in vivo by macrophages and by activated T-lymphocytes (Patarca et al., 1989; Miyazaki et al., 1990; Craig and Denhardt, 1991).

1.2.2.2 Osteopontin in hematopoiesis

OPN suppresses the proliferation of hematopoietic stem cells in vitro and is assumed to regulate the hematopoietic stem cell pool (Liersch et al., 2012). OPN has been found to negatively regulate the stem cell pool (Stier et al., 2005). OPN-deficient mice exhibit a significant increase of stem cells in the BM compared to wild-type controls (Stier et al.,

2005). Osteopontin binds to the inner surface of the bone (endosteum) and is present in high concentrations of the hematopoietic niche, which is located in close proximity to the osteoblasts on the endosteum (Stier et al., 2005). A c-terminal fragment of OPN has been found to bind to two different variants of the CD44 protein (V6 and V3), although CD44v3 might bind through a heparin bridge (Rangaswami et al., 2006). Furthermore OPN binds to other receptors of the alpha and beta-integrins (Furger et al., 2001).

A study demonstrated that injection of OPN led to an accumulation of macrophages and, to a lesser extent, polymorphonuclear leukocytes (Singh et al., 1990). In addition, OPN has been found to affect B cell and macrophage function (Lampe et al., 1991), and is assumed to act as a cytokine for T cells, as well as a chemoattractant for macrophages (Weber and Cantor, 1996).

Elevated expression of OPN has been found in a number of pathologies and in some developmental or stress-responsive physiological situations. OPN expression has been implicated in cancer, immune and inflammatory responses, vascular remodelling, renal diseases, lactation, calcification and remodelling of mineralized tissues (Furger et al., 2001).

In a patient study of 84 newly diagnosed AML patients researchers found enhanced OPN expression-levels in leukemic blasts (immature white blood cells), in serum and in BM (on m-RNA-level and on protein level) (Liersch et al., 2012). This study found a correlation between elevated OPN-levels and a highly decreased survival rate of the AML patients (Liersch et al., 2012). Different cytokines have the ability to increase the expression of osteopontin in the bone marrow, which has an impact on the functioning of stem cells (Stier et al., 2005).

Besides being ubiquitously expressed in several originated tissues, the function of OPN in many of these contexts remains poorly understood (Furger et al., 2001) and needs further investigation.

1.3 Former results of OPN$^{-/-}$ mice

Osteopontin, being part of the ECM and playing a role in the formation and homeostasis of the bone marrow microenvironment, has been found to be a negative regulator for the

hematopoietic stem cell pool (Stier et al., 2005). Prior research of our working group has shown that OPN leads to a reduced reconstitution ability of HSCs in aging mice (Li et al., 2018). Aging of $OPN^{-/-}$ mice led to increased lymphocytes and a decline of erythrocytes in the peripheral blood (Li et al., 2018). In a bone marrow transplantation setting, aged OPN-deficient stem cells showed reduced reconstitution ability likely due to insufficient differentiation of HSCs into more mature cells (Li et al., 2018). In the transplantation setting aged $OPN^{-/-}$ bone marrow cells fail to adequately reconstitute red blood cells and platelets, resulting in severe anaemia and thrombocytopenia as well as premature deaths of recipient mice (Li et al., 2018). OPN has different effects on HSCs in aged and young animals and is particularly important to maintain stem cell function in aging mice (Li et al., 2018).

1.4 The interaction between CD44 and OPN

Weber et al., 1996 stated that the ability of OPN to regulate inflammation (Singh et al., 1990), bone formation (Reinholt et al., 1990) and angiogenesis (Yue et al., 1994), which has been attributed mainly to ligation of $\alpha_v\beta_3$integrins, may also depend on an interaction with CD44. This study revealed the capability of OPN (but not hyaluronic acid (HA)) to induce CD44-dependent chemotaxis, whereas HA (but not OPN) induces CD44-dependent cell aggregation (Weber et al., 1996). The researchers assumed that the interaction between OPN and CD44 on activated lymphocytes and monocytes may mediate migration out of the bloodstream into sites of inflammation, where additional interactions between CD44 and HA may induce homotypic cellular aggregation of emigrant cells (Weber et al., 1996). The hypothesis that an interaction of OPN and CD44 has an impact on migration abilities of specific cells has been made even more plausible, by the revealing of a co localization of intracellular OPN with CD44 and ERM (ezrin, radixin and moesin) proteins in migrating embryonic fibroblastic cells, activated macrophages and metastatic breast cancer cells (Sodek et al., 2000; Zohar et al., 2000). OPN has been found to stimulate CD44 expression on the osteoclast surface, and CD44 has been shown to be required for osteoclast motility and bone resorption (Chellaiah et al., 2003).

Osteopontin and CD44 seem not to be essential for mouse survival, because existing CD44 and OPN knockout mouse strains are fertile, normal in size and do not display any gross physical or behavioural abnormalities. But interestingly, the knockout of OPN has been found to lead to an enriched HSC population in the referring mice (Stier et al., 2005), and $CD44^{-/-}$ mice exhibit irregularities in lymphocyte trafficking (Protin et al., 1999). Summarizing, very little is known about the exact roles of CD44 and osteopontin in hematopoietic processes.

1.5 Mouse strains within this PhD thesis

1.5.1.1 $CD44^{-/-}$ mouse origin

The $CD44^{-/-}$ mice (*$CD44^{tm1Hbg}$*; [$CD44^{-/-}$]) from Stock No. 005085 were originally purchased from the Jackson Laboratory (postal address: 600 Main Street. Bar Harbor, ME USA 04609) and backcrossed for at least 6 generations into the C57Bl6-SJL (black six wild-type mice) strain background prior to use in this study. The *$CD44^{tm1Hbg}$* mouse colony has already been in the possession of the working group of Dr. Viktor Janzen for several years, before the experiments for this PhD thesis have started. The colony was also crossed to the congenic strain Bl6-SJL CD45.1 to obtain CD45.1 or CD45.1:CD45.2 heterozygous offspring for transplantation experiments. Mice heterozygous for the CD44 transgene ($CD44^{+/-}$) were mated to obtain littermates with the following genotypes: wild-types ($CD44^{+/+}$), CD44 knockout ($CD44^{-/-}$) or being heterozygous for CD44 ($CD44^{+/-}$). All animal experiments were approved by the federal office for Nature, Environment and Consumer Protection, North Rhine Westphalia (Landesamt für Natur, Umwelt und Verbraucherschutz NRW) and local authorities at the University of Bonn (animal experiment protocol: AZ 84–02.04.2016.A423). All experiments were performed in accordance with relevant guidelines and regulations. The mice were housed at the University of Bonn animal facility (Haus für Experimentelle Therapie) according to institutional guidelines.

1.5.1.2 CD44$^{-/-}$ mouse strain description

CD44$^{-/-}$ mice are viable, fertile and exhibit a comparable body size to wild-type mice (Protin et al., 1999). Lymphocyte development is unimpaired, but irregularities in lymphocyte trafficking are observable (Protin et al., 1999). In case of tail injection of CD44$^{-/-}$ derived lymphocytes into wild-type mice, those lymphocytes show impaired traffic ability to the peripheral lymph nodes and the thymus (Protin et al., 1999).

A study of CD44$^{-/-}$ mice showed that an elimination of all isoforms of CD44 did not impact embryonic development of the referring mice, which implies that CD44 is dispensable and does not have a crucial impact on mouse development (Protin et al., 1999).

1.5.2.1 OPN$^{-/-}$ mouse origin

OPN129/C57BL/6$^{-/-}$ transgenic mice (inbred mouse strain (black six)) were previously described (Stier et al., 2005). The OPN129/C57BL/6$^{-/-}$ mouse colony has already been in the possession of the working group of Dr. Viktor Janzen for several years, before the referring mice were used in experiments for this PhD thesis. These mice were backcrossed into the C57BL/6 background for at least six generations. The colony was also crossed to the congenic strain Bl6-SJL CD45.1 to obtain CD45.1 homozygous offspring for transplantation experiments. Mice heterozygous for the OPN transgene (OPN$^{+/-}$) were mated to obtain littermates with the following genotypes: WT (OPN$^{+/+}$), OPN-knockout (OPN$^{-/-}$), or being heterozygous for OPN (OPN$^{+/-}$). All animal experiments were approved by the federal office for Nature, Environment and Consumer Protection, North Rhine Westphalia (Landesamt für Natur, Umwelt und Verbraucherschutz NRW) and local authorities at the University of Bonn (animal experiment protocol: AZ 84–02.04.2016.A423). All experiments were performed in accordance with relevant guidelines and regulations. The mice were housed at the University of Bonn animal facility (Haus für Experimentelle Therapie) according to institutional guidelines.

1.5.2.2 OPN$^{-/-}$ mouse strain description

The OPN$^{-/-}$ homozygous mutant mice were phenotypically normal, and no abnormalities were noted in growth, external morphology, or behaviour compared with wild-type littermates (Liaw et al., 1998). In addition, both male and female mutants were completely fertile, giving birth to normal sized litters, and raising offspring successfully prior to weaning (Liaw et al., 1998).

1.5.3 Cross breeding of CD44$^{-/-}$ and OPN$^{-/-}$ mice for generation of CD44$^{-/-}$:OPN$^{-/-}$ mice

In the animal-facility of the Universitätsklinikum Bonn, CD44$^{-/-}$ mice (origin: The Jackson Laboratory) and OPN$^{-/-}$ mice, both being backcrossed over 6 generations into the C57Bl/6N background, have been in the possession of our working group (head of group: Dr. Viktor Janzen). I started crossbreeding of those single-knockout mice and generated CD44$^{-/-}$:OPN$^{-/-}$ mice (Protocol: AZ: 84-02.04.2016.A423 under LANUV (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein Westfalen) approval.

1.6 Malignancies of hematopoiesis

A disturbance of the equilibrium between self-renewal and differentiation potential of HSCs, which are controlled by extrinsic and intrinsic factors, can cause an imbalance of hematopoiesis, which may lead to hematological diseases (Li and Clevers, 2010; Doulatov et al., 2012). Mutations which may lead to cancer development can not only occur in somatic cells, but are also able to assimilate in progenitor cells like HSCs and MPPs within the BM (Passegué et al., 2003). Because of their long persistence time in combination with their omnipresence in various tissues of multicellular adult organisms (Shipitsin and Polyak, 2008), stem cells are prone for tumour initiating transforming events, that can accumulate over long time periods (Reya et al., 2001). The “stem cell origin of cancer”-hypothesis, states, that stem cells (or other cells) that acquired the ability for self-renewal, accumulate genetic changes over time (Shipitsin and Polyak, 2008). Those genetic mutations in stem cells have the potential to lead to alterations in form and function of progenitors in the BM and adult blood cells in the peripheral blood,

which can be the starting point of haematological cancerous development (Shipitsin and Polyak, 2008).

1.7 Goal of this thesis

The aim of this PhD thesis is to identify the impact of the absence of CD44 and OPN in mice on hematopoietic processes, especially in the HSC department. Of special interest is the influence of $CD44^{-/-}$:$OPN^{-/-}$ double-deficiency in the BM environment on the proportion and number of hematopoietic stem and progenitor cells (HSPC: Lin^- $cKit^+$ Sca^+ [LKS]), the size of the highly enriched HSC population (Lin^- $cKit^+$ Sca^+ $CD48^-$ $CD150^+$) and the number of multipotent progenitors (MPP: Lin^- $cKit^+$ Sca^+ $CD48^+$ $CD150^-$). Furthermore, the impact of $CD44^{-/-}$:$OPN^{-/-}$ double-deficiency on blood cell populations under steady- and stress-state experimental conditions was under investigation.

Prior results of our working group have shown that OPN plays a role in stem cell related hematopoiesis. Furthermore, intracellular OPN has been found to be co localized with CD44 and ezrin, radixin and moesin proteins (ERM proteins) in migrating embryonic fibroblastic cells, activated macrophages and metastatic breast cancer cells (Sodek et al., 2000; Zohar et al., 2000) and stimulates CD44 expression on the osteoclast surface, which impacts osteoclast motility and bone resorption (Chellaiah et al., 2003).

The finding, that the ligand-receptor-binding between CD44 and OPN is not exclusive, but can be mediated through OPN binding to integrins, which enables signaling to hematopoietic cells even without the presence of CD44, raises the question if a double knockout of CD44 and OPN would have a more severe impact on HSC functioning or could be compensated through different binding proteins.

Close proximity of OPN and CD44 in the BM environment, the circumstance that OPN is a negative regulator of the stem cell pool (Stier et al., 2005) and the finding that CD44 acts as a negative regulator in adult BM with increased stem and progenitor cell pools (Cao et al., 2016) lead to the hypothesis that the double-deficiency of CD44 and OPN

might have a stronger impact on hematopoiesis that the single deficiency of OPN referring to wild-type mice.

For unraveling gene impact of CD44 and OPN on hematopoiesis, two C57BL/6N mice strains, one being deficient in the CD44 gene and the other one being deficient in the OPN gene, have been crossbred. The resulting $CD44^{-/-}$:$OPN^{-/-}$ double-deficient offspring, as well as $OPN^{-/-}$ deficient and double-heterozygous mice ($CD44^{+/-}$:$OPN^{+/-}$) were used for blood and bone marrow analyses under steady- and stress-state conditions.

Steady-state conditions define analyses of blood and BM composition of mice under unaffected living conditions during their whole lifespan (3-21 months), whereas stress-state conditions are represented by the impact of a serial stem cell transplantation, G-CSF and Poly(I:C) stress on BM and peripheral blood compositions of mice at a certain age cohort.

The serial stem cell transplantation experiment is aimed to investigate the repopulation potential of $CD44^{-/-}$:$OPN^{-/-}$ deficient HSCs in comparison to wild-type HSCs.

Polyinosinic:polycytidylic acid (Poly(I:C)) is an immunostimulant, which is used to mimic viral infections in animals in order to evaluate the innate immunity system response of the Poly(I:C) receiving animal (Fortier et al., 2004). In vivo Poly(I:C) application triggers an interleukin-1-dependant mechanism and induces fever in rats (Fortier et al., 2004). Poly(I:C) interacts with toll-like receptor 3 (TLR3) (Li et al., 2015), which is expressed in the membrane of B cells (Browne, 2012), macrophages (Gallego et al., 2011) and dendritic cells (Matsumoto et al., 2017). In stress-state experiments within this PhD thesis, Poly(I:C) treatment has been used to find out if viral immune response in $CD44^{-/-}$:$OPN^{-/-}$ mice is altered in comparison to wild-types.

The hematopoietic growth factor granulocyte colony-stimulating factor (G-CSF) is a peptide hormone (Matchett et al., 2007), which is involved in inflammatory immune responses (Martins et al., 2010). It is used for the stimulation of granulopoiesis to shorten the period of severe neutropenia, which often follows high-dose chemotherapy (Sheridan et al., 1992). It stimulates the production of granulocytes and stem cells in BM and enhances their release into the peripheral blood (Deotare et al., 2015). G-CSF

experiments within this PhD thesis are aimed to unravel if the immune response of $CD44^{-/-}$:$OPN^{-/-}$ mice differs from the immunological reaction of wild-type mice in case of a simulated bacterial infection.

Summarized, the absence of CD44 and OPN in double-knockout mice should unravel gene impact in experimental steady- and stress-state settings, if blood- and BM- status were compared to wild-types, which possess fully functional CD44 and OPN genes and all mice were treated under equal conditions.

Following questions are aimed to answer within this PhD thesis:

1. What influence has $CD44^{-/-}$:$OPN^{-/-}$ deficiency on the size of the stem cell pool, on proliferation and differentiation capacity of HSCs?

2. Is the elimination of $CD44^{-/-}$:$OPN^{-/-}$ in HSCs in reference to wild-types more severe referring to hematopoiesis than $OPN^{-/-}$ deficiency?

3. How does induced stress impact the functioning of HSCs in $CD44^{-/-}$:$OPN^{-/-}$ deficient mice?

2. Material and Methods

2.1 Materials

2.1.1 Laboratory Equipment

Table 1. Laboratory equipment, model and supplier.

Equipment	Model	Supplier
Autoclave	CX-150	Systec, Wettenberg, Germany
blood analyzer	Hemavet 950	Drew Scientific, USA
blood analyzer	BC-5000 Vet	(Mindray)
Centrifuge	5810R, 5430R, miniSpin plus	Eppendorf, Hamburg, Germany
Centrifuge	Mini Star	VWR, Radnor, PA
Centrifuge	Varifuge 3.0R	Heraeus, Hanao, Germany
Electrophoresis chamber	PerfectBlue Gel System	Peqlab,Erlangen, Germany
Electrophoresis chamber	Xcell SureLock Mini-Cell	Thermo Scientific, Waltham, MA
Eppendorf Pipettes	0,1-2,5 µl; 2-20 µl; 20-200 µl; 100-1000 µl; 10-100 µl	Eppendorf, Hamburg, Germany
Flow cytometer	FACS Canto II	BD, Heidelberg, Germany
Gel imaging system	ChemiDoc XRS+	Bio-Rad, Hercules, CA
Laminar flow hood	MSC-Advantage	Thermo Scientific, Waltham, MA
Microwave	NN-E245W	Panasonic, Kadoma, Japan
PCR cycler	Mastercycler pro	Eppendorf, Hamburg, Germany
Roller mixer	STR9	Stuart, Staffordshire, UK
Vortexer	ZX3 Vortex mixer	VELP Scientifica, Usmate, Italy
Vortexer	ZX3 Vortex mixer	VELP Scientifica, Usmate, Italy
Waterbath	WNB 7-45	Memmert, Nuremberg, Germany
X-ray generator	RS-2000 biological irradiator	Rad source, Suwanee, GA

2.1.2 Consumable materials

Table 2. Consumable materials and supplier.

Item	Supplier
Alcohol pads	Braun
BD Micro-Fine Insulinspritzen U40	Becton Dickinson
BD Micro-Fine Insulinspritzen U40 12,7 mm	BD
Cell strainer (35µm) in 6ml facs tube	LB OCI Falcon
Cell strainer 70 µm Nylon	Corning
Disposable scalpel	pfm medical ag
Dissecting scissors straight 110 mm	Labomedic
Eclipse Sicherheitskanüle 25Gx5/8 0,5x16mm	BD
Eppendorf Tube® 5.0 ml	VWR
FACS tubes	Falcon
Falcon tube with cell stainer snap cap	Falcon
Falcon tubes 15 ml	Corning
Falcon tubes 50 ml	Greiner Bio-one
Filter tips (10 µl, 200 µl, 1000 µl)	Nerbe Plus
Hypodermic safety needle	Magellan
Leukosilk	Hansaplast
Micro tube 1,3 ml (with EDTA K3)	Sarstedt
Microtainer K2E Tubes	BD
Microtubes (1 ml; 1,5 ml)	Sarstedt
Monoject Magellan Sicherheitskanülen 25G x 5/8 inch	Magellan
Mortar and Pestle, porcellain, 20mL	Labrobedarf Carl Roth
Needles	BD
Parafilm	Pechiney
PCR reaction tubes	Biozym Scientific
PCR-eppis (8er Stripes), PP, 0,2 ml	Aesculap AG
Pincette 18/10 steel	Labomedic
Pipette (5ml; 10 ml)	Greiner Bio-one
Refill pipette tips (10 µl, 200 µl)	Nerbe Plus
Röhre 5 ml 75x12mm PS FACS Canto	LB Sarstedt AG und Co
Safety glasses	UVEX Super Fit SV Perform
Scapell Fig.10	pfm medical ag
Sterile filter 0,22 µm	Millipore
Syringe (2 ml, 5 ml, 10 ml)	BD

2.1.3 Chemicals, reagents and kits

Table 3. Chemicals, reagents and kits and supplier.

Item	Supplier
1-Step Fix/Lyse Solution	eBioscience/Thermo Fisher
1-step Fix/Lyse solution (10x)	LB Mercateo
1-step Fix/Lyse solution (10x)	eBioscience
2 x M-PCR OPTI^{TM} Mix (Dye Plus)	Biotool
Agarose basic	PanReac AppliChem
APC BrdU Flow Kit	Becton Dickinson
Bromodeoxyuridine (BrdU)	Becton Dickinson
BSA (Bovine serum albumin)	Invitrogen
Cotrim K Sirup f Kinder	Ratiopharm
Direct PCR Tail Lysing Reagent	FreitextPeqlab
Distilled Water Sterile Tissue Culture tested 500 mL	SigmaAldrich
DMSO	SigmaAldrich
DNA ladder (100pb-1000bp)	Peqlab
DPBS	Thermo Fisher Scientific/
DPBS	Life Technologies
Ethanol 70 %, 99 %	Otto Fischar
Ethylendiamidtetraacetat (EDTA)	Sigma
FACS Lysing Solution/Buffer	BD
Fetal bovine serum (FBS)	PAA
Filgrastim/Neupogen 30 (FertigspritzenKonzentr)	Amgen
G-CSF	Amgen
Helipur H plus N Desinfect	Braun
Lysing solution	Becton Dickinson
Methanol	Merk
PCR Tail Lysing Reagent	Peqlab
Penicillin-streptavidin, 100x	Gibco, Life Technologies
Perm Buffer III	Becton Dickinson
Perm/Wash Buffer	BD
Perm/Wash Buffer 100ml	LB Frei BD Biosciences
Perm/Wash Buffer I	BD
Perm/Wash Buffer III	BD
Permeabilization Buffer Plus	Becton Dickinson
Pipette (5mL, 10mL)	Greiner Bio-one

Poly(I) Poly (C) Double Strand	GE healthcare
Pre-Separation Filter, 30 µm	Miltenyi Biotec
Proteinase K	Roche
Proteinase K 100 mg	thermofisher
RBC Lysis Buffer (10X)	LB Frei BioLegend
Roti-Safe GelStain	Fiers
ß-Mercaptoethanol	Sigma
tissue glue	surgibond
tissue glue (Histoacryl)	aesculap

2.1.4 Antibodies for flow cytometry

Table 4. Antibodies for flow cytometry and supplier.

Specificity	Fluorochrome	Dilution	Supplier
BrdU	FITC	1:50	BD
CD117/c-Kit	APC	1:100	eBioscience
CD11b/Mac1	eFluor450	1:500	eBioscience
CD135/Flt-3	PE	1:500	eBioscience
CD150	Percp-Cy5.5	1:100	eBioscience
CD19	APC	1:500	eBioscience
CD24	PE-Cy7	1:200	eBioscience
CD34	FITC	1:200	eBioscience
CD3e	PE-Cy7	1:500	eBioscience
CD43	PE	1:500	eBioscience
CD44	PE	1:500	eBioscience
CD45	PE	1:1000	eBioscience
CD45.1	PE	1:200	eBioscience
CD45.2	FITC	1:200	eBioscience
CD45R/B220	PerCP-Cyanine5.5	1:500	eBioscience
CD48	PE	1:700	eBioscience
DAPI		1:2000	eBioscience
Gr1	eFluor660	1:500	eBioscience
Ki67	FITC	1:50	eBioscience
Lineage cocktail	Biotin	1:50	BD
Sca-1	PE-Cy7	1:300	eBioscience
Sca-1	eF450	1:500	eBioscience
Sca-1	PE	1:1000	eBioscience
Streptavidin	PerCP-Cy5.5	1:500	eBioscience
Ter119	APC-eFluor780	1:500	eBioscience

2.1.5 Primers for genotyping

Table 5. CD44 and OPN genotyping primers.

Primers for genotyping	
Oligoname	**Sequence 5' -> 3'**
CD44_KO_for (target CD44)	CCT GGG TGG AGA GGC TAT TC
CD44_KO_rev (target CD44)	AGG TGA GAT GAC AGG AGA TC
CD44_WT_for (target CD44)	GGC GAC TAG ATC CCT CCG TT
CD44_WT_rev (target CD44)	ACC CAG AGG CAT ACC AGC TG
Neo1360 (target OPN)	CGT CCT GTA AGT CTG CAG AA
OPIn3 (target OPN)	CCA TAC AGG AAA GAG AGA CC
OPIn4 (target OPN)	AAC TGT TTT GCT TGC ATG CG

OPN primers designed by (Liaw et al., 1998).

2.1.6 Buffers and solutions

Table 6. Buffers, solutions, ingredients and storage conditions.

Item	**Composition**	**Storage**
Proteinase K 20 mg/ml	100 mg Proteinase K 5 ml 10 mM Tris buffer	-20°C
Staining buffer	500 µl FBS 49.5 ml PBS	4°C
Tris buffer 10 mM	0.08 g Tris-HCl 50 ml H_2O	room temperature
TAE buffer 50x	242g Tris 57,1 ml Acetic Acid 100 ml EDTA (0,5 M, pH 8,0) fill up to 1000 ml with water	room temperature
TAE buffer 1x	40 ml TAE buffer 50x 1960 ml H_2O	room temperature
TBS-T stock 10x	24.2 g Tris 80 g NaCl adjust pH to 7.6 700 ml H_2O	room temperature

2.1.7 Software

Table 7. Software and application.

Software	Application
BD FACS Diva	Application setup, data acquisition and analysis on BD FACSCanto II
Flowjow	Viewing and analyzing flow cytometric data
Nanodrop	Quantification of DNA
Pyrat	database for documentation, recording and breeding of laboratory animals
Realplex	Thermal cycling program setup and data acquision
TierBase	database for documentation, recording and breeding of laboratory animals

2.2 Methods

2.2.1 Mice breeding and housing

The mice were housed at the University of Bonn animal facility (Haus für Experimentelle Therapie) according to institutional guidelines. They were sacrificed by increasing CO_2 concentrations, as recommended by the corresponding authorities.

All animal experiments were approved by the federal office for Nature, Environment and Consumer Protection, North Rhine Westphalia (Landesamt für Natur, Umwelt und Verbraucherschutz NRW) and local authorities at the University of Bonn (animal experiment protocol: AZ 84–02.04.2016.A423). All experiments were performed in accordance with relevant guidelines and regulations. Setting of breeding couples, separating of offspring, and genotyping has been performed by me. Housing of animals (cleaning of cages, renewing of cage bedding, food- and water-supply) has been performed by staff members of the HET. Health status of mice was checked regularly by me and HET employees including veterinarians of the facility.

2.2.2 Mouse strains

For the experiments within this PhD thesis, wild-type mice (C57BL/6 black six mice being genetically equipped with a functional version of the OPN and CD44 gene), OPN knockout mice (OPN129/C57BL/6$^{-/-}$ [also termed as OPN$^{-/-}$ mice] being deficient in the OPN gene) and CD44 knockout mice (*CD44^{tm1Hbg}* [also termed as CD44$^{-/-}$ [miceJAX stock #005085]] being deficient in the CD44 gene) have been used.

CD44$^{-/-}$ mice and OPN$^{-/-}$ mice have been used in breeding for offspring being double-deficient of CD44 and OPN (CD44$^{-/-}$:OPN$^{-/-}$ mice).

2.3 Genotyping

2.3.1 DNA extraction

DNA extraction of tail tip cuts (0,1mm), which have been collected after separating offspring from breeding couple, has been performed as described in manual “DirectPCR Lysis Reagent Tail” (Peqlab). After DNA extraction from tail tissue, the DNA extract has been ready for usage in PCR.

2.3.2 PCR

The oligonukleotide primers have been purchased from Eurofins MWG/Operon (Ebersberg). These primers (Table 5.) have successfully been used for the identification of CD44 and OPN knockout mice for several years since these strains have been in the possession of the working group of Dr. Viktor Janzen. When I started working on this PhD project, I proceeded using these primers for genotyping discriminations.

Genotyping PCRs of the CD44 gene locus have been performed in following pipetting scheme:

Table 8. Pipetting scheme of CD44 KO primer PCR master mix.

Chemical	µl / sample
H_2O dest	9
2*M-PCR Mix	10
Primer 1 forward	0,25
Primer 2 reverse	0,25
Template	**1**
Total volume:	**20,5**

PCR program settings for the amplification of the CD44 knockout gene locus have been adjusted like shown in Table 9.

Table 9. PCR-cycler-settings for the amplification of the CD44 knockout gene locus.

temperature in ºC	time	number of cycles
94º C	5 min	1
94º C (Denaturierung)	20 sec	38
49º C (Annealing)	30 sec	
72º C (Elongation)	40 sec	
72º C (Final Elongation)	5 min	

PCR program settings of the CD44 wild-type gene locus have been as described like shown in Table 10.

Table 10. PCR-cycler settings for the amplification of the CD44 wild-type locus.

temperature in ºC	time	number of cycles
94º C	5 min	1
94º C (Denaturierung)	20 sec	38
52º C (Annealing)	30 sec	
72º C (Elongation)	40 sec	
72º C (Final Elongation)	5 min	

The pipetting scheme for the OPN master mix has been performed like described in Table 11.

Table 11. Pipetting of Osteopontin-knockout and wild-type primer PCR master mix.

Chemical	µl / sample
H_2O dest	8
PCR Mix	10
OPN 3 primer	0,5
OPN 4 primer	0,5
Neo 1360 primer	0,5
Template	1
Mastermix per eppi:	19,5
Total volume per eppi	20,5

PCR program settings for the amplification of the OPN gene locus have been performed as described in Table 8.

Table 12. PCR-cycler settings for the amplification of the OPN locus.

temperature in ºC	time	cycle number
94º C	5 min	1
94º C (Denaturierung)	20 sec	40
52 °C (Annealing)	30 sec	
72º C (Elongation)	1 min	
72º C	5 min	1
4º C	∞	1

2.3.3 Gel electrophoresis

PCR products, which resemble multiple copies of primer defined DNA fragments of a certain length, are segregated by the gel electrophoreses process.

1- 1,5 % agarose in 100 ml of either 1x TAE buffer or 1x TB buffer within a Erlenmeyer flask was cooked in a microwave for 3 minutes. After cooking, 5µl of dye (Roti-Safe

GelStain [Fiers]) were added and the viscous liquid was pour into the gel chamber. After 20 minutes polymerization, the gel, was loaded with the PCR products and a defined fragment marker (DNA ladder (100-1000 base pairs (bp)) [Peqlab]). The electrophoresis process was performed at 100 mA, 200 V and 40 W for 35 minutes. The gel examination and photo documentation has been implemented with a gel imaging system (ChemiDoc XRS+ [Bio-Rad, Hercules, CA]).

2.4 Peripheral blood analyses

2.4.1 Peripheral blood analyses with BC 5000-Vet Blood Analyzer

Blood collection has been performed by minimal punctual incision with scalpel on lateral tail vein of mice, after prior heating of cage (while mouse being within cage) with a warming light for 3 minutes. 5 droplets of blood (80-100 µl) from lateral tail vein have been collected in a microtainer EDTA tube (BD) and the lesion was sealed with Histoacryl tissue glue (Aesculap) to prevent further blood loss. Blood samples were analyzed with the BC 5000-Vet Blood Analyzer (Mindray) for the determination of the white blood cell count (WBC), erythrocyte-, hemoglobin- and thrombocyte- cell counts. For further definition of white blood cells and a discrimination of B-, T-, granulocyte and monocytes blood values, the remaining blood was used for analyses on BD FACS Canto II.

2.4.2 Peripheral blood analyses with BD FACS Canto II

The after BC 5000-Vet analyses remaining blood samples were treated with FACS Lysing Solution/Buffer (BD) for erythrocyte lyses and fixation, following manufacturer's instructions. Afterwards samples were washed with 1 ml staining buffer (SB) (containing PBS with 1% fetal bovine serum (FBS)) per sample. The staining with differentially colored dye conjugated antibodies against granulocytes (Gr-1 (Ly6G) eF660), B cells (B220 Percp Cy5.5), Monocytes/Macrophages (CD11b (Mac1) eF450 Pac. Blue) and T cells (CD3e PE-Cy7) has been performed. In case of serial transplantation blood analyses, the pan-hematopoietic surface markers CD45.1 (PE conjugated) and CD45.2 (FITC conjugated) have been used, for the discrimination of donor and recipient-derived

blood cells. 50 µl per sample of the antibody mix were added. After 20 minutes of incubation in the dark, samples were washed with 1 ml staining buffer and measured on BD FACS Canto II.

2.5 BM analysis

2.5.1 Mouse preparation prior to BM staining and measurement on FACS

Mice were killed via CO_2 - infiltration in a closed chamber according to LANUV guidelines. Both hind legs per mouse were extracted. Two femurs and two tibula and fibula (both hind legs) of each mouse were flushed with a pipette and a hypodermic needle (27GMagellan) in 3 ml PBS. This BM-PBS-suspension was filtered through a 35 µm mesh filter on a FACS tube (FALCON), 100 µl were extracted to an eppi tube and used for WBC measurement on the BC 5000 VET analyzer. The rest of the remaining BM-PBS-suspension per sample was used for BM stainings and FACS analyses.

2.5.2 Staining of BM samples

Stem and progenitor cells were first-step-stained with an antibody mix consisting of Mac-1a (CD11b), Gr-1(Ly-6G und Ly-6C), Ter119 (Ly-76), and B220 (CD45R) (lineage-cocktail). Afterwards all samples were washed with 1 ml SB, centrifuged for 5 minutes (300g for 5 minutes) and the supernatant was discarded. In the second-staining-step all matured cells were dyed with either Streptavidin eFlour450 (in case of LKS staining) or Streptavidin PerCP-Cy5.5 (in case of progenitor staining). Furthermore, samples were stained with c-Kit-APC-, Sca1-PE-Cy7-, CD150 Percp-Cy5.5- and CD48-PE antibodies. In case of the serial BM analyses, the pan-hematopoietic surface markers CD45.1 (PE conjugated) and CD45.2 (FITC conjugated) have been additionally used within the antibody mix, for the discrimination of donor and recipient-derived BM cells. In case of progenitor subset discrimination, samples were additionally stained with c-Kit-APC, Sca-1-PE-Cy7, CD34-FITC, CD16/32-PE und CD127-APC-eF780 besides the lineage cocktail (with Streptavidin PerCP-Cy5.5 as 2^{nd} antibody).

2.5.3 BrdU proliferation assay

5-Bromo-2´deoxyuridine (BrdU) is a chemically synthesized base analogue to thymidine, which is incorporated instead of thymidine into newly synthesized DNA during the S-phase of the cell cycle (Nowakowski et al., 1989). BrdU labeling is a common technique for the study of adult neurogenesis (Lehner et al., 2011).

To assess proliferation of HSCs and MPPs in BM of mice the APC BrdU Flow Kit (BD) has been used, following manufacturer`s instructions. One day before the experiment, each mouse received an intraperitoneal (ip) injection of BrdU (3 mg/ml) in 200 µl sterile PBS per mouse. Additionally, mice received 0,8 mg/ml BrdU in drinking water. One mouse was not treated with BrdU (control).

Bone marrow cells were stained with lineage antibodies, antibodies specific for c-Kit, Sca-1, CD150, CD48 and BrdU.

2.6 Serial stem cell transplantation of $CD44^{-/-}$:$OPN^{-/-}$-BM-donor cells into wild-type-recipient mice

For the stem cell transplantation assays, a congenic mouse system was utilized: donor cells expressing the pan-hematopoietic surface marker CD45.1 and were transplanted into CD45.2-bearing hosts. The wild-type female C57BL/6 recipients were obtained from Charles River Laboratories. Wild-type- ($CD44^{+/+}$:$OPN^{+/+}$) and $CD44^{-/-}$:$OPN^{-/-}$donors, being CD45.1 positive for the pan-hematopoietic surface marker, were bred in-house.
One day prior the first round of serial stem cell transplantation, twenty recipient mice (WT Black6: $CD45.2^{+}$) were irradiated 2 times at 4,75 Gy for 226 seconds. Between each radiation, mice were able to rest for 4-5 hours. The following day, two $CD44^{-/-}$:$OPN^{-/-}$ mice (age 3 months: being $CD45.1^{+}$) were chosen as donors for 10 recipient mice (age: 2 months: being $CD45.2^{+}$). Two wild-type mice ($CD44^{+/+}$:$OPN^{+/+}$, age 3 months: being $CD45.1^{+}$) were chosen as donors for 10 recipient mice (age: 2 months: being $CD45.2^{+}$). Donor mice were killed, followed by extraction of femur, fibula und tibia. Bones were flushed in 3 ml PBS with hypodermic needle to extract BM. Suspension was filtered and 100 µl were separated for cellularity measurement with BC-

5000Vet. One femur was used for staining of LKS-SLAM, Lineage and Progenitor staining. White blood cell counts were measured per sample, extracted BM cells per genotype were pooled in 30.000.000 cells in 7500µl sterile PBS. One recipient mouse received 2.000.000 cells in 500µl sterile PBS, via transplantation into lateral tail vein. Engraftment efficiency in recipients was monitored by donor contribution of $CD45.1^{+}$ positive cells using FACS analysis. Peripheral blood cell and BM analyses have been performed at different time points after transplantation to assess short- and long-term reconstitution ability. For serial transplantation, eight weeks after the first round of transplantation, recipients were used as donors for the next transplantation cycle to assess the self-renewal ability of repetitive transplants. Transplants were discontinued after the third round of transplantation due to a lack of peripheral blood reconstitution in the third-round recipients of aged bone marrow.

2.7 G-CSF stress

Three months old mice have been used in G-CSF treatment experiment. Following genotype cohorts were included: 12 wild-type mice ($CD44^{+/+}$:$OPN^{+/+}$) of which 10 were treated with G-CSF and 2 with sterile NaCl (control mice), 7 $CD44^{-/-}$:$OPN^{-/-}$ mice and 11 $OPN^{-/-}$ mice. Peripheral blood status of wild-type, $CD44^{-/-}$:$OPN^{-/-}$ and $OPN^{-/-}$ mice has been measured before G-CSF treatment and three, four and six days after daily G-CSF treatment. Daily G-CSF (Filgrastim/Neupogen 30; Amgen) injections (ip) of 0,4 µg/g (per body weight of mouse), which has been diluted with sterile NaCl to the calculated dosage per mouse, have been applied to treated animals for the duration of 6 days. Control mice (wild-types ($CD44^{+/+}$:$OPN^{+/+}$)) received daily injections of sterile NaCl. After 6 days of treatment, mice have been killed and their BM has been analyzed.

2.8 Poly(I:C) stress

Six months old mice (being $CD44^{-/-}$:$OPN^{-/-}$, $OPN^{-/-}$, $CD44^{+/-}$:$OPN^{+/-}$ and $CD44^{+/+}$:$OPN^{+/+}$ (wild-type)) were divided into two treatment groups (cohorts). All mice were weighted. One day before Poly(I:C) treatment, mice were bled and analyzed for granulocyte-, monocyte-, T-cell- and B-cell- values in the peripheral blood with BC-5000 Vet (Mindray)

and FACS (FACS Canto II; BD) measurement. One cohort was treated with 100% Poly(I:C) (concentration of stock solution: 1,47 mg/ml in sterile PBS) according to their body weight (12.5 µg/g body weight); the other cohort was treated with equivalent amount of sterile PBS (mock group). After 24 hours, mice were bled and blood- and bone marrow status were measured after treatment.

2.9 Statistical analyses

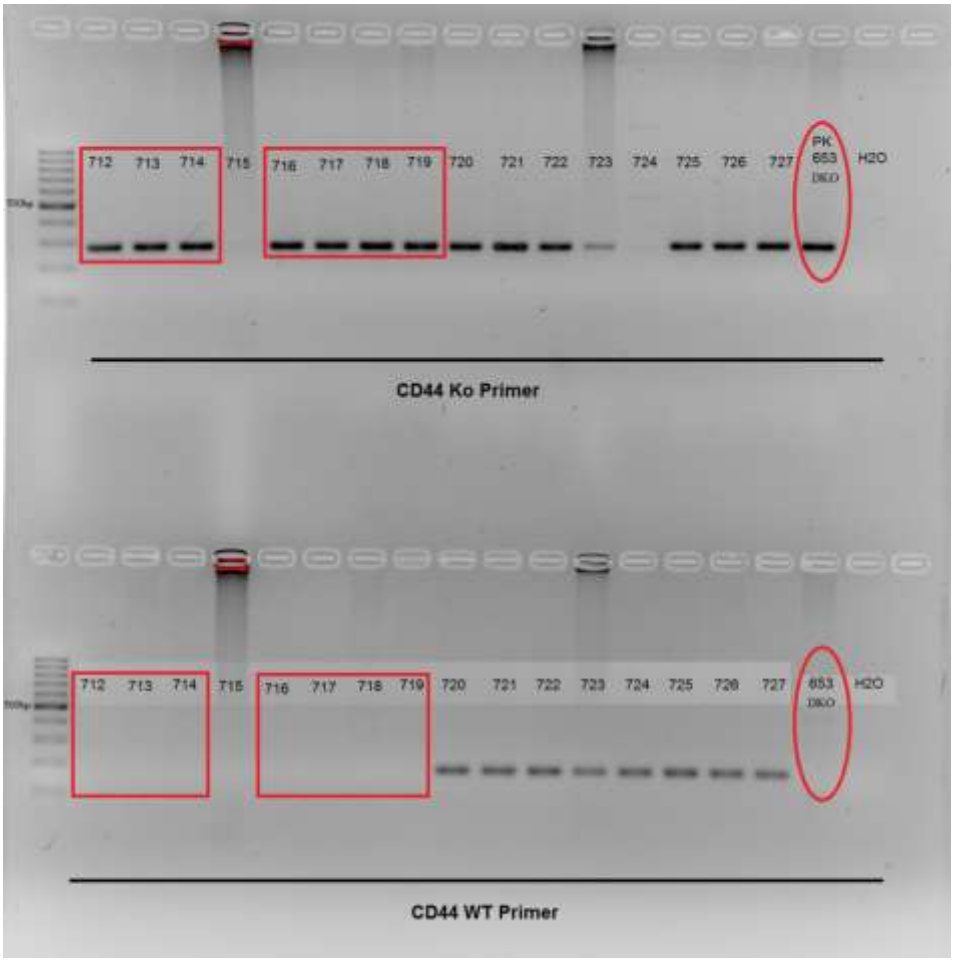
Statistical analysis was performed using the unpaired two-tailed student's t test; significance was defined as a P value of less than 0.05.

3. Results

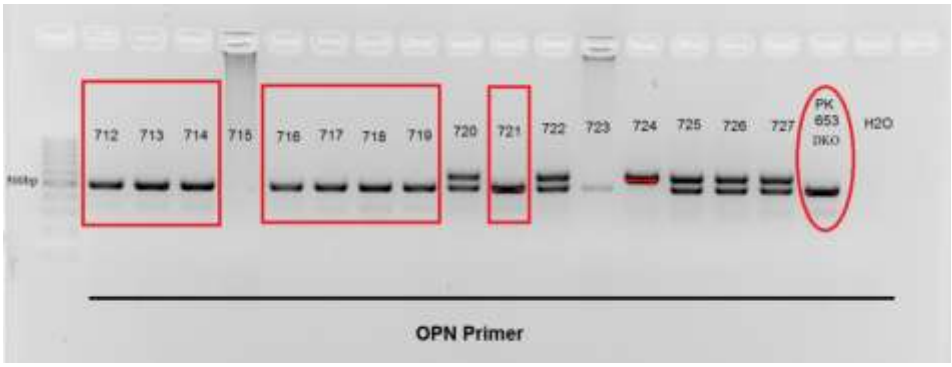
3.1 Mouse strains

3.1.1 Breeding of $CD44^{-/-}$:$OPN^{-/-}$ mice

Cross breeding of $CD44^{-/-}$ and $OPN^{-/-}$ mice resulted in offspring being double-deficient in CD44 and OPN ($CD44^{-/-}$:$OPN^{-/-}$ mice). One example of a typical genotyping result is shown in figure 4.



A



B

Figure 4. Gel-picture of genotyping result of CD44$^{-/-}$ and OPN$^{-/-}$ cross-bred mice after PCR. PCR products have been applied on Agarose-gel (2%). **(A)** CD44 PCR products being generated with knockout primers within the PCR (top of gel picture) and CD44 PCR products, which have been generated with wild-type primers within the PCR (bottom of gel picture). **(B)** OPN PCR products, which have been amplified with the three OPN primers (view Table 5).The first gel-slot of each row has been filled with the standard (100pb-1000bp DNA ladder Peqlab, 500bp being the most prominent band). Numbers above bands represent mouse-id-numbers. Mouse within the red circle is a CD44$^{-/-}$:OPN$^{-/-}$ mouse (PK: (positive control); 653; DKO) and has been used as a positive control within the referring PCRs. Mice in the red square are CD44$^{-/-}$:OPN$^{-/-}$ (DKO = double-knockout mice). H_2O represents the PCR sample in which water has been used as template in the PCR master mix; it represents the negative target control.

The (red circled) mouse number 653 (Fig.4.(A and B)) was a CD44 and OPN double-deficient mouse ($CD44^{-/-}$:$OPN^{-/-}$ mouse), which has been the offspring of one $CD44^{-/-}$ and $OPN^{-/-}$ cross-breeding mating couple. The double-knockout of CD44 and OPN in mouse 653 is shown by the presence of the 280bp long CD44-mutant-allele, the lack of the CD44-wild-type-allele, the presence of the 500bp long osteopontin-knockout-allele and the missing of the OPN-wild-type-allele. The mice with the id-numbers 712, 713, 714, 716, 717, 718 and 719 had the identical genetic profile in the CD44 and the OPN loci like the $CD44^{-/-}$:$OPN^{-/-}$ mouse number 653 and were therefore defined as being double-deficient for both loci. Mice 720, 722, 725, 726 and 727 were heterozygous for CD44 (showing the wild-type and the mutant allele) and heterozygous for OPN (showing the wild-type and the mutant allele). Mouse 721 was heterozygous in the CD44 locus (showing the wild-type and the mutant allele) and deficient in the OPN locus (showing the 500bp mutant allele of OPN and the lacking of the wild-type OPN allele). Mouse 724 was a wild-type mouse (showing the 175bp long wild-type allele of CD44 as well as the 600bp long wild-type allele of OPN). Within this particular genotyping result, allele composition within the CD44 and OPN loci of mouse 715 and 723 has been unclear, because no PCR-products of mouse number 715 have been found and bands of mouse 723 have been considered as being too weak (Fig. 4 (A and B)). Therefore, genotyping for mouse 715 and 723 has been repeated for allelic clarification.

3.1.2 The $CD44^{-/-}$:$OPN^{-/-}$-phenotype in C57Bl6 mice

$CD44^{-/-}$:$OPN^{-/-}$ mice showed identical morphological features and normal species behavior compared to wild-type controls until they were older than 12 months. At an age of over 12 months some (about 30% per genotype) of the $CD44^{-/-}$:$OPN^{-/-}$- and $CD44^{+/-}$:$OPN^{+/-}$ mice started to show hair loss. In most cases hair loss began round the eyes, snout and then continued to spread over the whole mouse body in the time schedule of a few weeks. $CD44^{-/-}$:$OPN^{-/-}$ mice exhibited impaired wound healing properties in case of injury. It sometimes occurred that male siblings, which lived within the same cage, had fights, which resulted in open wounds. In those cases, these males were separated instantly for the prevention of further fights and injury. Open wounds of those separated $CD44^{-/-}$:$OPN^{-/-}$ males took longer before being closed within the healing

process compared to those of wild-types. In wild-type mice, fight related wounds healed within a time range of 10 to 14 days, whereas the healing process in $CD44^{-/-}$:$OPN^{-/-}$ mice often took more than 21 days. $CD44^{-/-}$:$OPN^{-/-}$-, $OPN^{-/-}$- and $CD44^{+/-}$:$OPN^{+/-}$ mice did not show an earlier mortality or prolonged lifetime compared to wild-type controls. But significant differences in peripheral blood composition and in bone marrow cell populations were recognizable in the referring genotypes in reference to wild-type mice.

3.2 Steady-state results

3.2.1 Blood values of aging $CD44^{-/-}$:$OPN^{-/-}$ mice

3.2.1.1 Elevation of thrombocytes in peripheral blood of $CD44^{-/-}$:$OPN^{-/-}$ mice with emphasis on the older age

Over a blood status monitoring time frame of 21 months (performing blood analyses every 3 months, starting with 12 weeks aged mice), differences in blood values between the 4 different genotypes ($CD44^{-/-}$:$OPN^{-/-}$-, $CD44^{+/-}$:$OPN^{+/-}$-, $OPN^{-/-}$- and wild-type control mice ($CD44^{+/+}$:$OPN^{+/+}$)) were recognizable.

During their lifespan $CD44^{-/-}$:$OPN^{-/-}$ mice showed elevated thrombocyte levels in their peripheral blood compared to wild-type control mice, with the highest count of 1.702.000 cells/µl ($P \leq 0{,}0005$) in the 18-21 months old cohort (Fig.6.(B)). Wild-type controls displayed thrombocyte levels between 907.444 to 1.200.000 cells/µl in peripheral blood during their livespan (compare Fig.5. (A-D); Fig.6. (A and B)).

Average thrombocyte cell counts in $CD44^{-/-}$:$OPN^{-/-}$ mice (1.200.000 cells/µl peripheral blood) were significantly elevated ($P \leq 0{,}0005$) compared to wild-type controls (900.000 cells/µl peripheral blood) in the 9 months cohort (Fig.5.(C)), in the 15 months cohort (Fig.6.(A)) and in the 18 till 21 months cohort (Fig.6.(B)). In the age cohort of 18-21 months, $CD44^{-/-}$:$OPN^{-/-}$ mice exhibited the highest thrombocyte count of all age cohorts, with a more than 1,6 times elevated level of thrombocytes (1.702.000 cells/µl) ($P \leq 0{,}0005$) compared to wild-type controls (1.045.230 cells/µl) (Fig.6.(B)). $OPN^{-/-}$ mice revealed a comparable high thrombocyte count (1.645.667 cells/µl; $P \leq 0{,}0005$) in comparison to $CD44^{-/-}$:$OPN^{-/-}$ mice within this age cohort (Fig.6.(B)). Three, six and

twelve months aged animals exhibited (Fig.5(A); (B); (D)) no significant differences in thrombocyte cell counts between all observed genotypes. Mice being double-heterozygous for CD44 and OPN ($CD44^{+/-}$:$OPN^{+/-}$) showed similar thrombocyte levels from 926.250 till 1.137.333 cells/µl blood at the age of 6 (Fig.5.(B)), 9 (Fig.5.(C)) and 15 months (Fig.6. (A)) compared to wild-type controls, without being significant. In the age of 3 (Fig.5.(A)) and 12 months (Fig.5.(D)), $CD44^{+/-}$:$OPN^{+/-}$ mice exhibited a tendency of slightly decreased thrombocyte counts (870.812 cells/µl; 1.134.500 cells/µl) referring to wild-type controls (951.227 cells/µl; 1.237.666 cells/µl). In the age cohort of 15 months $CD44^{+/-}$:$OPN^{+/-}$ mice show comparable thrombocyte levels in peripheral blood (1.137.333 cells/µl) than in wild-type controls (1.116.000 cells/µl) (Fig.6.(A)).

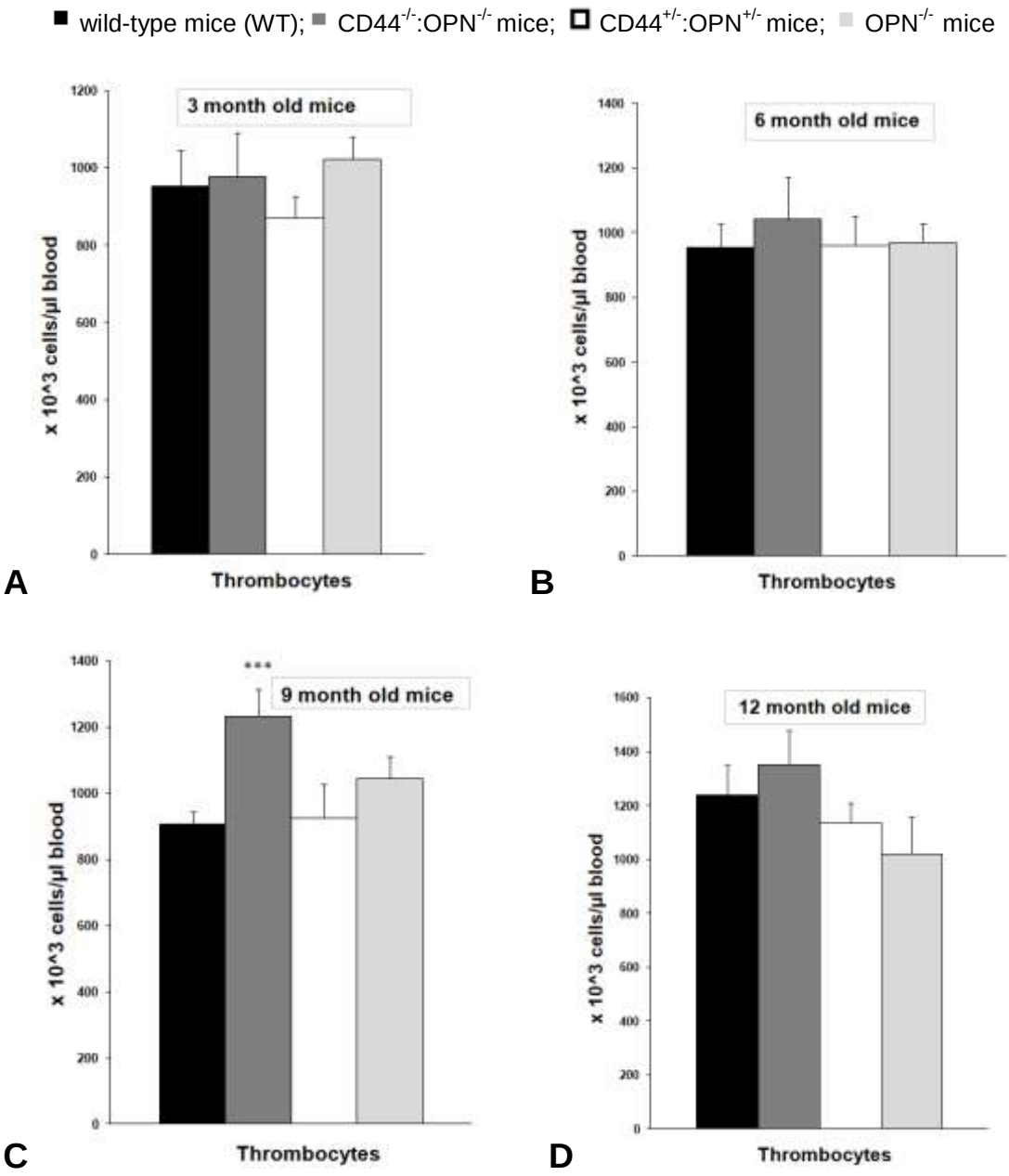


Figure 5. CD44$^{-/-}$:OPN$^{-/-}$ double-deficiency elevates thrombocyte cell counts in peripheral blood of 9 months old mice. (A) 3 months old mice; wild-types: n = 22; CD44$^{-/-}$:OPN$^{-/-}$: n = 30; CD44$^{+/-}$:OPN$^{+/-}$: n = 16; OPN$^{-/-}$: n = 19. **(B)** 6 months old mice; wild-types: n = 18; CD44$^{-/-}$:OPN$^{-/-}$: n = 16; CD44$^{+/-}$:OPN$^{+/-}$: n = 16; OPN$^{-/-}$: n = 20. **(C)** 9 months old mice; wild-types: n = 9; CD44$^{-/-}$:OPN$^{-/-}$: n = 21; CD44$^{+/-}$:OPN$^{+/-}$: n = 8; OPN$^{-/-}$: n = 8. **(D)** 12 months old mice; wild-types: n = 12; CD44$^{-/-}$:OPN$^{-/-}$: n = 22; CD44$^{+/-}$:OPN$^{+/-}$: n = 6; OPN$^{-/-}$: n = 14. ■ Black bar: wild-type mice (WT); ■ dark grey bar: CD44$^{-/-}$:OPN$^{-/-}$ mice; □ white bar: CD44$^{+/-}$:OPN$^{+/-}$ mice; ■ light grey bar: OPN$^{-/-}$ mice. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM; ***$P \leq 0{,}0005$.

At the age of 18-21 months, CD44$^{+/-}$:OPN$^{+/-}$ mice displayed a tendency of a 1,3 times higher thrombocyte cell count (1.383.500 cells/µl) than in controls (1.045.230 cells/µl)

(Fig.6.(B)). Throughout the observed lifespan, thrombocyte levels of $CD44^{+/-}$:$OPN^{+/-}$ mice were always slightly lower than those of $CD44^{-/-}$:$OPN^{-/-}$- and $OPN^{-/-}$ mice (compare Fig.5.(A-D) and Fig.6. (A-B)), without being sifnificant.

At the age of 3 months, $OPN^{-/-}$ mice showed a slightly elevated thrombocyte count in the periperal blood (1.022.231 cells/µl) referring to WT mice (951.227 cells/µl) (Fig.5.(A)). In the 6 months mouse cohort, thrombocyte levels in peripheral blood of $OPN^{-/-}$ mice exhibited an elevated tendency (969.550 cells/µl) referring to controls (953.333 cells/µl) (Fig.5.(B)). 15 months aged $OPN^{-/-}$ mice showed elevated peripheral blood thrombocyte counts (1.228.500 cells/µl) compared to their wild-type counterparts (1.116.000 cells/µl), without being significant (Fig.6.(A)).

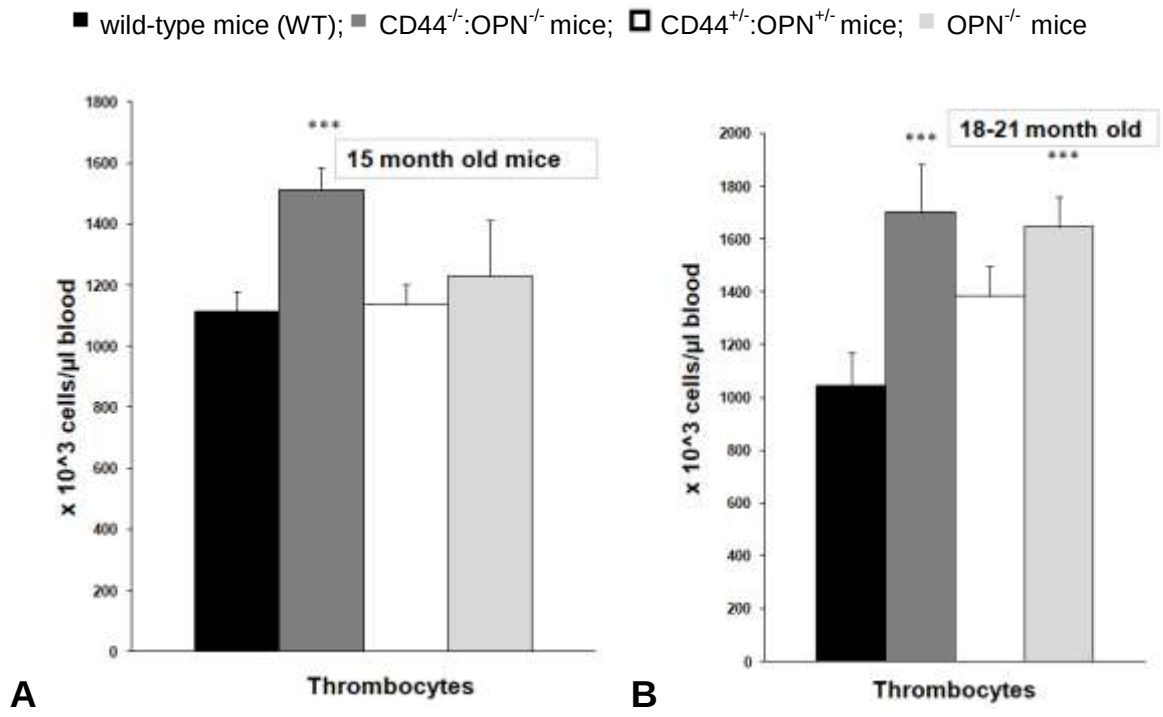


Figure 6. $CD44^{-/-}$:$OPN^{-/-}$ double-deficiency and $OPN^{-/-}$ deficiency elevate thrombocyte cell counts in peripheral blood of old mice. (A) 15 months old mice; WT: n = 11; $CD44^{-/-}$:$OPN^{-/-}$: n = 16; $CD44^{+/-}$:$OPN^{+/-}$: n = 3; $OPN^{-/-}$: n = 10 . **(B)** 18 till 21 months old mice; WT: n = 13; $CD44^{-/-}$:$OPN^{-/-}$: n = 26; $CD44^{+/-}$:$OPN^{+/-}$: n = 4; $OPN^{-/-}$: n = 9. ■Black bar: wild-type mice (WT); ■dark grey bar: $CD44^{-/-}$:$OPN^{-/-}$ mice; □ white bar: $CD44^{+/-}$:$OPN^{+/-}$ mice; ■light grey bar: $OPN^{-/-}$ mice. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM; ***$P \leq 0{,}0005$.

3.2.1.2 CD44$^{-/-}$:OPN$^{-/-}$ double-deficiency affects leukocyte numbers in peripheral blood

Notably, during an investigation of peripheral blood values in the different age cohorts, CD44$^{-/-}$:OPN$^{-/-}$ mice exhibited a significant increased T- and B cell count compared to controls in all evaluated aging stages, except for the 3 months cohort, in which only T cell values were significantly elevated (compare Fig. 7-9).

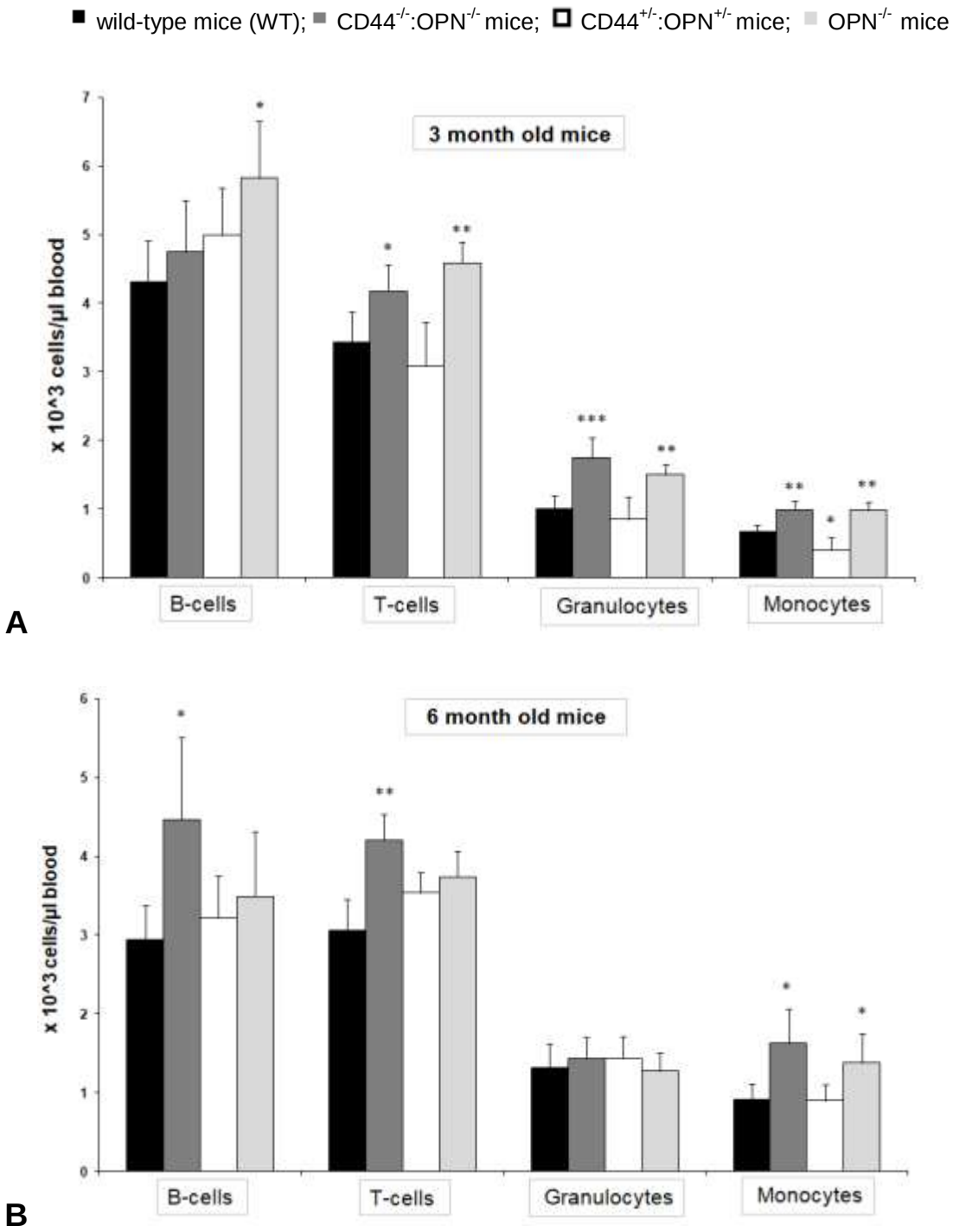


Figure 7. CD44$^{-/-}$:OPN$^{-/-}$ double-deficiency and OPN$^{-/-}$ single-deficiency elevate white blood cell counts in peripheral blood of young mice. Absolute leukocyte cell counts in peripheral blood are shown. **(A)** 3 months old mice. **(B)** 6 months old mice. ■ Black bar: wild-type mice (WT); ■ dark grey bar: CD44$^{-/-}$:OPN$^{-/-}$ mice; □ white bar: CD44$^{+/-}$:OPN$^{+/-}$ mice; ■ light grey bar: OPN$^{-/-}$ mice. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM; n ≥ 16; *P ≤ 0,05; **P ≤ 0,005; ***P ≤ 0,0005.

At the age of 3 months, $CD44^{-/-}$:$OPN^{-/-}$- and $OPN^{-/-}$ mice showed a significantly (more than 1,2 times) higher T cell count (4176 cells/µl; $P \leq 0,05$ and 4590 cells/µl; $P \leq 0,005$) than WT mice (3440 cells/µl) (Fig.7.(A)). B cells levels in blood of $CD44^{-/-}$:$OPN^{-/-}$- and $OPN^{-/-}$ mice exhibited an elevated state compared to wild-types in the 3 months aged cohort without being statistically significant (Fig.7.(A)). Granulocyte and monocyte levels in $CD44^{-/-}$:$OPN^{-/-}$- (1742 cells/µl, $P \leq 0,0005$; 991 cells/µl, $P \leq 0,005$) and $OPN^{-/-}$ mice (1498 cells/µl, $P \leq 0,005$; 981 cells/µl, $P \leq 0,005$) were significantly higher than in WT controls (1017 cells/µl; 683 cells/µl) (Fig.7.(A)). In the 3 months age cohort, $CD44^{+/-}$:$OPN^{+/-}$ mice exhibited the lowest monocyte count of all four genotypes in peripheral blood (395 cells/µl, $P \leq 0,05$) (Fig.7.(A)).

In the 6 months cohort B cells (4464 cells/µl; $P \leq 0,05$), T cells (4207 cells/µl; $P \leq 0,005$) and monocyte levels (1626 cells/µl; $P \leq 0,05$) in peripheral blood of $CD44^{-/-}$:$OPN^{-/-}$ mice were higher than those of WTs (2945 cells/µl , 3060 cells/µl and 905 cells/µl) (Fig.7.(B)). Six months old mice of all 4 gentopyes showed no differences in granulocyte values in peripheral blood. Monocyte counts of $OPN^{-/-}$ mice were with 1378 cells/µl ($P \leq 0,05$) significant higher than in controls (905 cells/µl) (Fig.7.(B)).

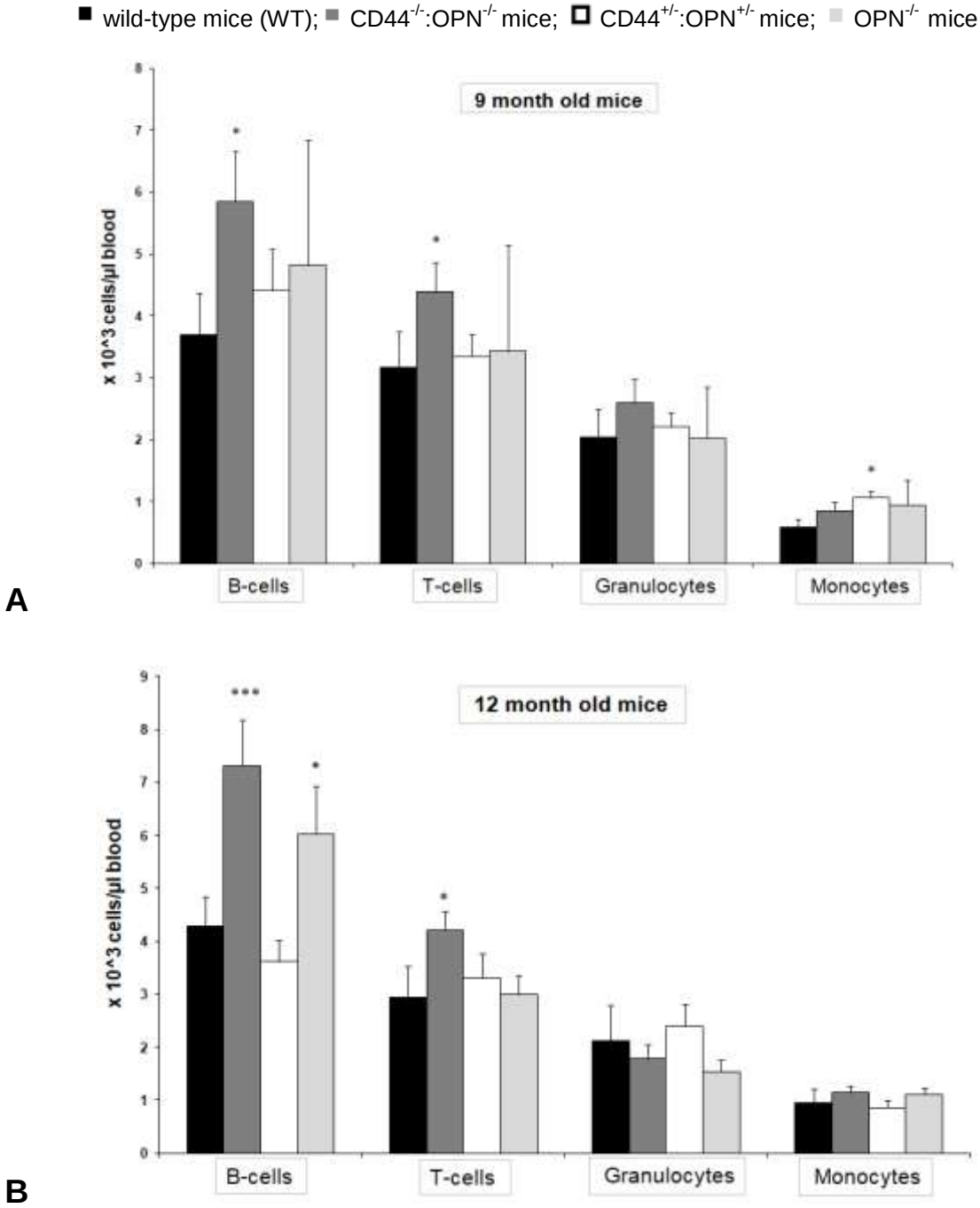
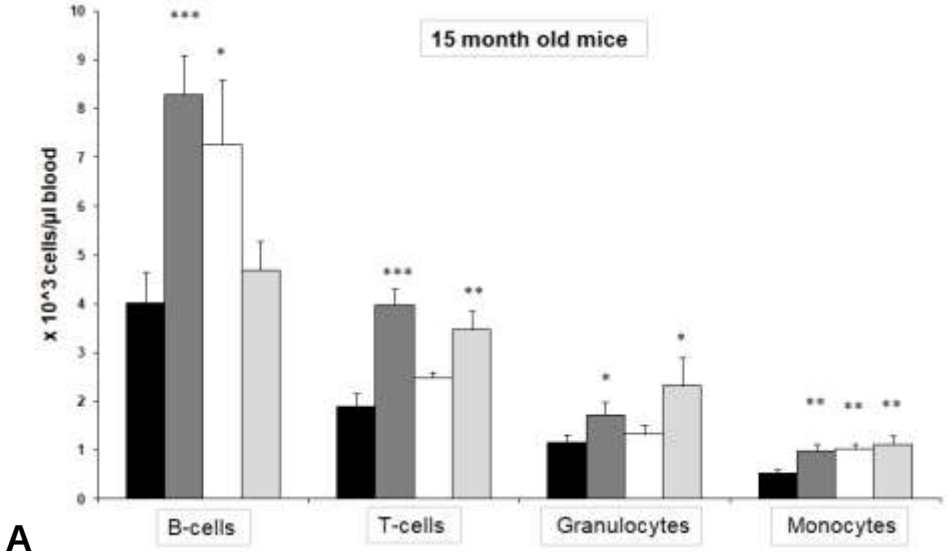


Figure 8. CD44$^{-/-}$:OPN$^{-/-}$ double deficiency elevates B and T cell values in peripheral blood of 9 and 12 months old mice. Absolute leukocyte cell counts in peripheral blood are shown. **(A)** 9 months old mice; WT: n = 9; CD44$^{-/-}$:OPN$^{-/-}$: n = 21; CD44$^{+/-}$:OPN$^{+/-}$: n = 8; OPN$^{-/-}$: n = 8. **(B)** 12 months old mice; WT: n = 12; CD44$^{-/-}$:OPN$^{-/-}$: n = 22; CD44$^{+/-}$:OPN$^{+/-}$: n = 6; OPN$^{-/-}$: n = 13. ■ Black bar: wild-type mice (WT); ■ dark grey bar: CD44$^{-/-}$:OPN$^{-/-}$ mice; □ white bar: CD44$^{+/-}$:OPN$^{+/-}$ mice; ■ light grey bar: OPN$^{-/-}$ mice. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM; *$P \leq 0{,}05$; ***$P \leq 0{,}0005$.

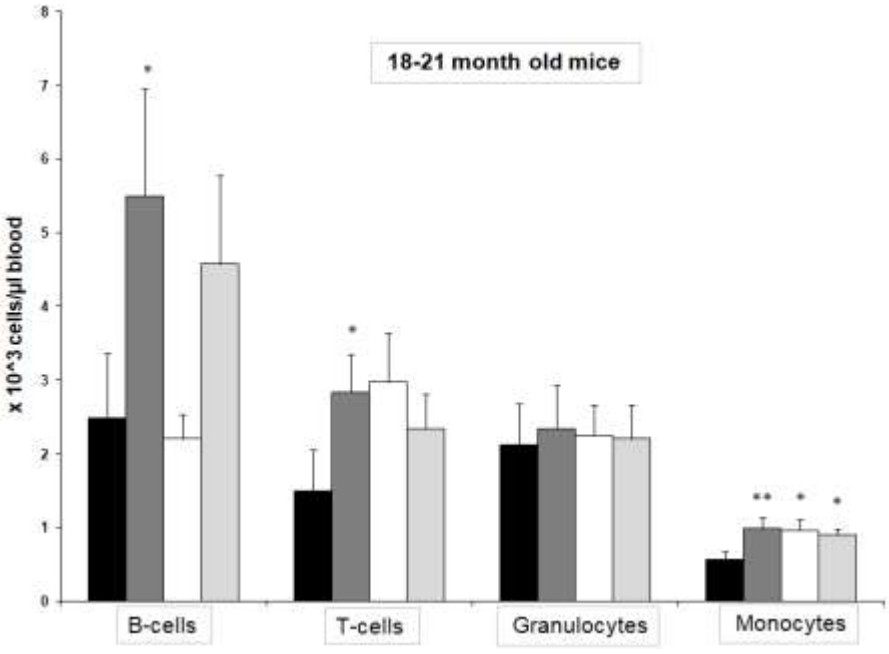
At the age of 9 months, $CD44^{-/-}$:$OPN^{-/-}$ mice displayed significantly more B- (5844 cells/µ; $P \leq 0,05$) and T cells (4390 cells/µl; $P \leq 0,05$) in peripheral blood than WTs (3688 and 3170 cells/µl) (Fig.8.(A)). In this age cohort, the tendency of elevated B- and T cells was also recognizable for $OPN^{-/-}$ mice without being significant. 9 months old $CD44^{+/-}$:$OPN^{+/-}$ mice showed with 1062 cells/µl ($P \leq 0,05$) the highest monocyte count in peripheral blood of the 4 observed genotypes. No significant differences in granulocyte blood counts between $CD44^{-/-}$:$OPN^{-/-}$-, $OPN^{-/-}$-, $CD44^{+/-}$:$OPN^{+/-}$- and control mice have been found (Fig.8.(A)).

In the 12 months cohort, $CD44^{-/-}$:$OPN^{-/-}$ mice exhibited significant more B- (7320 cells/µl; $P \leq 0,0005$) and T cells (4212 cells/µl; $P \leq 0,05$) in peripheral blood than WTs (4292 cells/µl; 2931 cells/µl) (Fig.8.(B)). Granulocyte and monocyte blood cell counts of all 4 observed genotypes within this age group displayed no significant differences (Fig.8.(B)). B cell counts in peripheral blood of $OPN^{-/-}$ mice were significantly elevated (6026 cells/µl) compared to wild-type values (4292 cells/µl; $P \leq 0,05$).

■ wild-type mice (WT); ■ $CD44^{-/-}$:$OPN^{-/-}$ mice; □ $CD44^{+/-}$:$OPN^{+/-}$ mice; ■ $OPN^{-/-}$ mice



A



B

Figure 9. $CD44^{-/-}$:$OPN^{-/-}$ double-deficiency elevates white blood cell counts in peripheral blood of old mice. Absolute leukocyte cell counts in peripheral blood are shown. **(A)** 15 months old mice; WT: n = 11; $CD44^{-/-}$:$OPN^{-/-}$: n = 16; $CD44^{+/-}$:$OPN^{+/-}$: n = 3; $OPN^{-/-}$: n = 10. **(B)** 18 till 21 months old mice; WT: n = 13; $CD44^{-/-}$:$OPN^{-/-}$: n = 26; het: 4; $OPN^{-/-}$: n = 9. ■ Black bar: wild-type mice (WT); ■ dark grey bar: $CD44^{-/-}$:$OPN^{-/-}$ mice; □ white bar: $CD44^{+/-}$:$OPN^{+/-}$ mice; ■ light grey bar= $OPN^{-/-}$ mice. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM; *$P \leq 0,05$; **$P \leq 0,005$; ***$P \leq 0,0005$.

Whereas in the 15 months age cohort only $CD44^{-/-}$:$OPN^{-/-}$ mice showed a more than doubled B cell count (8276 cells/µl, $P \leq 0{,}0005$) in peripheral blood compared to controls (4008 cells/µl), $CD44^{-/-}$:$OPN^{-/-}$- and $OPN^{-/-}$ mice both exhibited elevated T cell- (3972 cells/µl, $P \leq 0{,}0005$; 3460 cells/µl, $P \leq 0{,}005$), granulocyte- (1714 ells/µl, $P \leq 0{,}05$; 2316 cells/µl, $P \leq 0{,}05$) and monocyte counts (983 cells/µl, $P \leq 0{,}005$; 1111 cells/µl, $P \leq 0{,}005$) referring to wild-types (1896, 1158 and 525 cells/µl) (Fig.9.(A)). Only in the 15 months age cohort, $CD44^{+/-}$:$OPN^{+/-}$ mice displayed a 1,8 time higher B-cell blood count (7268 cells/µl; $P \leq 0{,}05$) than WTs (4008 cells/µl) (Fig.9.(A)). Granulocyte blood levels in $OPN^{-/-}$ (2316 cells/µl; $P \leq 0{,}05$) were highest of the observed 4 genotypes in this age group and showed a significantly risen value compared to wild-type controls (Fig.9.(A)). $CD44^{+/-}$:$OPN^{+/-}$ mice exhibited a similar granulocyte cell count (1181 cells/µl) in their peripheral blood than controls (1159 cells/µl).

In the 18-21 months cohort, $CD44^{-/-}$:$OPN^{-/-}$ mice showed elevated B cell (5493 cells/µl; $P \leq 0{,}05$) , T cell (2832 cells/µl; $P \leq 0{,}05$) and monocyte values (979 cells/µl; $P \leq 0{,}05$) in peripheral blood compared to wild-types (Fig.9.(B)). $OPN^{-/-}$ mice exhibited a tendency of B cell count elevation (4571 cells/µl) in comparison to controls, without being significant. Granulocyte counts of $CD44^{-/-}$:$OPN^{-/-}$- (2336 cells/µl), $CD44^{+/-}$:$OPN^{+/-}$- (2446 cells/µl) and $OPN^{-/-}$ (2201 cells/µl) mice were at comparable levels thant those of controls (2117 cells/µl).

3.2.1.3 Elevated erythrocyte values in peripheral blood of 3 months old $OPN^{-/-}$- and 15 months old $CD44^{-/-}$:$OPN^{-/-}$ mice

At the age of 3 months $OPN^{-/-}$ mice exhibited with $9{,}9 \times 10^6$ cells/µl ($P \leq 0{,}05$) the highest erythrocyte value in peripheral blood referring to WT controls ($9{,}1 \times 10^6$cells/µl) (Fig. 10.(A)). No significant differences in erythrocyte blood levels of $CD44^{-/-}$:$OPN^{-/-}$- ($9{,}3 \times 10^6$ cells/µl), $CD44^{+/-}$:$OPN^{+/-}$- ($8{,}9 \times 10^6$ cells/µl) and control mice have been found.

In the age cohorts of 6 (Fig.10.(B)), 9 (Fig.10.(C)) ,12 (Fig.10.(D)) and 18-21 months (Fig.10.(F)), no significant differences in erythrocyte blood cell levels between the 4 observed genotypes has been observed. A tendency of elevated erythrocyte blood

counts in $CD44^{-/-}$:$OPN^{-/-}$- and $CD44^{+/-}$:$OPN^{+/-}$ mice in comparion to wild-types has been observed in the 6- (Fig.10.(B)), 9- (Fig.10.(C)) and 12 months cohort (Fig.10.(D)).

15 months aged $CD44^{-/-}$:$OPN^{-/-}$ mice displayed the highest erythrocyte level ($8{,}8x10^6$ cells/µl; $P \leq 0{,}05$) in peripheral blood, $CD44^{+/-}$:$OPN^{+/-}$ mice a decreased tendency ($8{,}0x10^6$ cells/µl) and $OPN^{-/-}$ mice ($8{,}4x10^6$ cells/µl) an increased tendency referring to controls ($8{,}2x10^6$ cells/µl) (Fig.10.(E)).

18-21 aged mice of all 4 genotypes displayed no significant differences between erythrocyte levels in peripheral blood (WT: $8.5x10^6$ cells/µl; $CD44^{-/-}$:$OPN^{-/-}$: $8{,}7x10^6$ cells/µl; $CD44^{+/-}$:$OPN^{+/-}$ mice: $8{,}1x10^6$ cells/µl and $OPN^{-/-}$: $9{,}4x10^6$ cells/µl) (Fig.10.(F)).

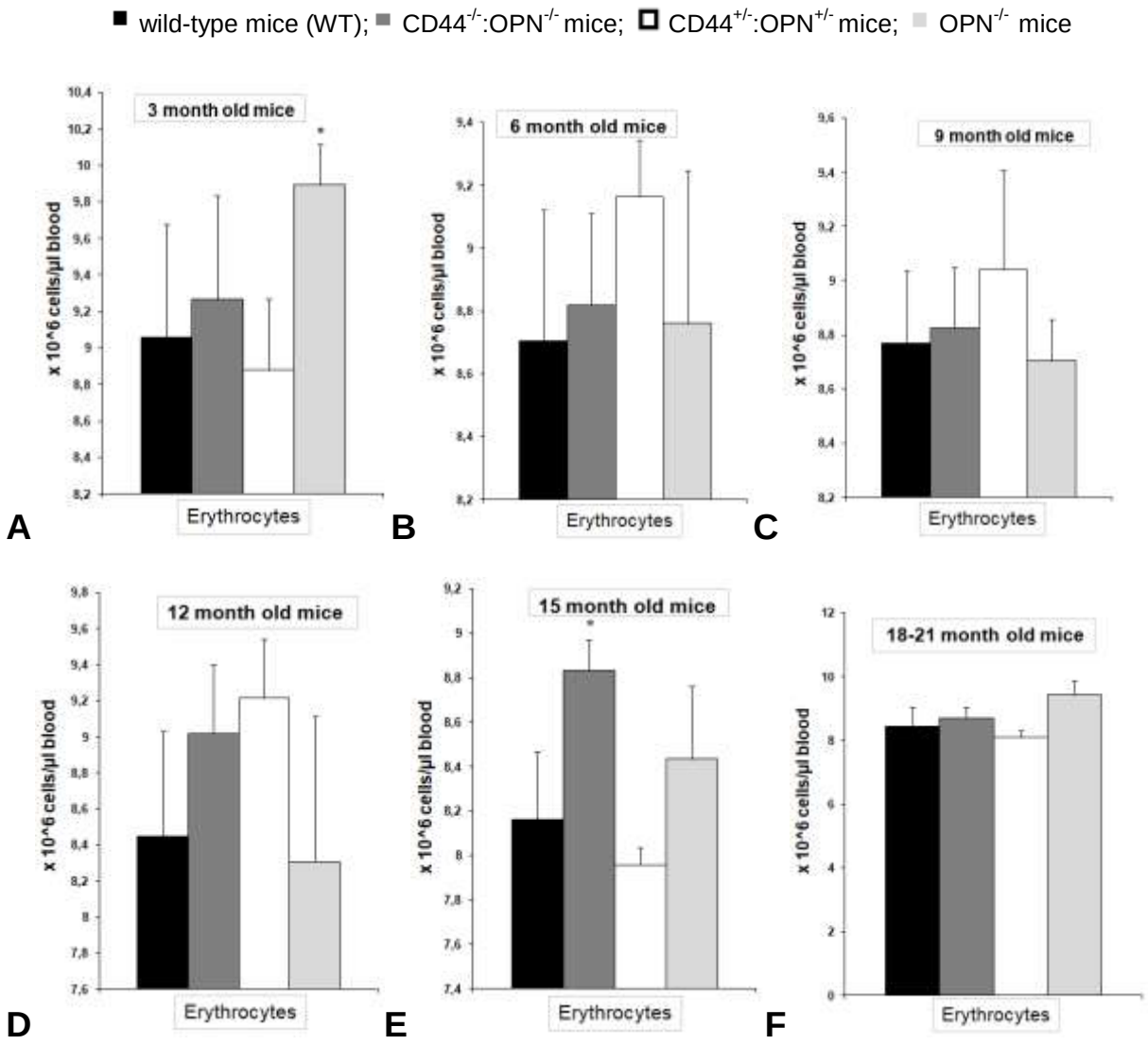


Figure 10. Elevated erythrocyte values in peripheral blood of 3 months old OPN$^{-/-}$- and 15 months old CD44$^{-/-}$:OPN$^{-/-}$ mice. Absolute erythrocyte values in peripheral blood are shown. **(A)** 3 months old mice; WT: n = 22; CD44$^{-/-}$:OPN$^{-/-}$: n = 30; CD44$^{+/-}$:OPN$^{+/-}$ mice: n = 16; OPN$^{-/-}$: n = 19. **(B)** 6 months old mice; WT: n = 18; CD44$^{-/-}$:OPN$^{-/-}$: n = 16; CD44$^{+/-}$:OPN$^{+/-}$ mice: n = 16; OPN$^{-/-}$: n = 20. **(C)** 9 months old mice; WT: n = 9; CD44$^{-/-}$:OPN$^{-/-}$: n = 21; CD44$^{+/-}$:OPN$^{+/-}$ mice: n = 8; OPN$^{-/-}$: n = 8. **(D)** 12 months aged mice; WT: n = 12; CD44$^{-/-}$:OPN$^{-/-}$: n = 22; CD44$^{+/-}$:OPN$^{+/-}$ mice: n = 6; OPN$^{-/-}$: n = 14. **(E)** 15 months old mice; WT: n = 11; CD44$^{-/-}$:OPN$^{-/}$: n = 16; CD44$^{+/-}$:OPN$^{+/-}$ mice: n = 3; OPN$^{-/-}$: n = 10. **(F)** 18 till 21 months old mice; WT: n = 13; CD44$^{-/-}$:OPN$^{-/-}$: n = 26; CD44$^{+/-}$:OPN$^{+/-}$ mice: n = 4; OPN$^{-/-}$: n = 9. ■ Black bar: wild-type mice (WT); ■ dark grey bar: CD44$^{-/-}$:OPN$^{-/-}$ mice; □ white bar: CD44$^{+/-}$:OPN$^{+/-}$ mice; ■ light grey bar= OPN$^{-/-}$ mice. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM; *$P \leq 0{,}05$.

3.2.1.4 $CD44^{-/-}$:$OPN^{-/-}$ double-deficiency decreases hemoglobin levels in peripheral blood of 9 months old mice

Within the age cohorts of 3 (Fig.11.(A)), 6 (Fig.11.(B)), 12 (Fig.11.(D)), 15 (Fig.11.(E)) and 18-21 months (Fig.11.(F)), no significant differences in hemoglobin levels of $CD44^{-/-}$:$OPN^{-/-}$-, $CD44^{+/-}$:$OPN^{+/-}$- and $OPN^{-/-}$ mice in peripheral blood compared to wild-types have been found. In the 9 months age-cohort $CD44^{-/-}$:$OPN^{-/-}$ mice exhibited the lowest hemoglobin count (13,5 µg/µl blood; ($P \leq 0,05$)) compared to $OPN^{-/-}$ (13,7µg/µl), $CD44^{+/-}$:$OPN^{+/-}$- (14,3 µg/µl) and wild-type mice (14,3 µg/µl) (Fig.11(C)).

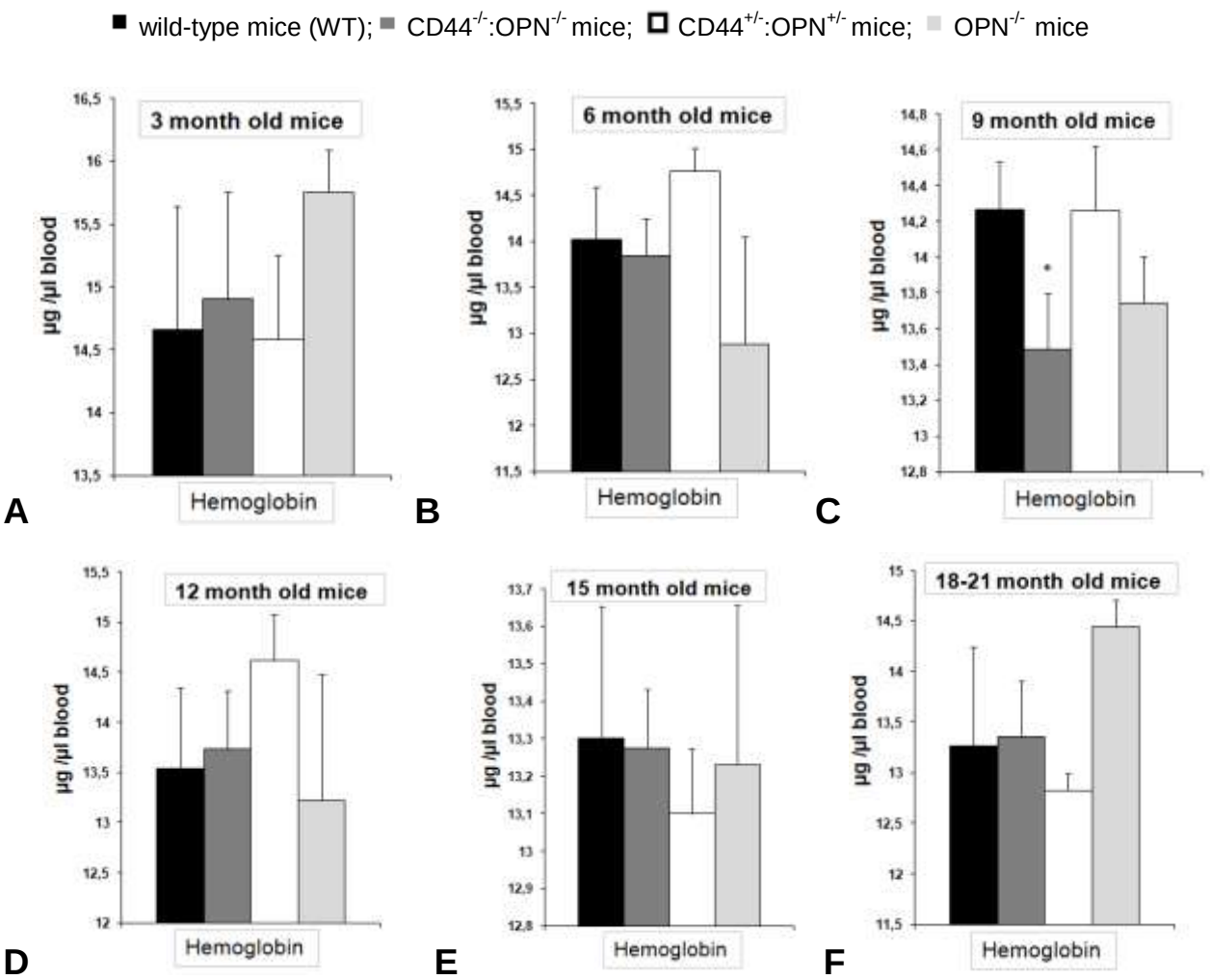


Figure 11. CD44$^{-/-}$:OPN$^{-/-}$ double-deficiency decreases hemoglobin levels in peripheral blood of 9 months old mice. Absolute hemoglobin levels in peripheral blood are bein shown **(A)** 3 months old mice; WT: n = 22; CD44$^{-/-}$:OPN$^{-/-}$: n = 30; CD44$^{+/-}$:OPN$^{+/-}$: n = 16; OPN$^{-/-}$: n = 19. **(B)** 6 months old mice; WT: n = 18; CD44$^{-/-}$:OPN$^{-/-}$: n = 16; CD44$^{+/-}$:OPN$^{+/-}$: n = 16; OPN$^{-/-}$: n = 20. **(C)** 9 months old mice; WT: n = 9; CD44$^{-/-}$:OPN$^{-/-}$: n = 21; CD44$^{+/-}$:OPN$^{+/-}$: n = 8; OPN$^{-/-}$: n = 8. **(D)** 12 months old mice; WT: n = 12; CD44$^{-/-}$:OPN$^{-/-}$: n = 22; CD44$^{+/-}$:OPN$^{+/-}$: n = 6; OPN$^{-/-}$: n = 14. **(E)** 15 months old mice; WT: n = 11; CD44$^{-/-}$:OPN$^{-/-}$: n = 16; CD44$^{+/-}$:OPN$^{+/-}$: n = 3; OPN$^{-/-}$: n = 10. **(F)** 18 till 21 months old mice; WT: n = 13; CD44$^{-/-}$:OPN$^{-/-}$: n = 26; CD44$^{+/-}$:OPN$^{+/-}$: n = 4; OPN$^{-/-}$: n = 9. ■ Black bar: wild-type mice (WT); ■ dark grey bar: CD44$^{-/-}$:OPN$^{-/-}$ mice; □ white bar: CD44$^{+/-}$:OPN$^{+/-}$ mice; ■ light grey bar= OPN$^{-/-}$ mice. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM; *$P \leq 0{,}05$.

3.2.2 BM cell populations of aging CD44$^{-/-}$:OPN$^{-/-}$ mice

3.2.2.1 CD44$^{-/-}$:OPN$^{-/-}$ double-deficiency decreases granulocyte numbers in BM of old mice

In comparison of BM composition between young CD44$^{-/-}$:OPN$^{-/-}$-, OPN$^{-/-}$- and CD44$^{+/-}$:OPN$^{+/-}$ mice with their old counterparts, there was no significant discrepancy between B cell, T cell and monocyte values referring to their wild-type controls (compare Fig.12.(A and B)).

Six months aged CD44$^{-/-}$:OPN$^{-/-}$-, OPN$^{-/-}$- and CD44$^{+/-}$:OPN$^{+/-}$ mice exhibited no significant differnces in B cells, T cells and monocyte values in the BM departement (Fig.12.(A)). BM of all 4 genotypes consisted to approximately 10% of B cells; 1% of T cells and 1,6 % of monocytes.

B cells numbers in BM of old CD44$^{-/-}$:OPN$^{-/-}$- (9,6%) and OPN$^{-/-}$ mice (9,8%) exhibited a tendency of elevation compared to their wild-type counterparts (5,8%) (Fig.12.(B)).

T- and monocyte counts in BM of old CD44$^{-/-}$:OPN$^{-/-}$- (1,6% and 2,6%) and OPN$^{-/-}$ mice (1,6% and 2 %) did not differ significantly from those of controls (1,6% and 2,0%) (Fig.12.(B)).

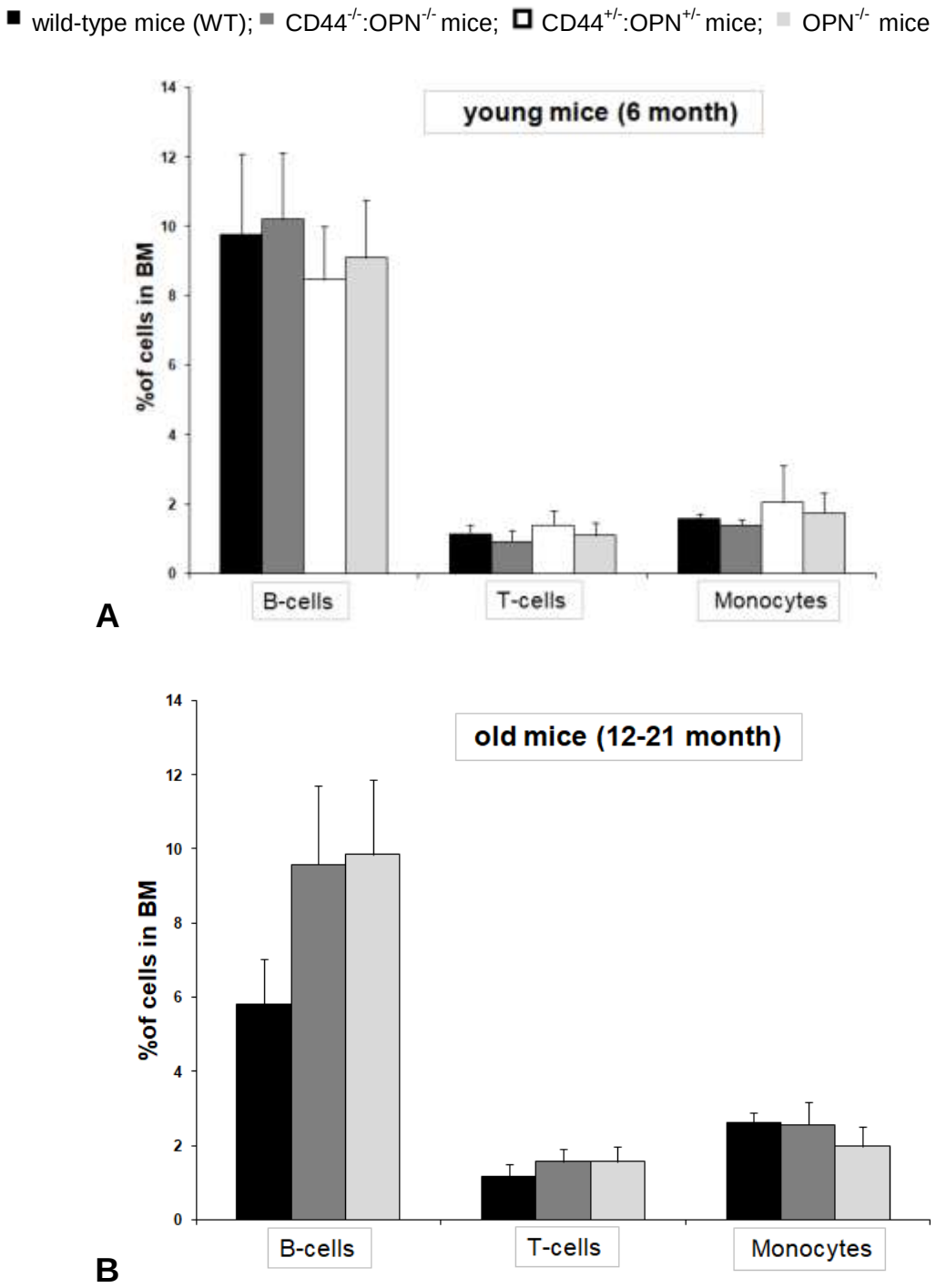


Figure 12. **CD44$^{-/-}$:OPN$^{-/-}$ double-deficiency does not alter B cell, T cell and monocyte numbers in BM of young and old mice. (A)** In young mice (6 months); WT: n = 9; CD44$^{-/-}$:OPN$^{-/-}$: n = 12; CD44$^{+/-}$:OPN$^{+/-}$: n = 8; OPN$^{-/-}$: n = 9. **(B)** In old mice (12-21 months); WT: n = 4; CD44$^{-/-}$:OPN$^{-/-}$: n = 9; OPN$^{-/-}$: n = 6. ■ Black bar: wild-type mice (WT); ■ dark grey bar: CD44$^{-/-}$:OPN$^{-/-}$ mice; □ white bar: CD44$^{+/-}$:OPN$^{+/-}$ mice; ■ light grey bar= OPN$^{-/-}$ mice. Percentage of cells in BM is referring to the extracted BM of 2 femurs, 2 tibiae and 2 fibulae per mouse. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM.

In young CD44$^{-/-}$:OPN$^{-/-}$-, CD44$^{+/-}$:OPN$^{+/-}$- and OPN$^{-/-}$ mice no significant differences in granulocyte and erythroid cells levels in BM referring to wild-types were observable (Fig.13.(A)). All 4 genotypes exhibited BM granulocyte values ranging betweeen 45,3% (CD44$^{-/-}$:OPN$^{-/-}$) and 50,8% (OPN$^{-/-}$). Erythroid cells in BM of young CD44$^{-/-}$:OPN$^{-/-}$ mice displayed a tendency of elevation (18,2%) referring to wild-types (14,1%), whereas CD44$^{+/-}$:OPN$^{+/-}$- (13,9%) and OPN$^{-/-}$ mice (13,1%) showed similar values than wild-types (Fig.13.(A)).

In BM of old CD44$^{-/-}$:OPN$^{-/-}$ mice granulocytes were significantly decreased in numbers (43,1%; $P \leq 0{,}0005$) referring to their wild-type controls (60%) (Fig.13.(B)). Old OPN$^{-/-}$ mice showed a tendency of granulocyte number reduction in BM (51,8%) compared to wild-types (60%). Erythroid cells in BM of old CD44$^{-/-}$:OPN$^{-/-}$- and OPN$^{-/-}$ mice displayed a slight tendency of being elevated (19,1% and 18,4% respectively) relating to controls (12,7%) (Fig.13.(B)).

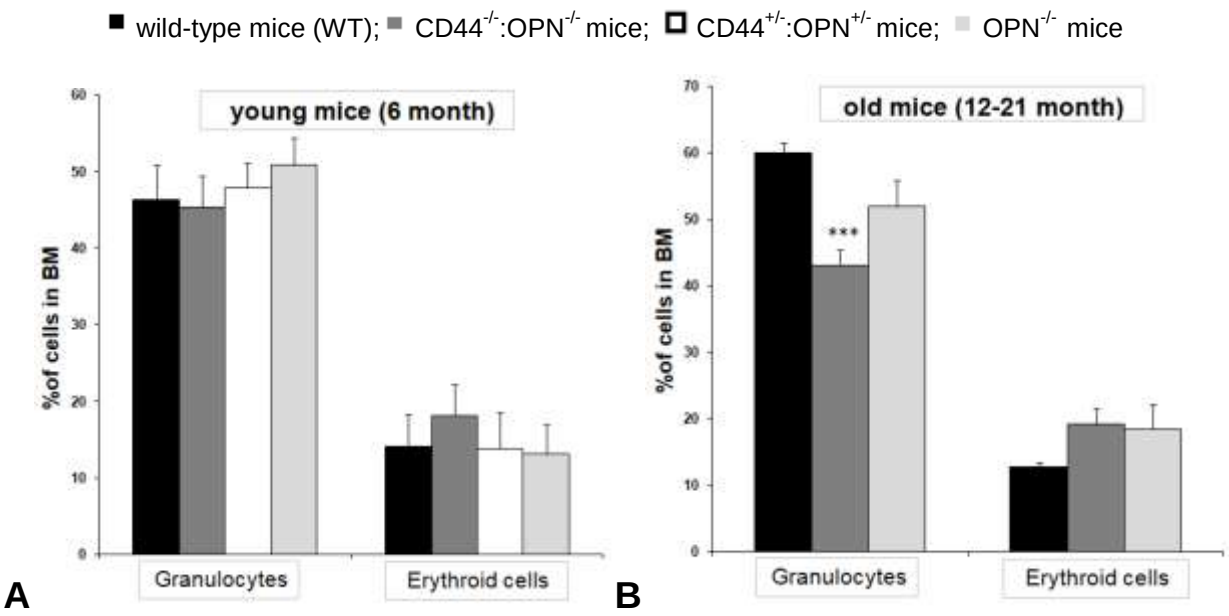


Figure 13. CD44$^{-/-}$:OPN$^{-/-}$ double-deficiency decreases granulocyte numbers in BM of old mice. (A) Granulocytes and erythroid cells in young mice (6 months old); WT: n = 9; CD44$^{-/-}$:OPN$^{-/-}$: n = 12; CD44$^{+/-}$:OPN$^{+/-}$: n = 8; OPN$^{-/-}$: n = 9. **(B)** Granulocytes and erythroid cells in old mice (12-21 months); WT: n = 4; CD44$^{-/-}$:OPN$^{-/-}$: n = 9; OPN$^{-/-}$: n = 6. ■Black bar: wild-type mice (WT); ■dark grey bar: CD44$^{-/-}$:OPN$^{-/-}$ mice; □ white bar: CD44$^{+/-}$:OPN$^{+/-}$ mice; ■light grey bar= OPN$^{-/-}$ mice. Percentage of cells in BM is referring to the extracted BM of 2 femurs, 2 tibiae and 2 fibulae per mouse. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM; ***$P \leq 0{,}0005$.

3.2.2.2 Diminished LKS counts in BM of old CD44$^{-/-}$:OPN$^{-/-}$ mice

Six months aged CD44$^{-/-}$:OPN$^{-/-}$-, OPN$^{-/-}$- and CD44$^{+/-}$:OPN$^{+/-}$ mice displayed no significant differences in LKS and MPP numbers within the BM departement compared to controls (Fig.14.(A)). Within this age cohort, LKS and MPP counts in BM of CD44$^{-/-}$:OPN$^{-/-}$- (0,4%; 0,3%) and OPN$^{-/-}$ mice (0,4%; 0,2%) were at comparable levels than those of wild-types (0,4%; 0,25%), whereas CD44$^{+/-}$:OPN$^{+/-}$ mice showed an elevated tendency (0,5%; 0,3%) (Fig.14.(A)).

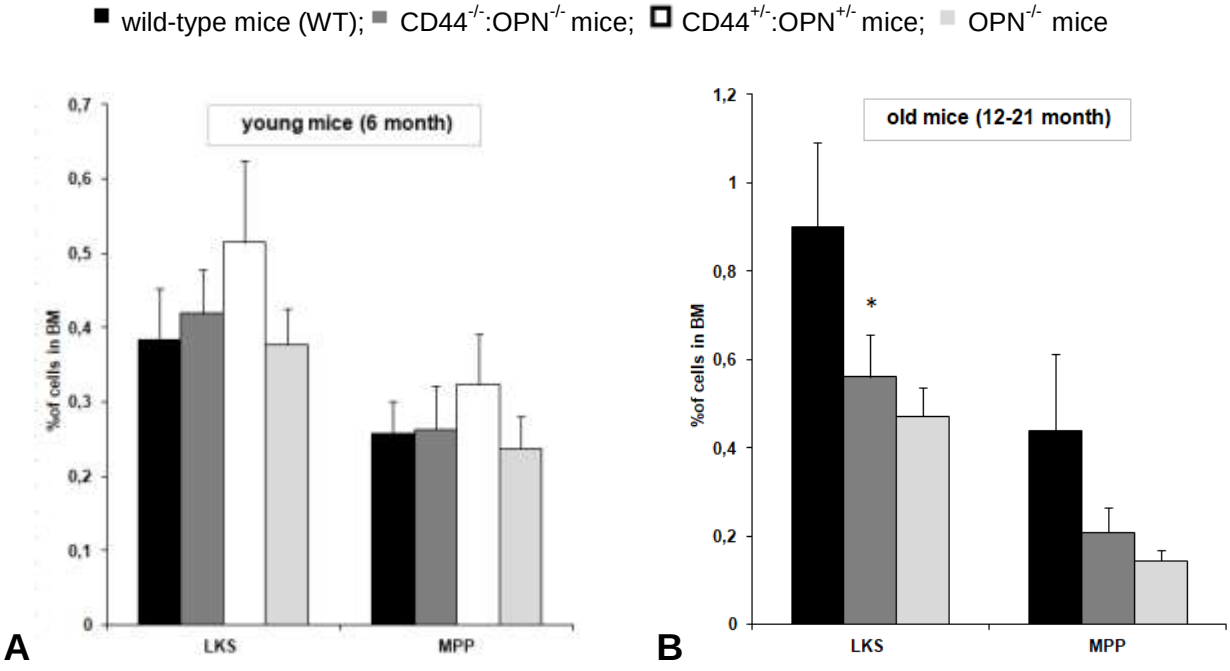


Figure 14. CD44$^{-/-}$:OPN$^{-/-}$ double-deficiency reduces LKS cell numbers in BM of old mice. (A) LKS and MPP of young mice (6 months); WT: n = 13; CD44$^{-/-}$:OPN$^{-/-}$: n = 19; CD44$^{+/-}$:OPN$^{+/-}$: n = 8; OPN$^{-/-}$: n = 13. **(B)** LKS and MPP of old mice (12-21 months); WT: n = 7; CD44$^{-/-}$:OPN$^{-/-}$: n = 15; OPN$^{-/-}$: n = 6. ■ Black bar: wild-type mice (WT); ■ dark grey bar: CD44$^{-/-}$:OPN$^{-/-}$ mice; □ white bar: CD44$^{+/-}$:OPN$^{+/-}$ mice; ■ light grey bar= OPN$^{-/-}$ mice. Percentage of cells in BM is referring to the extracted BM of 2 femurs, 2 tibiae and 2 fibulae per mouse. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM; *$P \leq 0,05$.

Old wild-type mice displayed a more than doubled LKS count (0,9%) in BM than young mice (0,4%) of this genotype (compare Fig.14.(A and B)). Old CD44$^{-/-}$:OPN$^{-/-}$ mice`s LKS cells in BM were significantly reduced to 0,6% ($P \leq 0,05$) compared controls (0,9%) (Fig.14.(B)). LKS cells in BM of old OPN$^{-/-}$ showed a tendency of reduction (0,5%) referring to controls (Fig.14.(B)). MPPs in BM of old CD44$^{-/-}$:OPN$^{-/-}$- (0,21%) and OPN$^{-/-}$

mice (0,14%) showed less than half the value of wild-types (0,44 %) without being significantly reduced (Fig.14.(B)).

Whereas in BM of young CD44$^{-/-}$:OPN$^{-/-}$- and OPN$^{-/-}$ mice LKS cell- and MPP numbers did not differ from those of wild-type controls, these two cell populations exhibited a tendency of reduction in the old mice cohort; but only LKS cell counts in BM of old CD44$^{-/-}$:OPN$^{-/-}$ mice were significantly downscaled (compare Fig.14.(A and B)).

3.2.2.3 CD44$^{-/-}$:OPN$^{-/-}$ double-deficiency does not alter HSC numbers in BM of young and old mice

HSCs counts in BM of young CD44$^{-/-}$:OPN$^{-/-}$- (0,04%), CD44$^{+/-}$:OPN$^{+/-}$- (0,04%) and OPN^{--} mice (0,04%) did not differ significantly from wild-types (0,04%) (Fig. 15. (A)). In the old mice cohort, no difference of HSCs numbers in BM of CD44$^{-/-}$:OPN$^{-/-}$- (0,13%), OPN$^{-/-}$- (0,16%) and wild-type control mice (0,1%) has been found (Fig. 15.(B)).

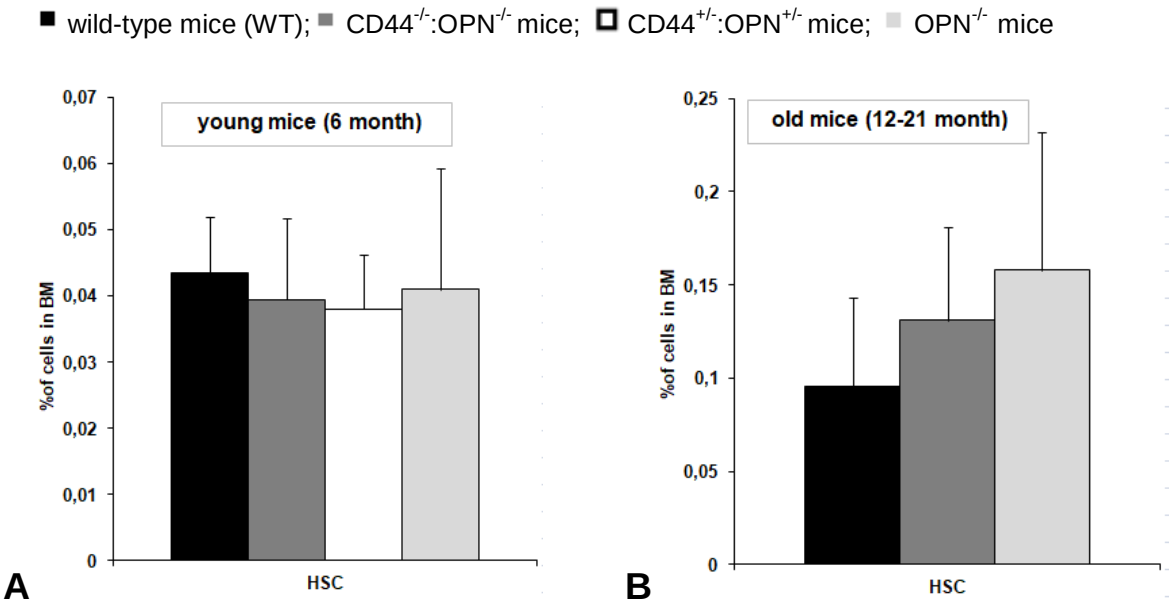


Figure 15. CD44$^{-/-}$:OPN$^{-/-}$ double-deficiency does not alter HSC numbers in BM of young and old mice. (A) HSC in young mice (6 months); WT: n = 13; CD44$^{-/-}$:OPN$^{-/-}$: n = 19; CD44$^{+/-}$:OPN$^{+/-}$: n = 8; OPN$^{-/-}$: n = 12. **(B)** HSC in old mice (12-21 months); WT: n = 7; CD44$^{-/-}$:OPN$^{-/-}$: n = 15; OPN$^{-/-}$: n = 6. ■ Black bar: wild-type mice (WT); ■ dark grey bar: CD44$^{-/-}$:OPN$^{-/-}$ mice; □ white bar: CD44$^{+/-}$:OPN$^{+/-}$ mice; ■ light grey bar= OPN$^{-/-}$ mice. Percentage of cells in BM is referring to the extracted BM of 2 femurs, 2 tibiae and 2 fibulae per mouse. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM.

3.2.2.4 $CD44^{-/-}$:$OPN^{-/-}$ double-deficiency does not alter progenitor composition in BM of young and old mice

No significant differences of CLP, GMP, CMP and MEP numbers in BM between $CD44^{-/-}$:$OPN^{-/-}$-, $OPN^{-/-}$-, $CD44^{+/-}$:$OPN^{+/-}$- and wild-type mice have been found in the young cohort (Fig.16.(A and B). Furthermore, CLP (Fig.16.(C)), GMP, CMP and MEP ((Fig.16.(D)) numbers in BM of 12- 21 old $CD44^{-/-}$:$OPN^{-/-}$- and $OPN^{-/-}$ mice were not significantly differing from wild-type control values.

In six months aged mice, common lymphoid progenitors (CLP) and granulocyte-monocyte progenitors (GMP) in the BM of $CD44^{-/-}$:$OPN^{-/-}$- (CLP: 0,32%; GMP: 0,34 %) and $OPN^{-/-}$ mice (CLP: 0,26%; GMP: 0,35%) showed a tendency of being elevated compared to WT controls (CMP: 0,23%; GMP: 0,28%) (Fig.16.(A and B)). Young $CD44^{+/-}$:$OPN^{+/-}$ mice exhibited the lowest value of CLP- (0,17%), GMP- (0,19%) and MEP numbers (0,1%) in BM compared to the three other genotypes within this age cohort. CMP values of the 4 analysed genotypes displayed being in a very similar range (WT: 0,17%; $CD44^{-/-}$:$OPN^{-/-}$: 0,13%; $CD44^{+/-}$:$OPN^{+/-}$: 0,16% and $OPN^{-/-}$: 0,21%).

Old $CD44^{-/-}$:$OPN^{-/-}$- and $OPN^{-/-}$ mice displayed comparable GMP ($CD44^{-/-}$:$OPN^{-/-}$: 0,07%; $OPN^{-/-}$: 0,07%) and MEP ($CD44^{-/-}$:$OPN^{-/-}$: 0,1%; $OPN^{-/-}$: 0,08%) values in BM than wild-type controls (GMP: 0,09% ; MEP: 0,1%) (Fig.16.(D)). CLP counts in BM of old $OPN^{-/-}$ mice (0,44%) were similar to those of wild-types (0,39%), while $CD44^{-/-}$:$OPN^{-/-}$ mice`s (0,29%) were slightly reduced (Fig.16.(C)). CMP counts in BM of old $CD44^{-/-}$:$OPN^{-/-}$- (0,11%) and $OPN^{-/-}$ mice (0,08%) exhibited lower numbers than those of old control mice (0,2%) (Fig.16.(D)).

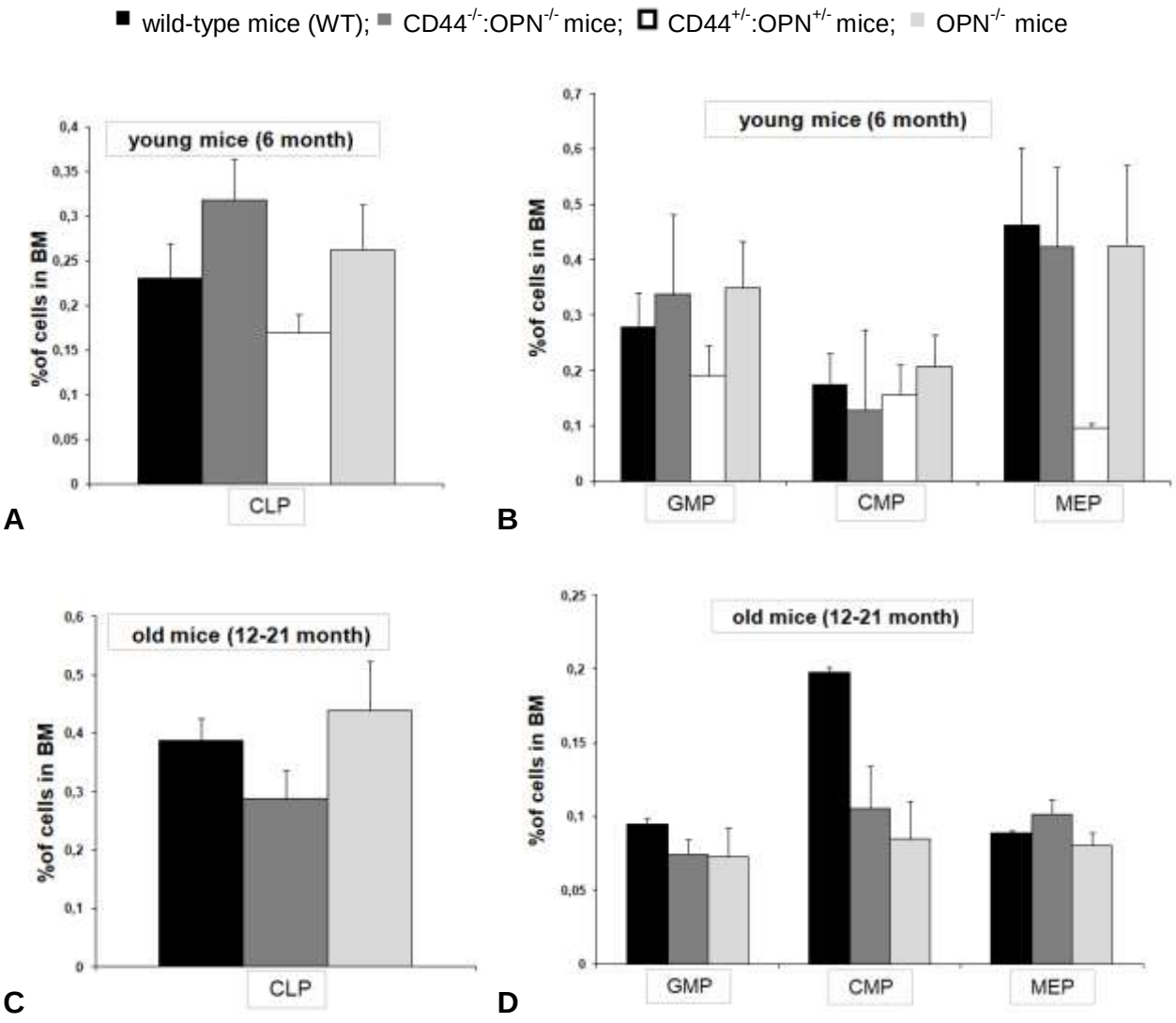


Figure 16. CD44$^{-/-}$:OPN$^{-/-}$ double-deficiency does not alter CLP, GMP, CMP and MEP numbers in BM of young and old mice. (A) Common lymphoid progenitors (CLP) in BM of young mice (age: 6 months); WT: n = 4; CD44$^{-/-}$:OPN$^{-/-}$: n = 7; CD44$^{+/-}$:OPN$^{+/-}$: n = 3; OPN$^{-/-}$: n = 4. **(B)** Granulocyte-monocyte progenitors (GMP), Common myeloid progenitors (CMP) and Megakaryocyte-erythroid progenitors (MEP) in BM of young mice (age: 6 months); WT: n = 8; CD44$^{-/-}$:OPN$^{-/-}$: n = 11; het: n = 3; OPN$^{-/-}$: n = 8 **(C)** Common lymphoid progenitors (CLP) in BM of old mice (age: 12-21 months); WT: n = 2; CD44$^{-/-}$:OPN$^{-/-}$: n = 5; OPN$^{-/-}$: n = 4. **(D)** Granulocyte-monocyte progenitors (GMP), Common myeloid progenitors (CMP) and Megakaryocyte-erythroid progenitors (MEP) in BM of old mice (age: 12-21 months); WT: n = 2; CD44$^{-/-}$:OPN$^{-/-}$: n = 5; OPN$^{-/-}$: n = 4. ■ Black bar: wild-type mice (WT); ■ dark grey bar: CD44$^{-/-}$:OPN$^{-/-}$mice; □ white bar: CD44$^{+/-}$:OPN$^{+/-}$ mice; ■ light grey bar: OPN$^{-/-}$ mice. Percentage of cells in BM is referring to the extracted BM of 2 femurs, 2 tibiae and 2 fibulae per mouse. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM.

3.2.2.5 OPN$^{-/-}$ deficiency enhances HSC proliferation in BM of young mice and shows a tendency of enhanced MPP proliferation in old mice

Young CD44$^{-/-}$:OPN$^{-/-}$- (9%), CD44$^{+/-}$:OPN$^{+/-}$- (9,2%) and OPN$^{-/-}$ mice (10%; $P \leq 0,05$) mice showed a two times higher proliferating HSC-rate than their wild-type counterparts (3,7%) (Fig.17.(A)). Old CD44$^{-/-}$:OPN$^{-/-}$- (12,2%), old CD44$^{+/-}$:OPN$^{+/-}$- (12,5%) and OPN$^{-/-}$ mice (14,8%) exhibited a tendency of elevated proliferation referring to wild-types (6,6%), without being significant (Fig.17.(B)).

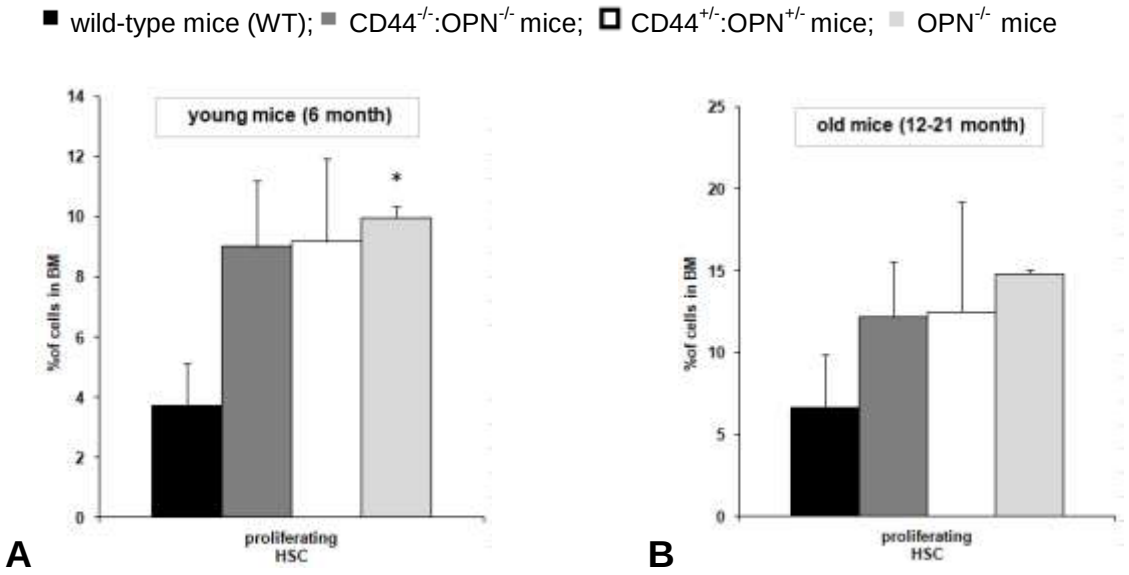


Figure 17. OPN$^{-/-}$ deficiency enhances HSC proliferation in BM of young mice. (A) proliferating of hematopoietic stem cells (HSC) in BM of young mice (age: 6 months); WT: n = 5; CD44$^{-/-}$:OPN$^{-/-}$: n = 9; het: n = 4; OPN$^{-/-}$: n = 3. **(B)** proliferating HSC in old mice (12-21 months old mice); WT: n = 5; CD44$^{-/-}$:OPN$^{-/-}$: n = 5; CD44$^{+/-}$:OPN$^{+/-}$: n= 2; OPN$^{-/-}$: n = 2. ■ Black bar: wild-type mice (WT); ■ dark grey bar: CD44$^{-/-}$:OPN$^{-/-}$ mice; □ white bar: CD44$^{+/-}$:OPN$^{+/-}$ mice; ■ light grey bar: OPN$^{-/-}$ mice. Percentage of cells in BM is referring to the extracted BM of 2 femurs, 2 tibiae and 2 fibulae per mouse. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM; *$P \leq 0,05$.

MPP proliferation in BM varies did not vary significantly between 6 months aged CD44$^{-/-}$:OPN$^{-/-}$- (33%), CD44$^{+/-}$:OPN$^{+/-}$- (36%) and OPN$^{-/-}$ mice (30%), referring to wild-types (33%) (Fig.18(A)). In BM of old OPN$^{-/-}$ mice MPPs displayed the highest proliferation (51 %) of all 4 observed genotypes (Fig.18(B)). This represented a 15 % higher proliferation of MPPs than controls (36%). BM Values of proliferating MPPs in

$CD44^{-/-}$:$OPN^{-/-}$- (32%) and $CD44^{+/-}$:$OPN^{+/-}$ mice (24%) exhibited a decreased tendency referring to controls (36%) without being significant (Fig.18.(B)).

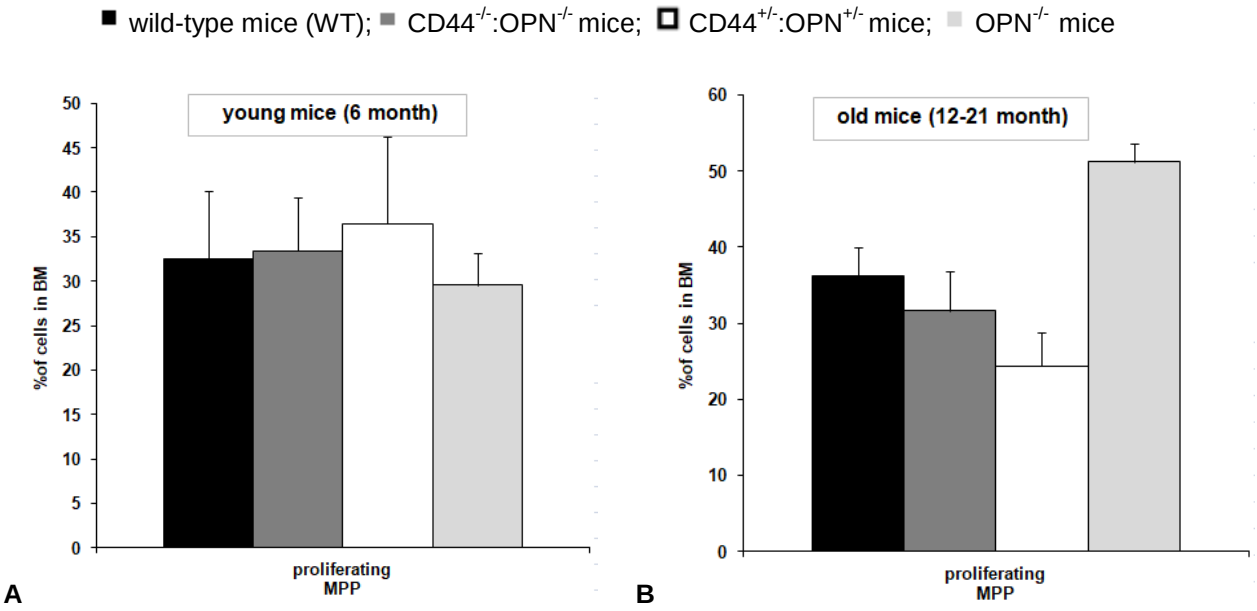


Figure 18. Tendency of enhanced MPP proliferation in BM of old $OPN^{-/-}$ deficient mice. (A) proliferating multipotent progenitors (MPP) in young mice (age: 6 months); WT: n = 5; $CD44^{-/-}$:$OPN^{-/-}$ mice: n = 9; $CD44^{+/-}$:$OPN^{+/-}$ mice: n = 4; $OPN^{-/-}$: n = 3. **(B)** proliferating MPP in old mice (12-21 months old mice); WT: n = 5; $CD44^{-/-}$:$OPN^{-/-}$ mice: n = 10; $CD44^{+/-}$:$OPN^{+/-}$: n = 2; $OPN^{-/-}$: n = 3. ■ Black bar: wild-type mice (WT); ■ dark grey bar: $CD44^{-/-}$:$OPN^{-/-}$ mice; □ white bar: $CD44^{+/-}$:$OPN^{+/-}$ mice; ■ light grey bar= $OPN^{-/-}$ mice. Percentage of cells in BM is referring to the extracted BM of 2 femurs, 2 tibiae and 2 fibulae per mouse. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM.

3.3 Stress-state results

3.3.1 Serial stem cell transplantation of young $CD44^{-/-}$:$OPN^{-/-}$-BM-donor cells into wild-type-BM-recipients

To investigate the impact of $CD44^{-/-}$:$OPN^{-/-}$ deficiency on the hematopoietic reconstitution ability of lethally irradiated mice, bone marrow cells from 3 months old $CD44^{-/-}$:$OPN^{-/-}$ mice have been transplanted into lethally irradiated wild-type recipients. A congenic mouse system was utilized: donor cells expressed the pan-hematopoietic surface marker CD45.1 and were transplanted into CD45.2-bearing hosts. Peripheral blood and bone marrow reconstitution ability has been monitored for the duration of three serial transplant rounds.

One $CD44^{-/-}$:$OPN^{-/-}$-BM-recipient mouse has died 11 days after the 3rd round of serial transplant. 4 weeks after transplant round 3, two of the $CD44^{-/-}$:$OPN^{-/-}$-BM-recipient mice have been very sick. One of these mice has been very thin and was sitting lethargic in the corner of the cage, while the other 4 mice in this cage were showing normal behavior. One other mouse showed neurological abnormal behavior (shaky movement). Therefore both sick $CD44^{-/-}$:$OPN^{-/-}$-BM recipient mice were killed instantly for relieving them.

3.3.2 Peripheral blood results

3.3.2.1 Inferior thrombocyte reconstitution ability in $CD44^{-/-}$:$OPN^{-/-}$-BM-recipient mice

Six (Fig.19.(A)) and 12 weeks (Fig.19. (B)) after the 1st round of serial transplantation, thrombocyte levels in peripheral blood of $CD44^{-/-}$:$OPN^{-/-}$-BM-recipient mice showed no significant differences to wild-type-BM-recipients (controls).

A significant ($P \leq 0,05$) reduction of $CD44^{-/-}$:$OPN^{-/-}$-BM-recipient mice`s thrombocyte levels (992.857 cells/µl) in peripheral blood, firstly occurred 16 weeks after 1st round of transplant referring to wild-types (1.104.714 cells/µl) (Fig.19.(C)). Reconstitution ability of $CD44^{-/-}$:$OPN^{-/-}$-BM-recipient mice`s thrombocytes has been impaired at this time point for the first time during a 3 round serial transplantation setting (Fig.19.(C)).

Four weeks after 2nd round of transplant, thrombocyte levels of WT-BM-recipients (251.600 cells/µl) and $CD44^{-/-}$:$OPN^{-/-}$-BM-recipients (282.610 cells/µl) were more than 50% lower than thrombocyte levels of those genotypes at 6 weeks after 1st round of transplant (584.600 cells/µl and 702.900 cells/µl, respectively) (compare Fig.19.(D) with (A)).

No significant differences in thrombocyte numbers within peripheral blood between $CD44^{-/-}$:$OPN^{-/-}$-BM-recipients (282.610 cells/µl) and control mice (251.600 cells/µl) have been found 4 weeks after 2nd round of serial transplantation (Fig.19.(D)).

■ wild-type-BM-recipient mice (controls); ■ CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice.

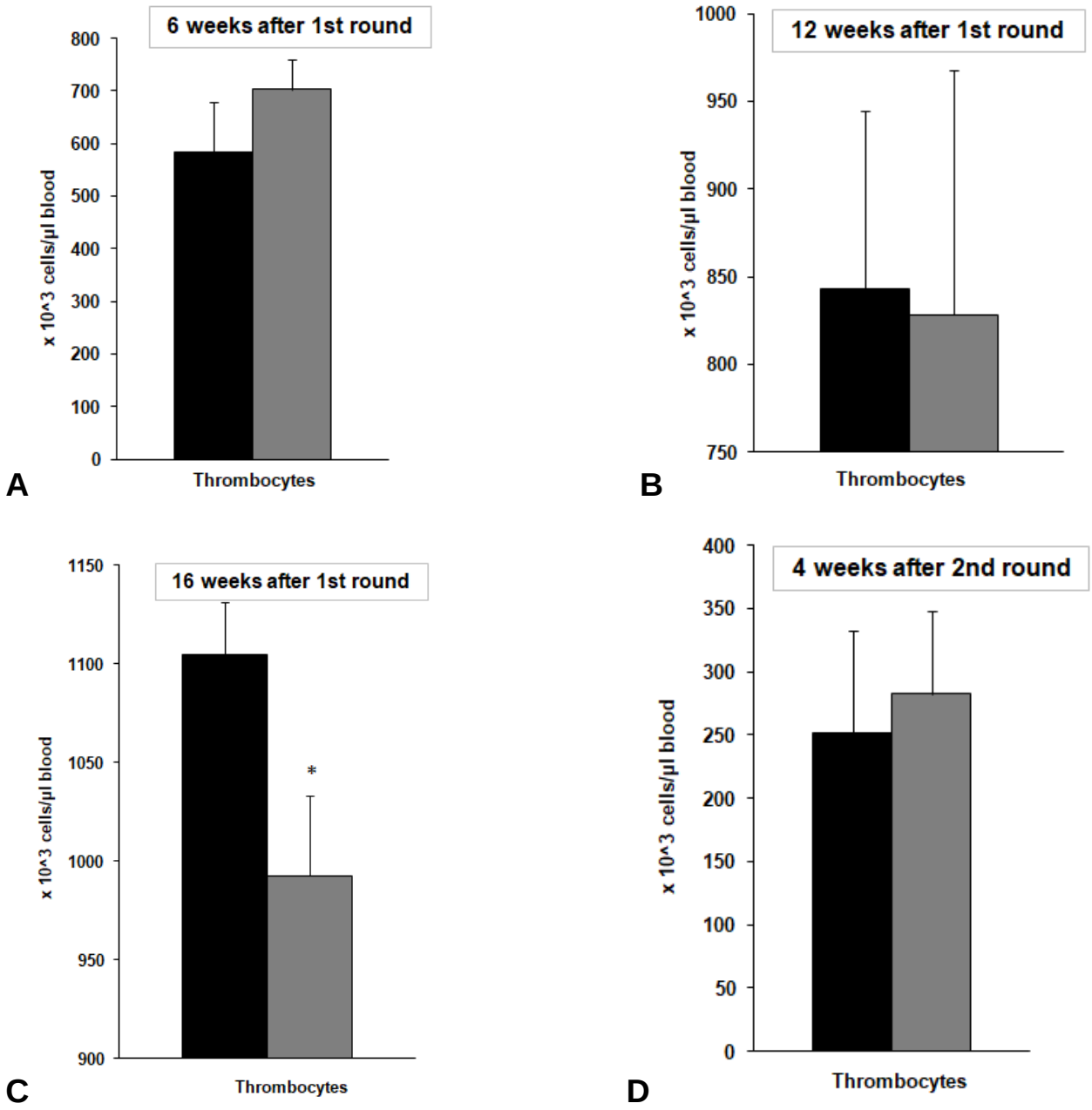


Figure 19. Inferior reconstitution of blood thrombocyte levels at 16 weeks after 1st round of serial transplantation in CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice. Absolute cell counts of donor cells expressing the pan-hematopoietic surface marker CD45.1 in peripheral blood of recipient mice are shown. **(A)** 6 weeks after 1st round of serial transplant; n = 10. **(B)** 12 weeks after 1st round of serial transplant; n = 7. **(C)** 16 weeks after 1st round of serial transplant; n = 7. **(D)** 4 weeks after 2nd round of serial transplant; n = 10. ■ Black bar: wild-type-BM-recipient mice (controls); ■ dark grey bar: CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM; *$P \leq 0{,}05$.

Eight, 12 and 16 after 2nd round of transplant, no differences in thrombocyte counts in peripheral blood of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice in comparison to controls were observable (Fig.20.(A, B and C)). 16 weeks after 2nd round of transplant thrombocyte levels of CD44$^{-/-}$:OPN$^{-/-}$ mice (836.140 cells/µl) were significant higher than in controls (541.000 cells/µl; $P \leq 0{,}05$) (Fig.20.(C)).

■ wild-type-BM-recipient mice (controls); ■ CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice.

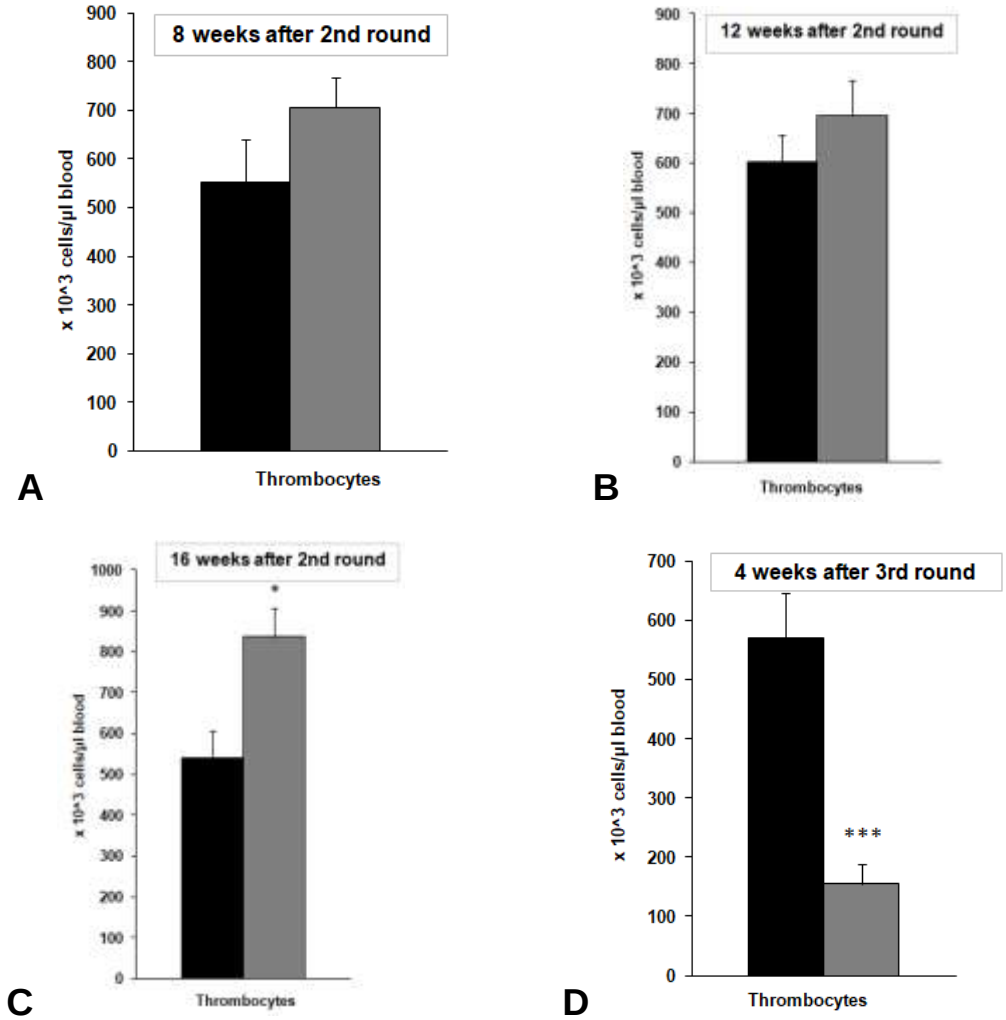


Figure 20. Inferior reconstitution of blood thrombocyte levels at 4 weeks after 3rd round of serial transplantation in CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice. Absolute cell counts of donor cells expressing the pan-hematopoietic surface marker CD45.1 in peripheral blood of recipient mice are shown. **(A)** 8 weeks after 2nd round of serial transplant; n = 10. **(B)** 12 weeks after 2nd round of serial transplant; n = 7. **(C)** 16 weeks after 2nd round of serial transplant; n = 7. **(D)** 4 weeks after 3rd round of serial transplant; WT BM recipients: n = 10; CD44$^{-/-}$:OPN$^{-/-}$-BM-recipients: n = 9. ■ Black bar: wild-type-BM-recipient mice (controls); ■ dark grey bar: CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM; *$P \leq 0{,}05$; ***$P \leq 0{,}0005$.

Transplantation round 3 underlined an impaired reconstitution ability of thrombocytes. $CD44^{-/-}$:$OPN^{-/-}$-BM-donor cells in the wild-type-BM-recipient environment failed to reconstitute thrombocytes in peripheral blood, resulting in severe thrombocytopenia in $CD44^{-/-}$:$OPN^{-/-}$-BM-recipient mice (Fig.20.(D); Fig.21.(B and C)).

Four (Fig.20.(D)), twelve (Fig.21.(B)) and 16 weeks (Fig.21.(C)) after serial transplantation round 3, thrombocyte counts in the peripheral blood of $CD44^{-/-}$:$OPN^{-/-}$-BM-recipient mice were significant lower than those of wild-type controls.

The lowest thrombocyte count in the whole serial transplantational setting, has been found in $CD44^{-/-}$:$OPN^{-/-}$-BM-recipient mice (154.556 cells/µl) at 4 weeks after round 3 (Fig.20.(D)); they show a significant 3,7 times lower thrombocyte level than wild-type controls (570.300 cells/µl; $P \leq 0{,}0005$). From 8 (Fig.21.(A)) over 12 (Fig.21.(B)) to 16 weeks (Fig.21.(C)) after 3rd round of serial transplantation, thrombocyte counts of WT-BM-recipients have risen from 800.000, over 1.200.000 to 1.300.000 cells/µl, whereas $CD44^{-/-}$:$OPN^{-/-}$-BM-recipient`s thrombocyte counts remained at a comparable level of ~ 600.000 cells/µl (being significant ($P \leq 0{,}0005$) at 12 and 16 weeks after 3rd round of transplant). 16 weeks after 3rd round of transplant $CD44^{-/-}$:$OPN^{-/-}$-BM-recipient`s thrombocyte count has risen to 739.571 cells/µl blood, whereas wild-type-BM-recipient`s exhibited an elevation to 1.331.100 cells/µl ($P \leq 0{,}0005$) (Fig.21(C)). In the time period from 12 to 16 weeks thrombocyte counts of WT-BM-recipient mice have risen in an amount of 160.300 cells/µl, while $CD44^{-/-}$:$OPN^{-/-}$-BM-recipient`s thrombocyte counts have only risen in an amount of 134.000 cells/µl. In the interval of 4 weeks, wild-type-BM-recipient mice have replenished 26.000 more cells/µl of thrombocytes compared to $CD44^{-/-}$:$OPN^{-/-}$-BM-recipient mice.

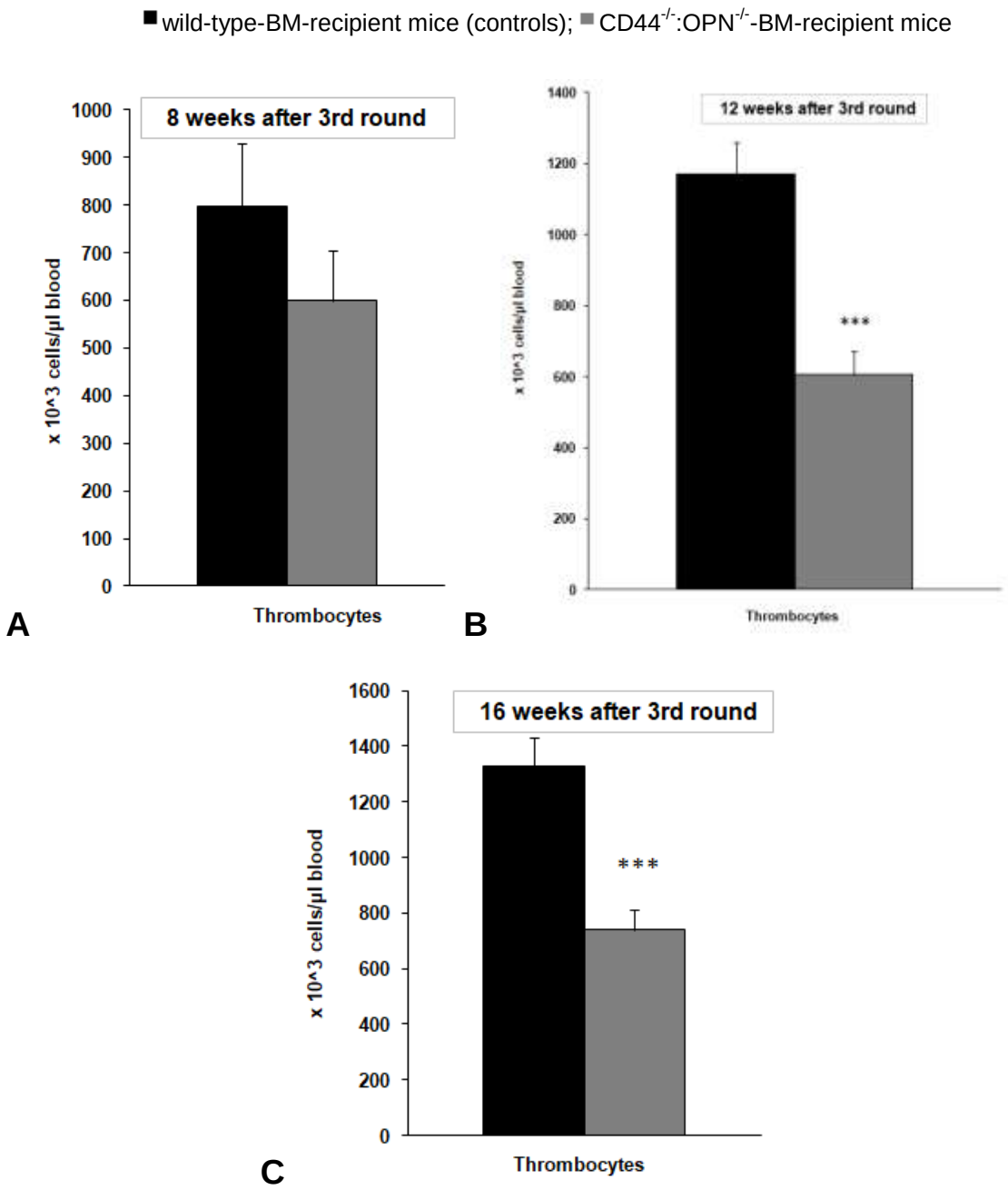


Figure 21. Thrombocytopenia in CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice at 3rd round of serial transplantation. Absolute cell counts of donor cells expressing the pan-hematopoietic surface marker CD45.1 in peripheral blood of recipient mice are shown. **(A)** 8 weeks after 3rd round of serial transplant; wild-type-BM-recipient mice (controls): n = 10; CD44$^{-/-}$:OPN$^{-/-}$-BM-recipients: n = 7. **(B)** 12 weeks after 3rd round of serial transplant; wild-type-BM-recipient mice (controls): n = 10; CD44$^{-/-}$:OPN$^{-/-}$-BM- recipients: n = 7. **(C)** 16 weeks after 3rd round of serial transplant; WT BM recipients: n = 10; CD44$^{-/-}$:OPN$^{-/-}$-BM-recipients: n = 7. ■ Black bar: wild-type-BM-recipient mice (controls); ■ dark grey bar: CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM; *** $P \leq 0{,}0005$.

3.3.2.2 Impaired B- and T cell reconstitution ability in absence of CD44 and OPN

Six weeks after 1st round of stem cell transplant no significant differences in B-, T-, granulocyte and monocyte cell counts in the peripheral blood between wild-type-BM-recipients and $CD44^{-/-}$:$OPN^{-/-}$-BM-recipient mice have been detected (Fig.22.(A)). A tendency of elevated B cell levels was observable in $CD44^{-/-}$:$OPN^{-/-}$-BM-recipients (5400 cells/µl blood) referring to wild-type controls (4020 clls/µl blood), while T cell- (1300 cells/µl blood), granulocyte- (1500 cells/µl blood) and monocyte- (1300 cells/µl blood) peripheral blood levels displayed to be comparable between both recipient cohorts (Fig.22.(A)).

12 weeks after 1st round of serial transplant leukocyte numbers within the peripheral blood of $CD44^{-/-}$:$OPN^{-/-}$-BM-recipient mice showed an elevated tendency compared to wild-type control levels, but only monocyte counts in blood of $CD44^{-/-}$:$OPN^{-/-}$-BM-recipients (1432 cells/µl blood) were significantly increased compared to wild-type (1000 cells/µl blood; $P \leq 0{,}05$) values (Fig.22(B)). B cell counts in peripheral blood of $CD44^{-/-}$:$OPN^{-/-}$-BM-recipient mice were at 5119 cells/µl blood (WT: 3702 cells/µl blood), T-cell counts at 3889 cells/µl (WT: 3380 cells/µl) blood and granulocytes at 2344 cells/µl blood (WT: 1798 cells/µl blood) (Fig.22(B)).

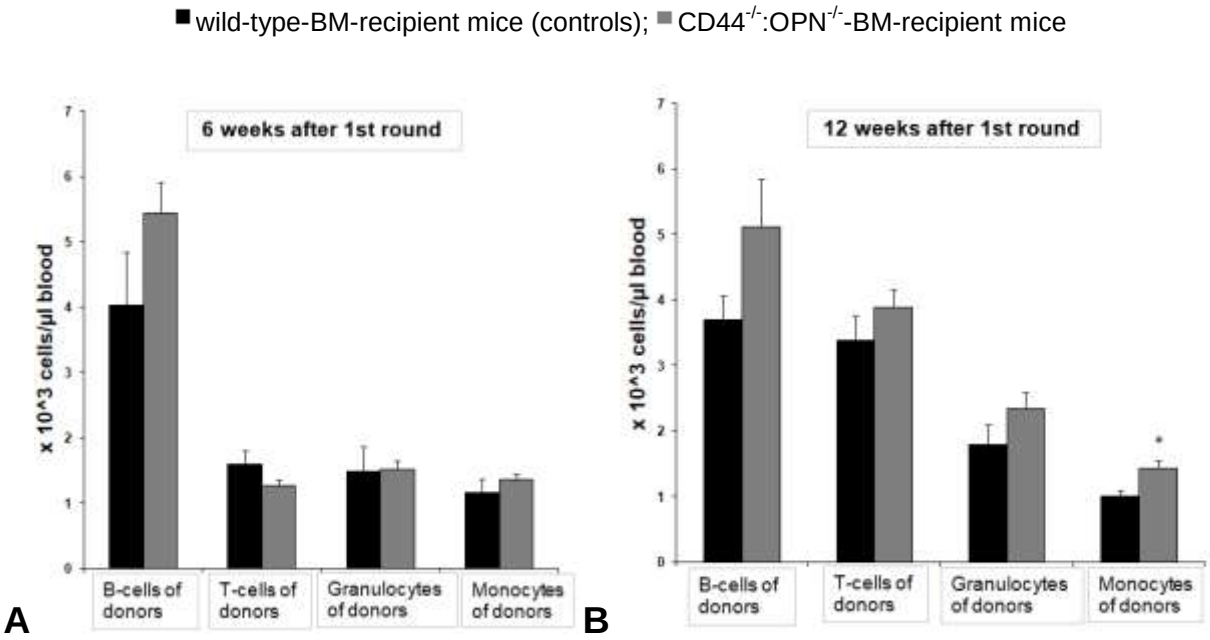


Figure 22. Enhanced monocyte reconstitution in peripheral blood of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice at 12 weeks after 1st round of serial transplantation. Absolute cell counts of donor cells expressing the pan-hematopoietic surface marker CD45.1 in peripheral blood of recipient mice are shown. **(A)** 6 weeks after 1st round of serial transplant; n = 10. **(B)** Absolute cell counts 12 weeks after 1st round of serial transplant; n = 7. ■ Black bar: wild-type-BM-recipient mice (controls); ■ dark grey bar: CD44$^{-/-}$:OPN$^{-/-}$-BM- recipient mice. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM; *$P \leq 0{,}05$.

16 weeks after 1st round of transplant, no significant differences between B, T, granulo- and monocyte counts in the peripheral blood of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipients and wild-type-BM-recipients were detectable (Fig.23(A)). B cells are present at ~1600 cells/µl, T cells at ~2800 cells/µl, granulocytes and monocytes at ~800 cells/µl in peripheral blood of both genotypes.

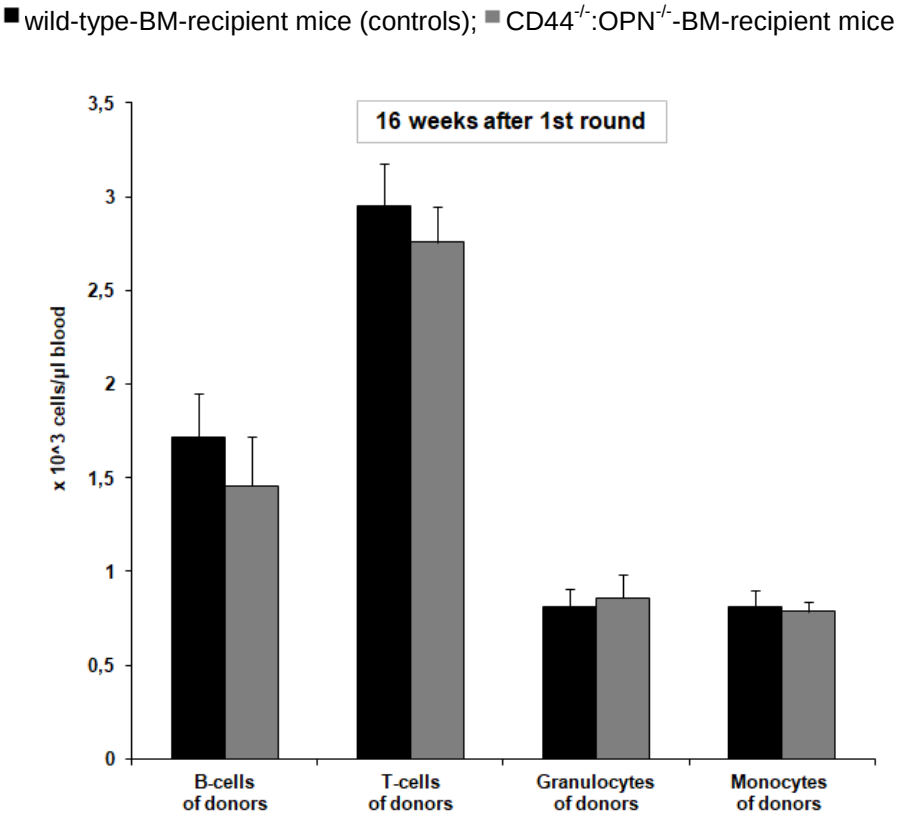


Figure 23. Unimpaired leukocyte blood cell reconstitution in peripheral blood of CD44$^{-/-}$:OPN$^{-/-}$- BM-recipients at 16 weeks after 1st round of serial transplantation. Absolute cell counts of donor cells expressing the pan-hematopoietic surface marker CD45.1 in peripheral blood of recipient mice are shown at 16 weeks after 1st round of serial transplant; n = 7. ■ Black bar: wild-type-BM-recipient mice (controls); ■ dark grey bar: CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM.

4 weeks after 2nd round, B cell levels in the peripheral blood of CD44$^{-/-}$:OPN$^{-/-}$-recipient mice (97 cells/µl blood) were nearly one third lower than in wild-type controls (263 cells/µl blood; $P \leq 0,05$) (Fig.24(A)). No significant differences of T-, granulocyte- and monocyte numbers in peripheral blood between CD44$^{-/-}$:OPN$^{-/-}$-BM recipients (171, 599, 249 cells /µl) and controls (250, 696, 312 cells/µl) have been found (Fig.24(A)).

8 weeks after 2nd round of transplant, leukocytes values in the peripheral blood of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice were significantly decreased referring to those of controls (Fig.24.(B)). B cell numbers in peripheral blood of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice were 2,6 times lower (521 cells/µl; $P \leq 0,005$), T cells 2 times lower (860 cells/µl; $P \leq 0,005$), granulocyte numbers 2,2 times lower (576 cells/µl blood; $P \leq 0,0005$) and monocyte values 2,1 times lower (414 cells/µl blood; $P \leq 0,005$) than in peripheral blood

of wild-type-BM-recipient mice (1377 cells/µl; 1714 cells/µl; 1254 cells/µl; 0,882 cells/µl) (Fig.24.(B)).

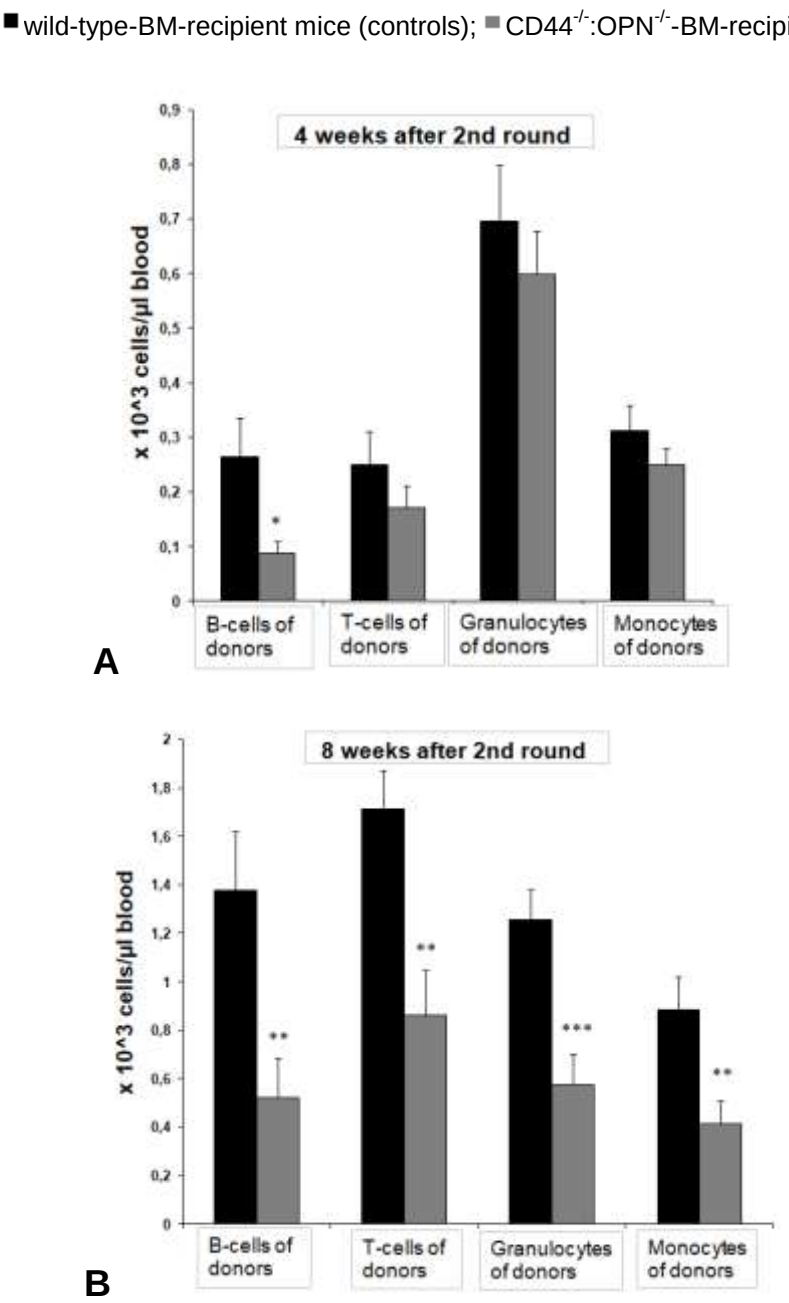


Figure 24. Reduced leukocyte blood cell reconstitution in peripheral blood of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice at 8 weeks after 2nd round of serial transplantation. Absolute cell counts of donor cells expressing the pan-hematopoietic surface marker CD45.1 in peripheral blood of recipient mice are shown. **(A)** 4 weeks after 2nd round of serial transplant; n = 10. **(B)** 8 weeks after 2nd round of serial transplant; n = 10. ■ Black bar: wild-type-BM-recipient mice (controls); ■ dark grey bar: CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM;*$P \leq 0,05$; *$P \leq 0,05$ **$P \leq 0,005$; ***$P \leq 0,0005$.

12 weeks after 2nd round of transplant, B- (289 cells/µl; $P \leq 0,05$) and T-cell counts (1134 cells/µl; $P \leq 0,05$) in peripheral blood of $CD44^{-/-}$:$OPN^{-/-}$-BM-recipient mice were significantly reduced compared to wild-type-BM-recipient values (B cell numbers: 1133 cells/µl; T cell numbers: 2612 cells/µl) (Fig.25.(A)). No significant differences in granulocyte and monocyte numbers were observable in peripheral blood of $CD44^{-/-}$:$OPN^{-/-}$-BM-recipient mice and wild-type controls at 12 weeks after 2nd round of serial transplantation (Fig.25.(A)).

16 weeks after 2nd round of transplant, $CD44^{-/-}$:$OPN^{-/-}$-BM-recipient mice exhibited a more than 3 fold reduction of B cells (429 cells/µl; $P \leq 0,05$) in peripheral blood relating to controls (1380 cells/µl)(Fig.25.(B)). T cells in $CD44^{-/-}$:$OPN^{-/-}$-BM-recipient mice`s peripheral blood showed a decreased count (1584 cells/µl; $P \leq 0,05$) relating to wild-type-BM-recipient control mice (3150 cells/µl). Additionally, monocytes of $CD44^{-/-}$:$OPN^{-/-}$-BM-recipient mice (290 cells/µl) were diminished significantly ($P \leq 0,05$) to less than half the amount of wild-type controls (722 cells/µ). $CD44^{-/-}$:$OPN^{-/-}$-BM-recipient mice's granulocyte numbers (784 cells/µl) in peripheral blood exhibited a decreased tendency compared to controls (1106 cells/µl), without being significant (Fig.25.(B)).

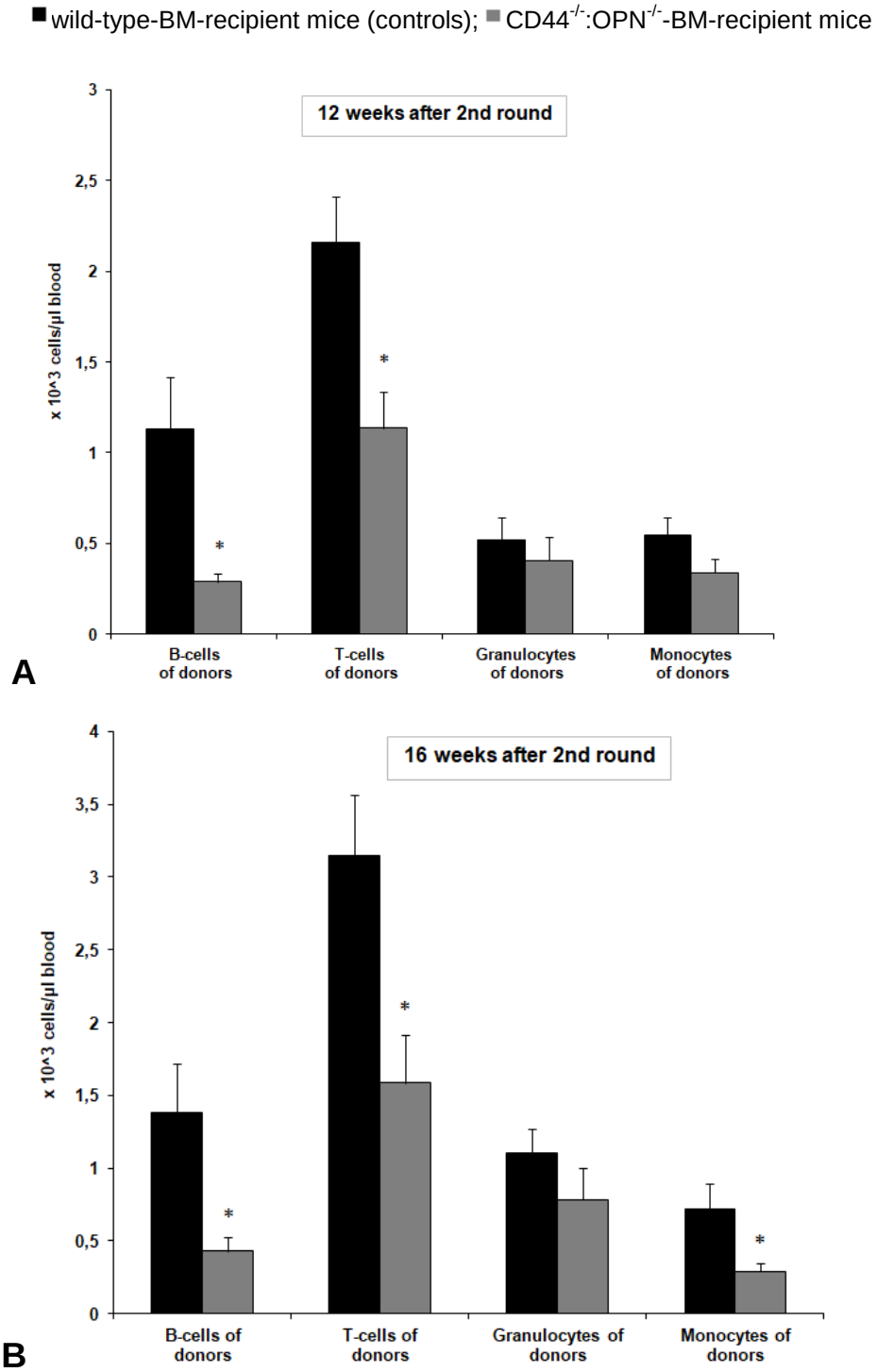


Figure 25. Reduced monocyte, B and T cell reconstitution in peripheral blood of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice at 16 weeks after 2nd round of serial transplantation. Absolute cell counts of donor cells expressing the pan-hematopoietic surface marker CD45.1 in peripheral blood of recipient mice are shown. **(A)** 12 weeks after 2nd round of serial transplantation; n = 7. **(B)** 16 weeks after 2nd round of serial transplantation; n = 7. ■ Black bar: wild-type-BM-recipient mice (controls); ■ dark grey bar: CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM; *P ≤ 0,05.

4 weeks after 3rd round of transplantation, the replensihment of leukocytes in the peripheral blood of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice and wild-type-BM-recipient mice was nearly depleted (Fig.26.(A)). CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice's B-, T-, granulocyte- and monocyte counts in the peripheral blood showed a significant reduction compared to controls (Fig.26.(A)). B cell levels in periperal blood of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice were 8,5 times decreased (4 cells/µl; $P \leq 0{,}005$), T cell levels 3,8 times reduced (23 cells/µl; $p \leq 0{,}005$), granulocyte levels (34 cells/µl; $P \leq 0{,}05$) 6,4 times lower and monocyte levels 15,5 times reduced (8 cells/µl; $P \leq 0{,}0005$) compared to wild-type-BM-recipient mice (34 cells/µl; 89 cells/µl; 218 cells/µl and 124 cells/µl) (Fig.26.(A)).

8 weeks after 3rd round of serial transplantation B- (7 cells/µl blood; $P \leq 0{,}0005$) and T-cell- (139 cells/µl; $P \leq 0{,}0005$) values in peripheral blood of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice were significantly decreased referring to controls (Fig.26.(B and C)). Granulocyte numbers in the peripheral blood of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice exhibited a tendecy of elevation to 189 cells/µl relating to WT-BM-recipient mice (11 cells/µl) without being significant (Fig.26.(B)). CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice`s monocyte counts in the peripheral blood (79 cells/µl) were elevated compared to controls (27 cells/µl) (Fig. 26.(B)).

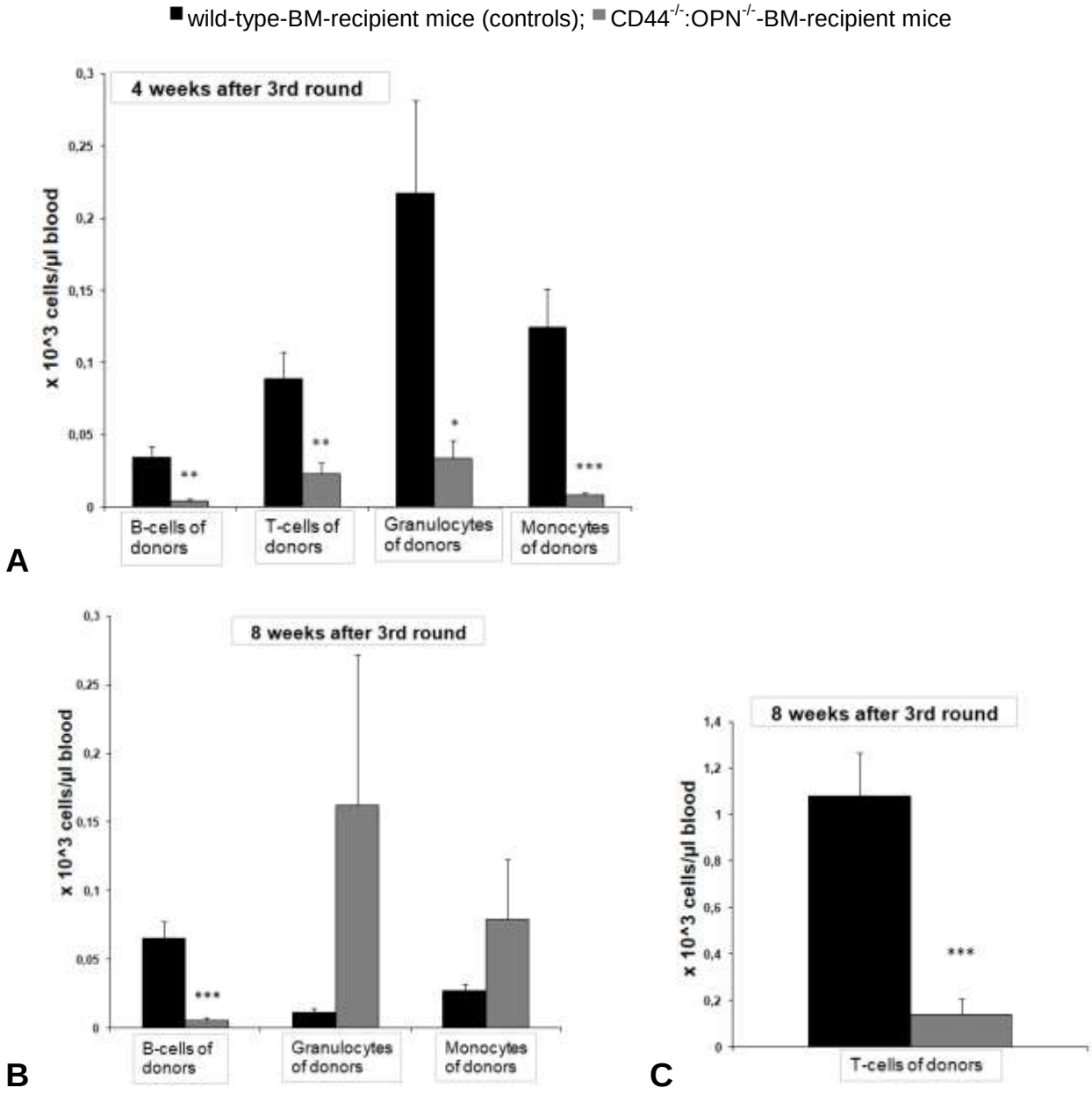
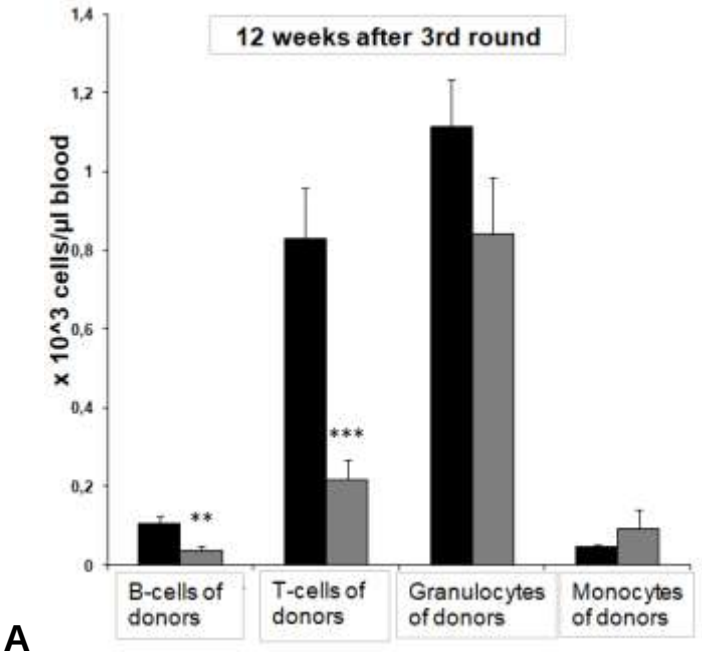


Figure 26. Leukocytopenia in peripheral blood of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice at 3rd round of serial transplantation. Absolute cell counts of donor cells expressing the pan-hematopoietic surface marker CD45.1 in peripheral blood of recipient mice are shown. **(A)** B and T cell, granulocyte and monocyte counts at 4 weeks after 3rd round of serial transplant; controls: n = 10; CD44$^{-/-}$:OPN$^{-/-}$-BM-recipients: n = 9. **(B)** B cell, granulocyte and monocyte cell counts at 8 weeks after 3rd round of serial transplant; controls: n = 10; CD44$^{-/-}$:OPN$^{-/-}$-BM-recipients: n = 7. **(C)** T cell numbers at 8 weeks after 3rd round of serial transplant; controls: n = 10; CD44$^{-/-}$:OPN$^{-/-}$-BM-recipients: n = 7. ■ Black bar: wild-type-BM-recipient mice (controls); ■ dark grey bar: CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM; *$P \leq 0,05$; **$P \leq 0,005$; *** $P \leq 0,0005$.

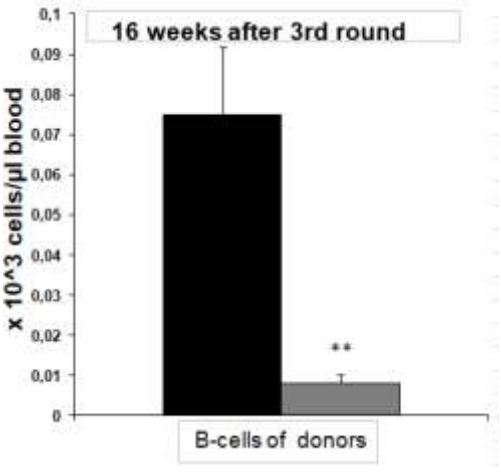
Peripheral blood cell counts 12 weeks after 3rd round of transplant revealed a significant reduction of B- (39 cells/µl blood; $P \leq 0{,}005$) and T cells (217 cells/µl blood; $P \leq 0{,}0005$) in $CD44^{-/-}$:$OPN^{-/-}$-BM-recipient mice relating to controls (107 cells/µ; 829 cells/µl) (Fig.27.(A)). Granulocyte- (1115 cells/µl blood) and monocyte levels (46 cells/µl blood) in peripheral blood of controls were in comparable numbers than those of $CD44^{-/-}$:$OPN^{-/-}$-BM-recipient mice (841 cells/µl blood; 93 cells/µl) (Fig.27.(A)).

16 weeks after 3rd round of transplant $CD44^{-/-}$:$OPN^{-/-}$-BM-recipient mice's B cell counts (8 cells/µl; $P \leq 0{,}005$) were 9 times lower than those of wild-type controls (75 cells/µl) (Fig.27.(B)). T cells of $CD44^{-/-}$:$OPN^{-/-}$-BM-recipient mice showed a significant reduction to 320 cells/µl ($P \leq 0{,}05$), while their granulocyte count (189 cells/µl; $P \leq 0{,}05$) was more than doubled compared to wild-type controls (779 cells/µl; 79 cells/l)(Fig.27.(B)). No significant difference of monocyte levels has been observed between $CD44^{-/-}$:$OPN^{-/-}$-BM-recipient mice (70 cells/µl) and wild-type-BM-recipient mice (55 cells/µl) (Fig.27.(B)).

■ wild-type-BM-recipient mice (controls); ■ CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice



A



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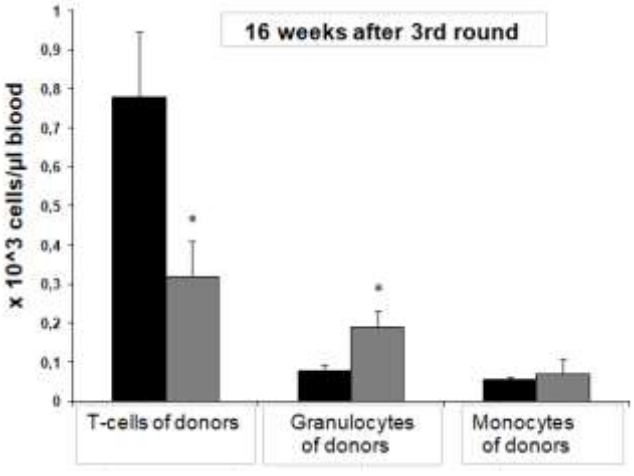


C

Figure 27. Depletion of B and T cell reconstitution in peripheral blood of CD44$^{-/-}$:OPN$^{-/-}$-BM recipient mice at the end of 3rd round of serial transplantation. Absolute cell counts of donor cells expressing the pan-hematopoietic surface marker CD45.1 in peripheral blood of recipient mice are shown. **(A)** B cell, T cell, granulocyte and monocytes cell counts at 12 weeks after 3rd round; controls: n = 10; CD44$^{-/-}$:OPN$^{-/-}$-BM-recipients: n = 7. **(B)** B cells at 16 weeks after 3rd round; controls: n = 10; CD44$^{-/-}$:OPN$^{-/-}$-BM-recipients: n = 7. **(C)** T cell, granulocyte and monocyte cell counts at 16 weeks after 3rd round; controls: n = 10; CD44$^{-/-}$:OPN$^{-/-}$-BM-recipients: n = 7. ■ Black bar: wild-type-BM-recipient mice (controls); ■ dark grey bar: CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM; *$P \leq 0,05$; **$P \leq 0,005$; ***$P \leq 0,0005$.

3.3.2.3 $CD44^{-/-}$:$OPN^{-/-}$ double-deficiency in BM donor cells impacts erythrocyte reconstitution ability

Six (Fig.28.(A)), twelve (Fig.28.(B)) and sixteen ((Fig.28.(C)) weeks after the first round of transplantation, erythrocyte levels in peripheral blood of $CD44^{-/-}$:$OPN^{-/-}$-BM-recipient mice did not differ significantly in numbers but exhibited an elevated tendency compared to those of controls.

First time a decreased tendency of erythrocyte levels in peripheral blood of $CD44^{-/-}$:$OPN^{-/-}$-BM-recipient mice (5,1x 10^6 cells/µl) compared to controls (5,6x10^6 cells/l) has been observed at 4 weeks after 2nd round of transplantation (Fig.28.(D)).

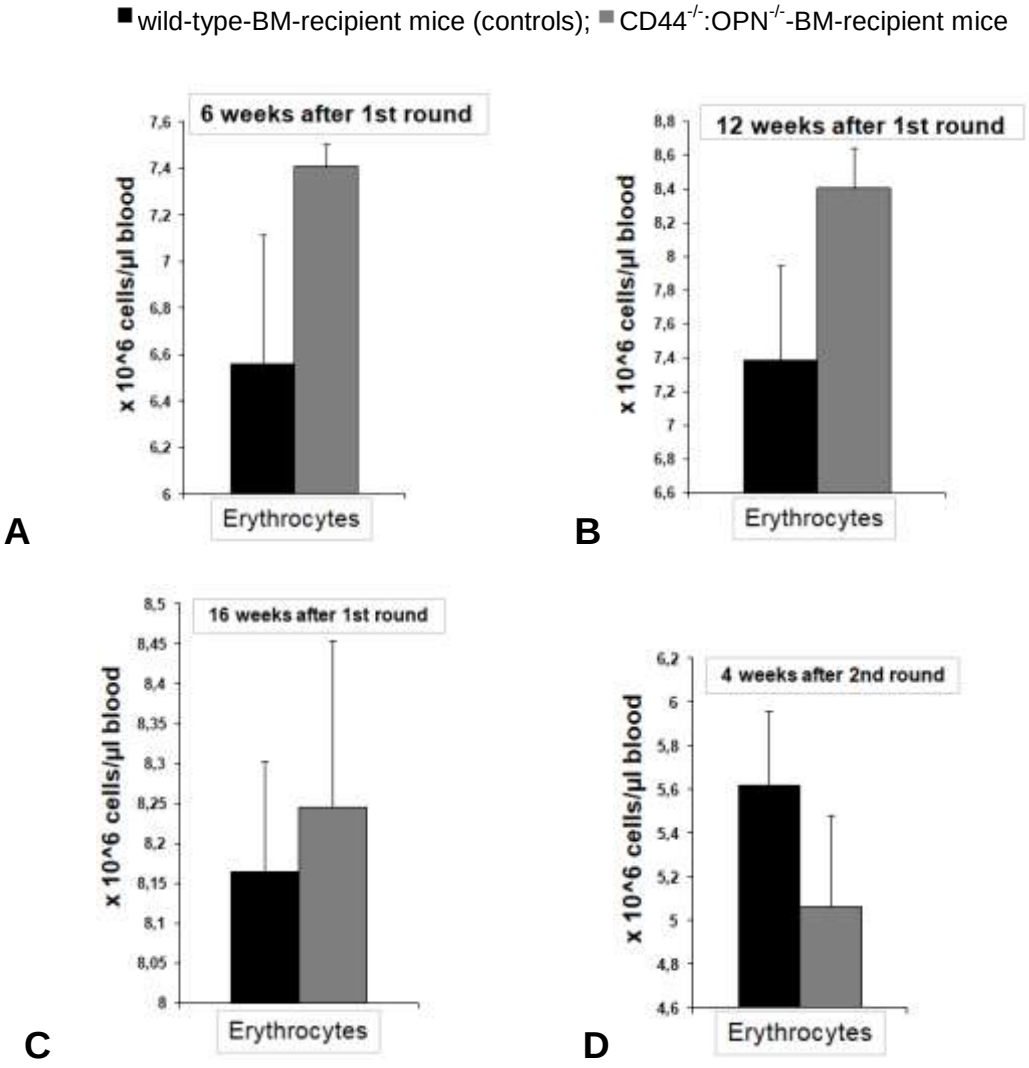


Figure 28. Unimpaired erythrocyte reconstitution in peripheral blood of CD44$^{-/-}$:OPN$^{-/-}$-BM- recipient mice within 1st round till 4 weeks after 2nd round of serial transplantation. Absolute erythrocyte cell counts of donor cells expressing the pan-hematopoietic surface marker CD45.1 in peripheral blood of recipient mice are shown. **(A)** 6 weeks after 1st round of serial transplant. **(B)** 12 weeks after 1st round of serial transplant. **(C)** 16 weeks after 1st round of serial transplant. **(D)** 4 weeks after 2nd round of serial transplant. ■Black bar: wild-type BM recipient mice (controls); ■dark grey bar: CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM.

The tendency of diminished erythrocyte counts in peripheral blood of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice compared to wild-type controls continued over 8 weeks after 2nd round (6,5x10^6 cells/µl vs. 6,9x10^6 cells/µl; Fig.29.(A)), 12 weeks after 2nd round (5,5 x10^6 cells/µl vs. 7,2x10^{6}cells/µl; $P \leq 0{,}005$; Fig.29.(B)), 16 weeks after 2nd round (7,5x 10^6 cells/µl vs. 8,7 x10^6 cells/µl; p≤0,005; Fig.29.(C)), 4 weeks after 3rd round (4,4 x10^6 cells/l vs. 5,5 x10^6 cells/µl; Fig.29.(D)), 8 weeks after 3rd round (6,7x10^6 cells/µl vs.

6,9x10^6 cells/µl; Fig.30.(A)), 12 weeks after 3rd (6,2x10^6 cells/µl vs. 7,1x10^6 cells/µl; Fig.30.(B)) round and 16 weeks after 3rd round (6,8x10^6 cells/l vs. 8,3x106 cells/µl; $P \leq 0,05$; Fig.30.(C)).

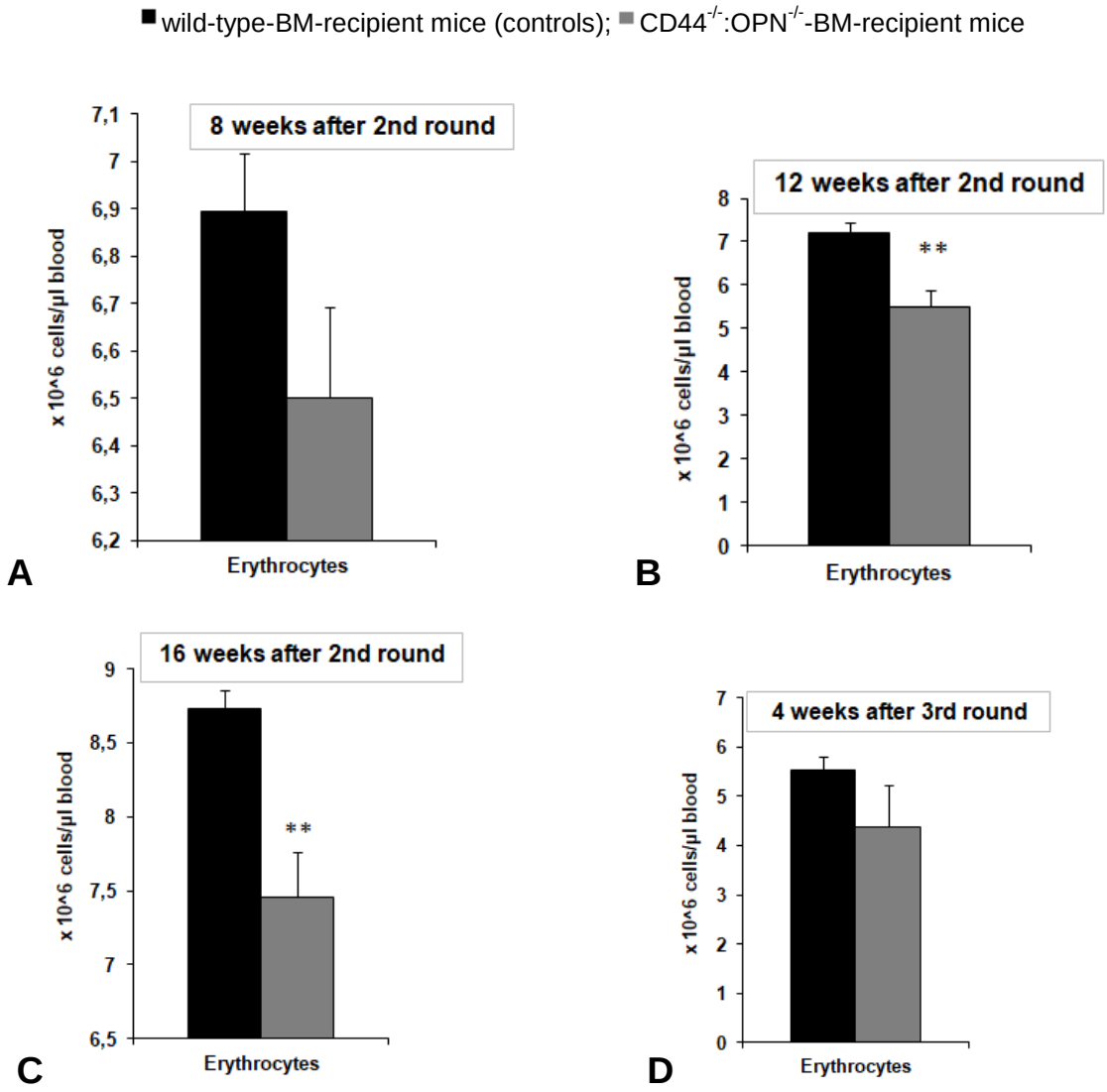


Figure 29. Reduced erythrocyte reconstitution in peripheral blood of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice at 12 and 16 weeks after 2nd round of serial transplantation. Absolute erythrocyte cell counts of donor cells expressing the pan-hematopoietic surface marker CD45.1 in peripheral blood of recipient mice are shown. **(A)** 8 weeks after 2nd round; n = 10. **(B)** 12 weeks after 2nd round; n = 7. **(C)** 16 weeks after 2nd round; n = 7. **(D)** 4 weeks after 3rd round; controls: n = 10; CD44$^{-/-}$:OPN$^{-/-}$- BM recipients: n = 9. ■ Black bar: wild-type-BM-recipient mice (controls); ■ dark grey bar: CD44$^{-/-}$:OPN$^{-/-}$- BM-recipient mice. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM; $^{**}P \leq 0,005$.

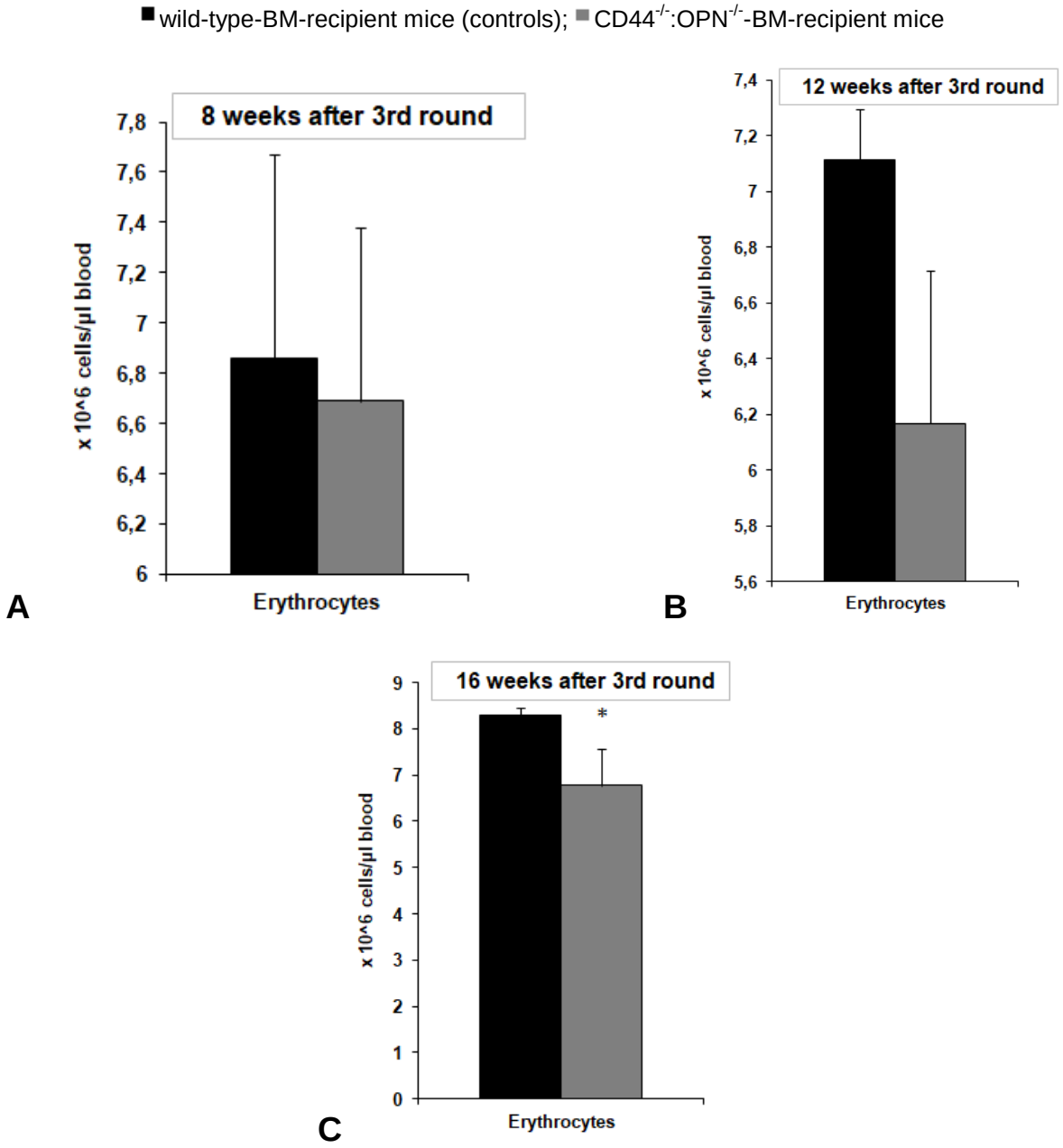


Figure 30. Reduced erythrocyte reconstitution in peripheral blood of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice after 3rd round of serial transplantation. Absolute erythrocyte cell counts of donor cells expressing the pan-hematopoietic surface marker CD45.1 in peripheral blood of recipient mice are shown. **(A)** 8 weeks after 3rd round; controls: n = 10; CD44$^{-/-}$:OPN$^{-/-}$- BM-recipients: n = 7. **(B)** 12 weeks after 3rd round; controls: n = 10; CD44$^{-/-}$:OPN$^{-/-}$- BM-recipients: n = 7 . **(C)** 16 weeks after 3rd round; controls: n = 10; CD44$^{-/-}$:OPN$^{-/-}$- BM-recipients: n = 7. ■ Black bar: wild-type-BM-recipient mice (controls); ■ dark grey bar: CD44$^{-/-}$:OPN$^{-/-}$- BM-recipient mice. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM; *$P \leq 0{,}05$.

3.3.2.4 Impaired hemoglobin replenishment in peripheral blood of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice

Hemoglobin levels in peripheral blood of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice exhibited an elevated tendency from 6 weeks after 1st round of transplant (12,7 µg/µl blood; Fig.31.(A)), over 12 weeks after 1st round (13,2 µg/µl blood; Fig.31.(B)) till 16 weeks after 1st round of transplant (13,1 µg/µl blood; Fig.31.(C)) referring to wild-type controls (11,3 µg/µl blood; 11,6 µg/µl blood; 12,9µg/µl blood).

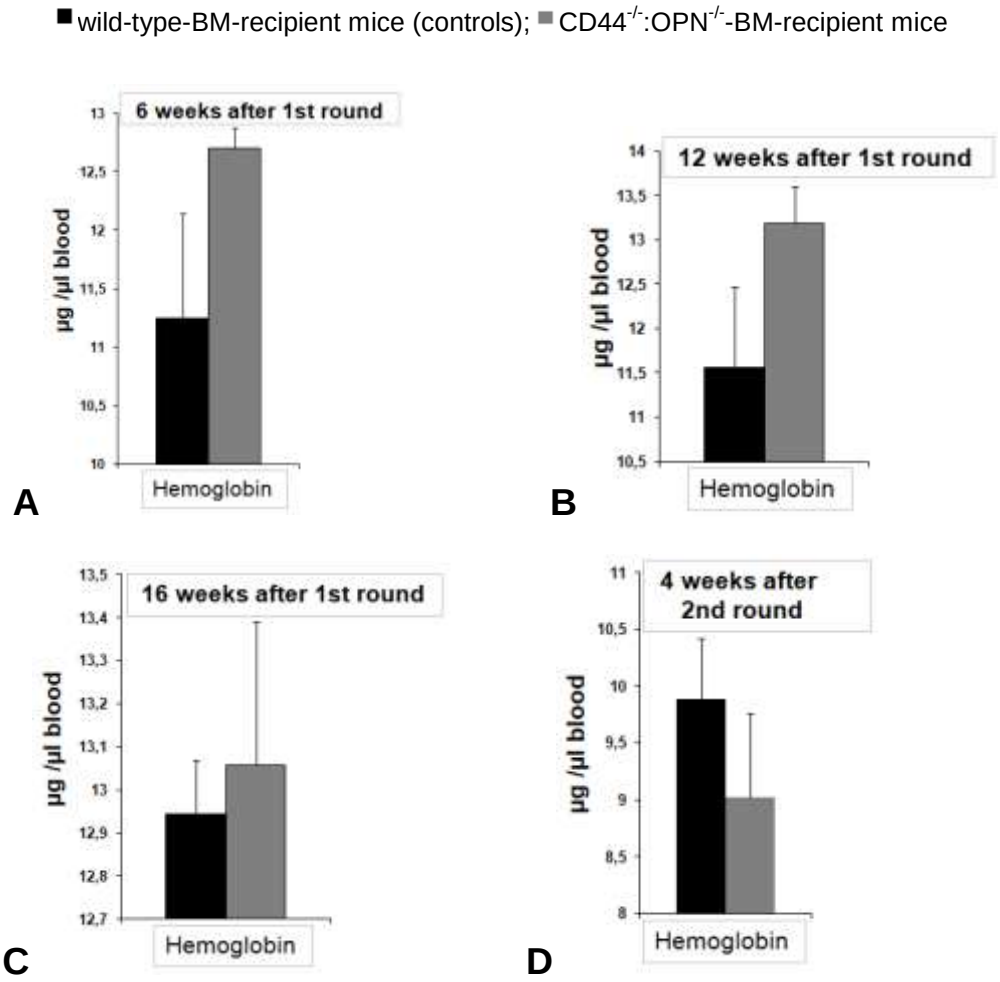


Figure 31. Unimpaired hemoglobin level reconstitution in peripheral blood of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient-mice during 1st round till 4 weeks after 2nd round of serial transplantation. Absolute hemoglobin levels of donor cells expressing the pan-hematopoietic surface marker CD45.1 in peripheral blood of recipient mice are shown. **(A)** 6 weeks after 1st round of serial transplant; n = 10; **(B)** 12 weeks after 1st round of serial transplant; n = 7. **(C)** 16 weeks after 1st round of serial transplant; n = 7. **(D)** 4 weeks after 2nd round of serial transplant; n = 10. ■ Black bar: wild-type BM recipient mice (controls); ■ dark grey bar: CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM.

Firstly, 4 weeks after 2nd round of serial transplantation, hemoglobin levels in peripheral blood of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice (9 µg/µl blood) were decreased compared to levels of controls (9,9 µg/µl blood) (Fig.31.(D)).

Since that time point, hemoglobin levels in peripheral blood of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice were lower than in controls; 8 weeks (11,2 µg/µl blood; Fig.32.(A)), 12 weeks (9,6 µg/µl blood; $P \leq 0,05$) (Fig. 32.(B)) and 16 weeks (12,8µg/µl blood $P \leq 0,05$) (Fig.32.(C)) after 2nd round and 4 weeks (8,7 µg/l blood) (Fig.32.(D)), 8 weeks (11,4 µg/µl blood) (Fig.33.(A)), 12 weeks (10,7 µg/µl blood) (Fig.33.(B)) and 16 weeks (11,5µg/µl blood) (Fig.33.(C)) after 3rd round of serial transplantation.

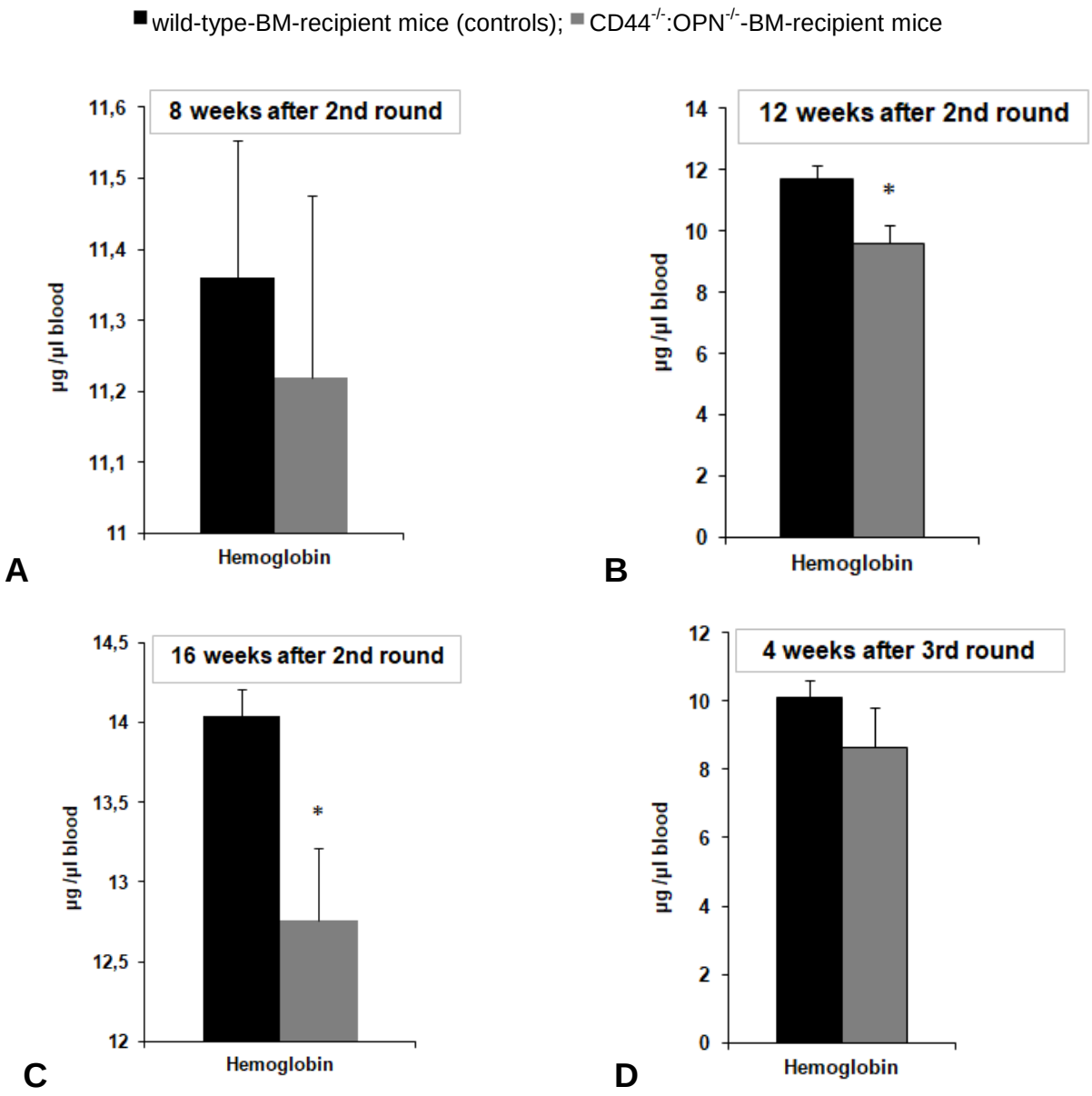


Figure 32. Decreased hemoglobin level reconstitution in peripheral blood of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice at 12 and 16 weels after 2nd round of serial transplantation. Absolute hemoglobin levels of donor cells expressing the pan-hematopoietic surface marker CD45.1 in peripheral blood of recipient mice are shown. **(A)** 8 weeks after 2nd round; n = 10. **(B)** 12 weeks after 2nd round; n = 7. **(C)** 16 weeks after 2nd round; n = 7. **(D)** 4 weeks after 3rd round; controls: n = 10; CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice: n = 9. ■ Black bar: wild-type-BM-recipient mice (controls); ■ dark grey bar: CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM; *$P \leq 0,05$.

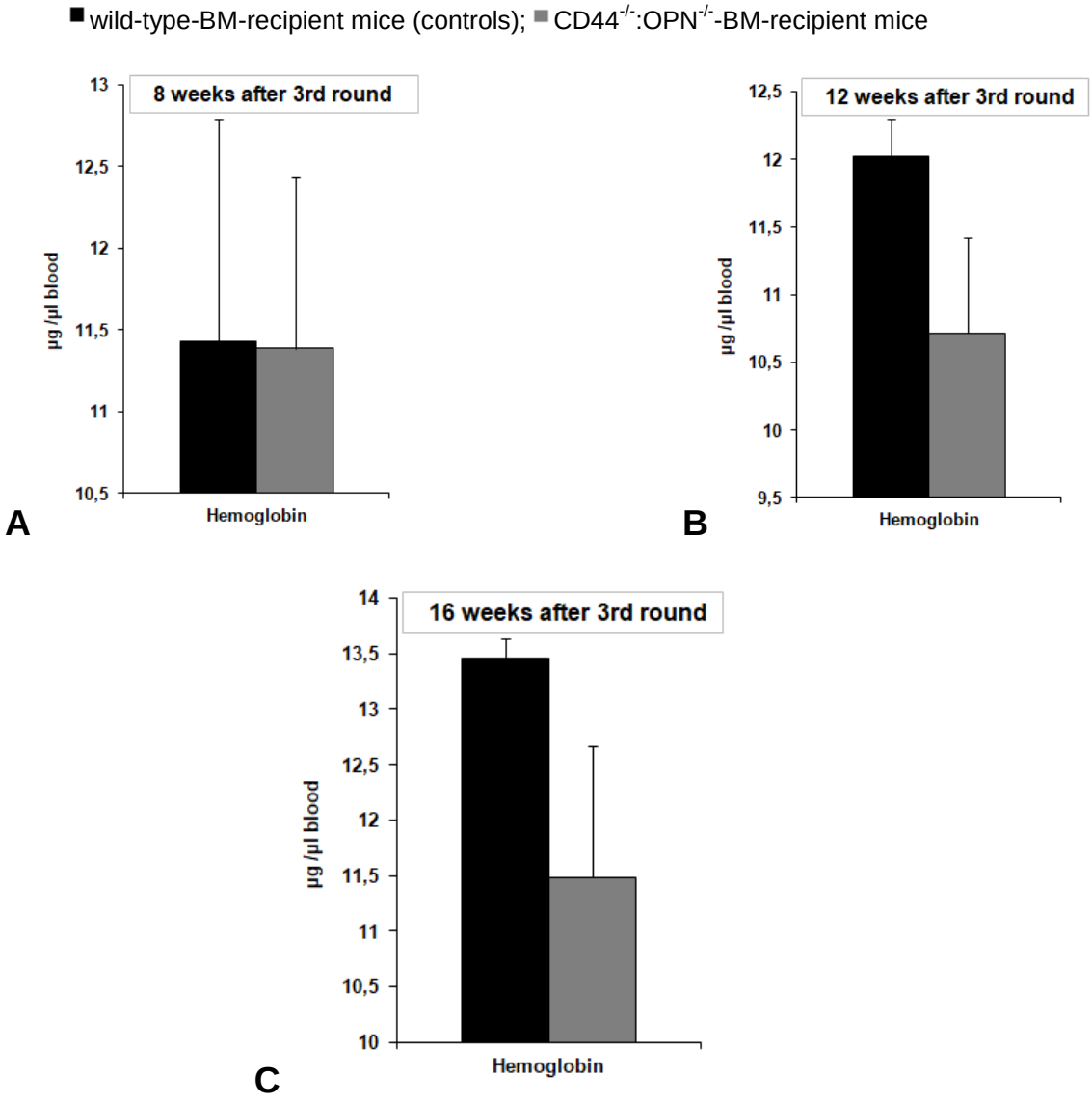


Figure 33. Unimpaired hemoglobin level reconstitution in peripheral blood of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice during 3rd round of serial transplantation. Absolute hemoglobin levels of donor cells expressing the pan-hematopoietic surface marker CD45.1 in peripheral blood of recipient mice are shown. **(A)** 8 weeks after 3rd round; controls: n = 10; CD44$^{-/-}$:OPN$^{-/-}$-BM-recipients: n = 7. **(B)** 12 weeks after 3rd round; controls: n = 10; CD44-/-:OPN-/- BM recipients: n = 7. **(C)** 16 weeks after 3rd round; controls: n = 10; CD44$^{-/-}$:OPN$^{-/-}$-BM-recipients: n = 7. ■ Black bar: wild-type-BM-recipient mice (controls); ■ dark grey bar: CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM.

3.3.3 BM cell populations after serial transplantation

3.3.3.1 Elevated B cell counts in BM of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice at 8 weeks after 1st and 16 weeks after 3rd round of serial transplantation

Relative B cells counts in BM of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice were significantly increased to 18,8% ($P \leq 0,05$) at 8 weeks after 1st round of transplantation relating to wild-type controls (12,5%) (Fig.34.(A)).

Relative B cell counts in the BM of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice exhibited a decreased tendency at 16 weeks after 1st round (10,2%; Fig.34.(B)), at 8 weeks after 2nd (4,9%; Fig.34.(C)) and at 16 weeks after 2nd round (1,6%; Fig.34.(D)) of transplantation compared to controls (10,9%; 5,1%; 3,0%).

16 weeks after 3rd round of transplant, B cell counts in BM of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice were significantly increased (17,2%; $P \leq 0,005$) relating to wild-type control values (5,6%) (Fig.34.(E)).

■ wild-type-BM-recipient mice (controls); ■ CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice

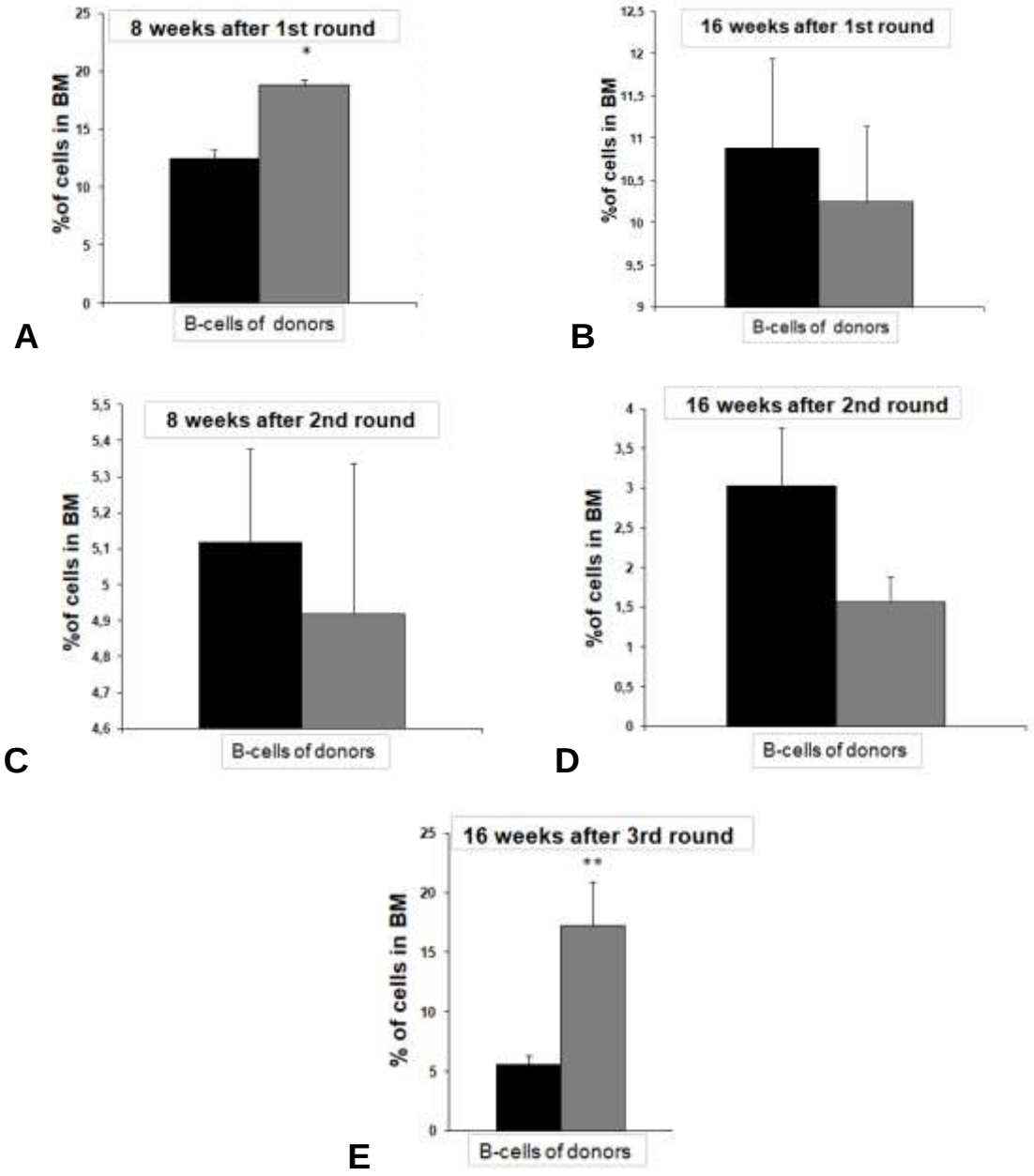


Figure 34. Enhanced B cell reconstitution in BM of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice at 8 weeks after 1st and 16 weeks after 3rd round of serial transplantation. Relative abundance of donor B cells expressing the pan-hematopoietic surface marker CD45.1 in BM of recipient mice are shown. **(A)** 8 weeks after 1st round; n = 3. **(B)** 16 weeks after 1stround; n = 7. **(C)** 8 weeks after 2nd round; n = 3. **(D)** 16 weeks after 2nd round; n = 7. **(E)** 16 weeks after 3rd round; controls: n = 7; CD44$^{-/-}$:OPN$^{-/-}$- BM-recipients: n = 7. ■ Black bar: wild-type-BM-recipient mice (controls); ■ dark grey bar: CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice. Percentage of cells is referring to the extracted BM of 1 femur per mouse. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM; *$P \leq 0,05$; **$P \leq 0,005$.

3.3.3.2 $CD44^{-/-}/OPN^{-/-}$ double-deficiency in BM donor cells does not impair T cell reconstitution in BM of wild-type recipient mice

T cell values in BM of $CD44^{-/-}$:$OPN^{-/-}$-BM-recipient mice showed an elevated tendency compared to those of wild-type controls, at 8 weeks after 1st round (3,7% vs. 2,9%; Fig.35.(A)) and 8 weeks after 2nd round (0,3% vs. 0,2%) of transplantation (Fig.35.(C)).

At 16 weeks after 1st (Fig.35.(B)), 16 weeks after 2nd (Fig.35.(D)) and 16 weeks after 3rd (Fig.35.(E)) round of serial transplantation B cell values of $CD44^{-/-}$:$OPN^{-/-}$-BM-recipient mice (10,2%; 1,6%; 0,9%) exhibited a reduced tendency referring to controls (10,9%; 1,7%; 1,5%).

■ wild-type-BM-recipient mice (controls); ■ $CD44^{-/-}$:$OPN^{-/-}$-BM-recipient mice

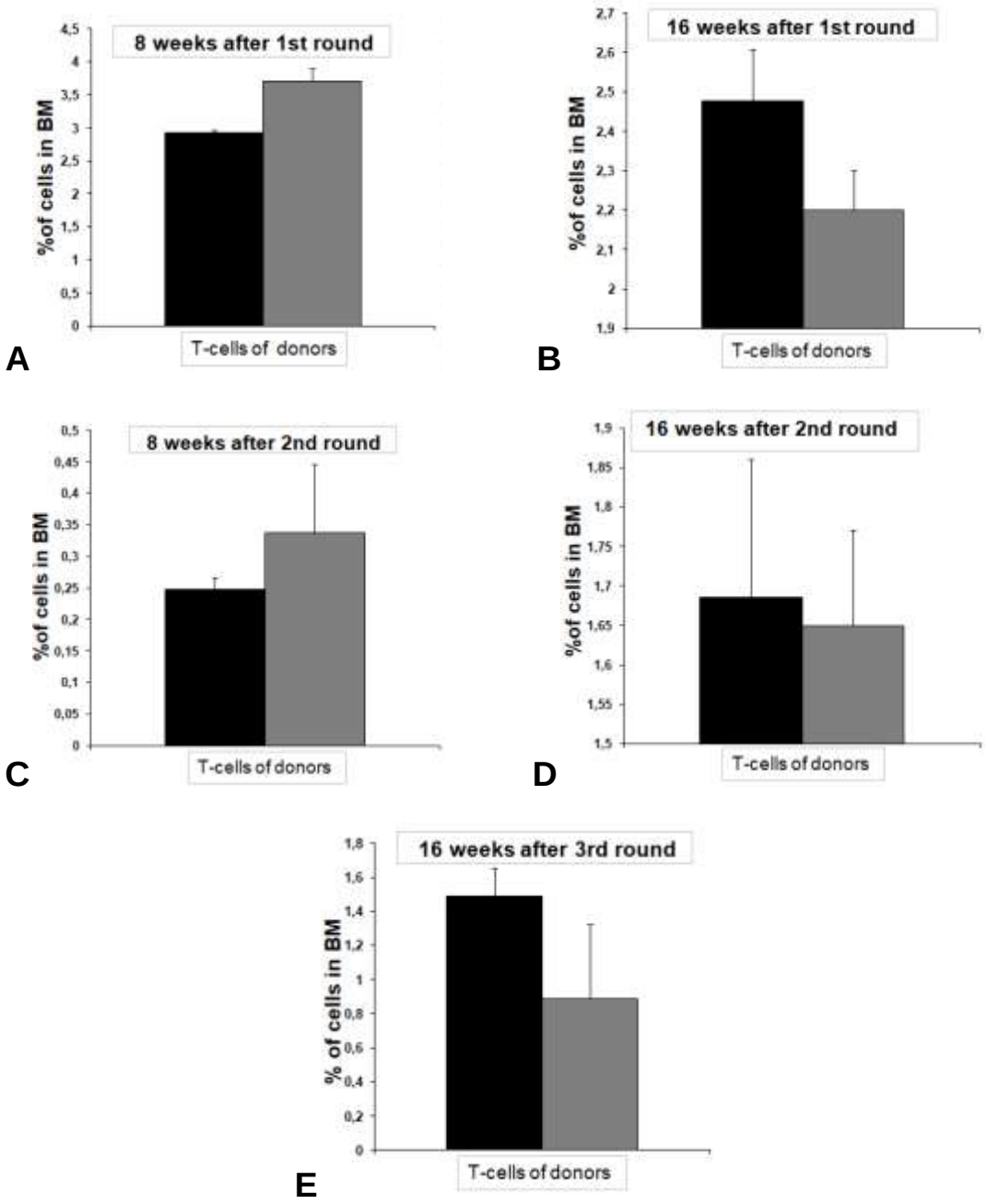


Figure 35. Unimpaired T cell reconstitution in BM of $CD44^{-/-}$:$OPN^{-/-}$-BM-recipient mice during 3 rounds of serial transplantation. Relative abundance of donor T cells expressing the pan-hematopoietic surface marker CD45.1 in BM of recipient mice are shown. **(A)** 8 weeks after 1st round; n = 3. **(B)** 16 weeks after 1st round; n = 7. **(C)** 8 weeks after 2nd round; n = 3. **(D)** 16 weeks after 2nd round; n = 7. **(E)** 16 weeks after 3rd round of transplant; controls: n = 10; $CD44^{-/-}$:$OPN^{-/-}$-BM-recipient mice: n= 7. ■ Black bar: wild-type-BM-recipient mice (controls); ■ dark grey bar: $CD44^{-/-}$:$OPN^{-/-}$-BM-recipient mice. Percentage of cells is referring to the extracted BM of 1 femur per mouse. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM.

3.3.3.3 Enhanced granulocyte reconstitution in BM of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice at 16 weeks after 1st round of serial transplantation

8 weeks after 1st (Fig.36.(A)), 8 weeks after 2nd (Fig.36.(C)) and 16 weeks after2nd (Fig.36.(D)) round of serial transplantation, CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice showed a tendency of diminisehd granulocyte numbers in BM (32,5%; 19%; 26,7%) referring to wild-type controls (35,6%; 33%; 30,1%).

16 weeks after 1st round of transplantation, CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice exhibited significantly elevated granulocyte counts in BM (45,3%; $P \leq 0,05$) compared to wild-type controls (34,1%) (Fig.36.(B)).

Sixteen weeks after 3rd round of transplantation granulocyte counts in BM were comparable in numbers of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient- and wild-type control mice (Fig.36.(E)). Sixteen weeks after 3 round of serial stem cell transplantation only 4,9 % of cells in BM of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice and 4,8% of cells in BM of wild-type control mice have been granulocytes (Fig.36.(E)). At 8 weeks after 1st round of transplantation granulocyte values in BM of both observed recipient groups have been between 32 and 35,5 % (Fig.36.(A)), while granulocyze levels in BM of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient- and wild-type control mice have both decreased to a level of about 5% after 3 rounds of serial transplantation (Fig.36.(E)). Therefore after 3 rounds of serial transplantation granulocytes have been reduced by 6,5 times compared to the BM status in the early transplant stage.

■ wild-type-BM-recipient mice (controls); ■ $CD44^{-/-}$:$OPN^{-/-}$-BM-recipient mice

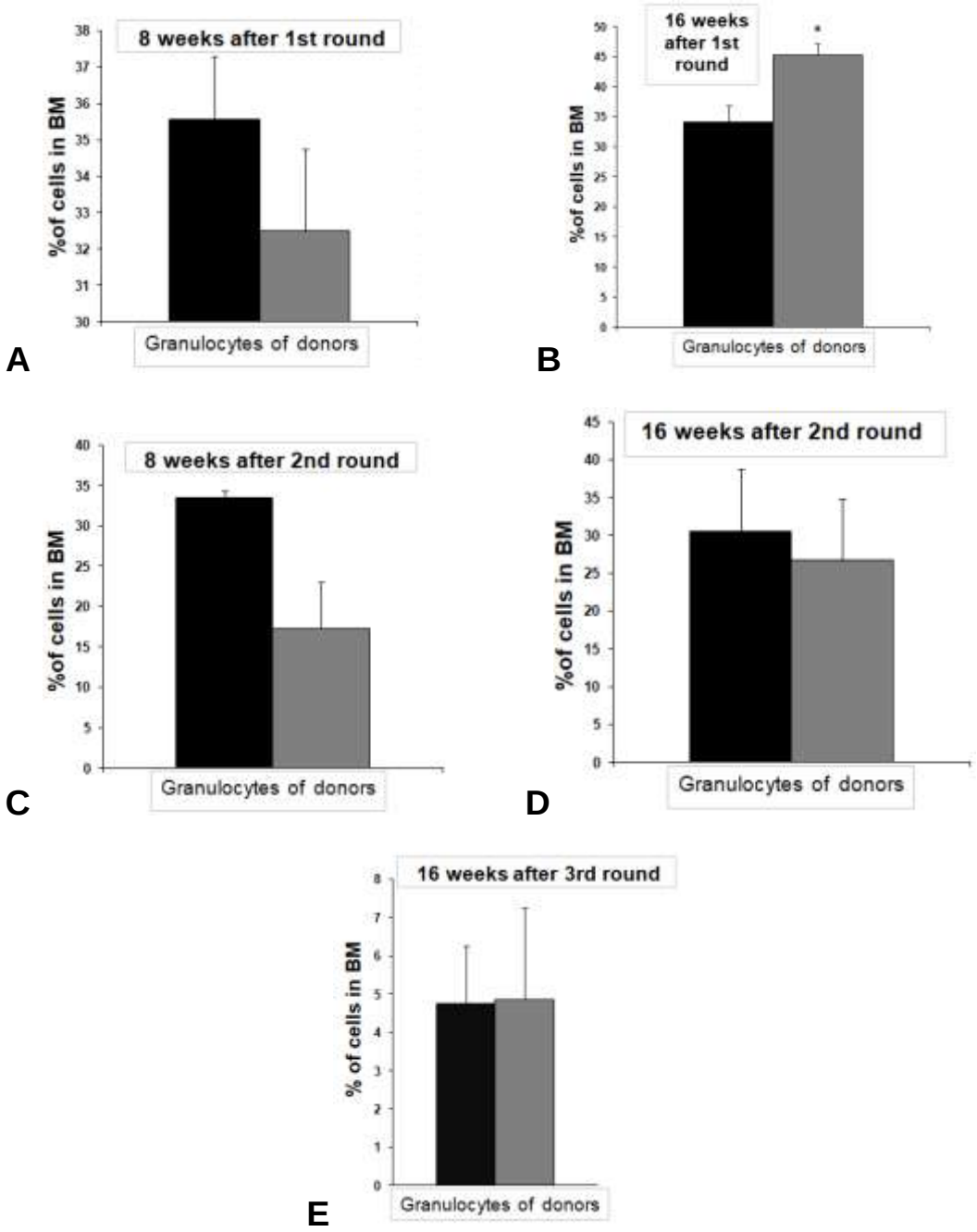


Figure 36. Enhanced granulocyte reconstitution in BM of $CD44^{-/-}$:$OPN^{-/-}$-BM-recipient mice at 16 weeks after 1st round of serial transplantation. Relative abundance of donor granulocyte cells expressing the pan-hematopoietic surface marker CD45.1 in BM of recipient mice are shown. **(A)** 8 weeks after 1st round; n = 3. **(B)** 16 weeks after 1st round; n = 7. **(C)** 8 weeks after 2nd round; n = 3. **(D)** 16 weeks after 2nd round; n = 7. **(E)** 16 weeks after 3rd round of transplant; controls: n = 10; $CD44^{-/-}$:$OPN^{-/-}$-BM-recipients: n= 7. ■ Black bar: wild-type-BM-recipient mice (controls); ■ dark grey bar: $CD44^{-/-}$:$OPN^{-/-}$-BM-recipient mice. Percentage of cells is referring to the extracted BM of 1 femur per mouse. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM; $*P \leq 0{,}05$.

3.3.3.4 Unimpaired monocyte levels in BM of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice during 3 rounds of serial transplantation

No significant differences in monocyte counts within BM of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice and control mice have been observed during the serial transplantaion setting.

Monocyte counts in BM of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice have shown an elevated tendency compared to controls at 8 weeks after 1st (1,8% vs. 1,6%; Fig.37.(A)) and 16 weeks after 3rd (0,6% vs. 0,5%) round of transplantation (Fig.37.(E)).

16 weeks after 1st (Fig.37.(B)), 8 weeks after 2nd Fig.37.(C)) and 16 weeks after 2nd (Fig.37.(D)) round of transplantation monocyte values in BM of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice showed a decreased tendency (1,6%; 0,8%; 1,5%) compared to controls (1,7%; 0,9%; 1,6%).

■ wild-type-BM-recipient mice (controls); ■ $CD44^{-/-}$:$OPN^{-/-}$-BM-recipient mice

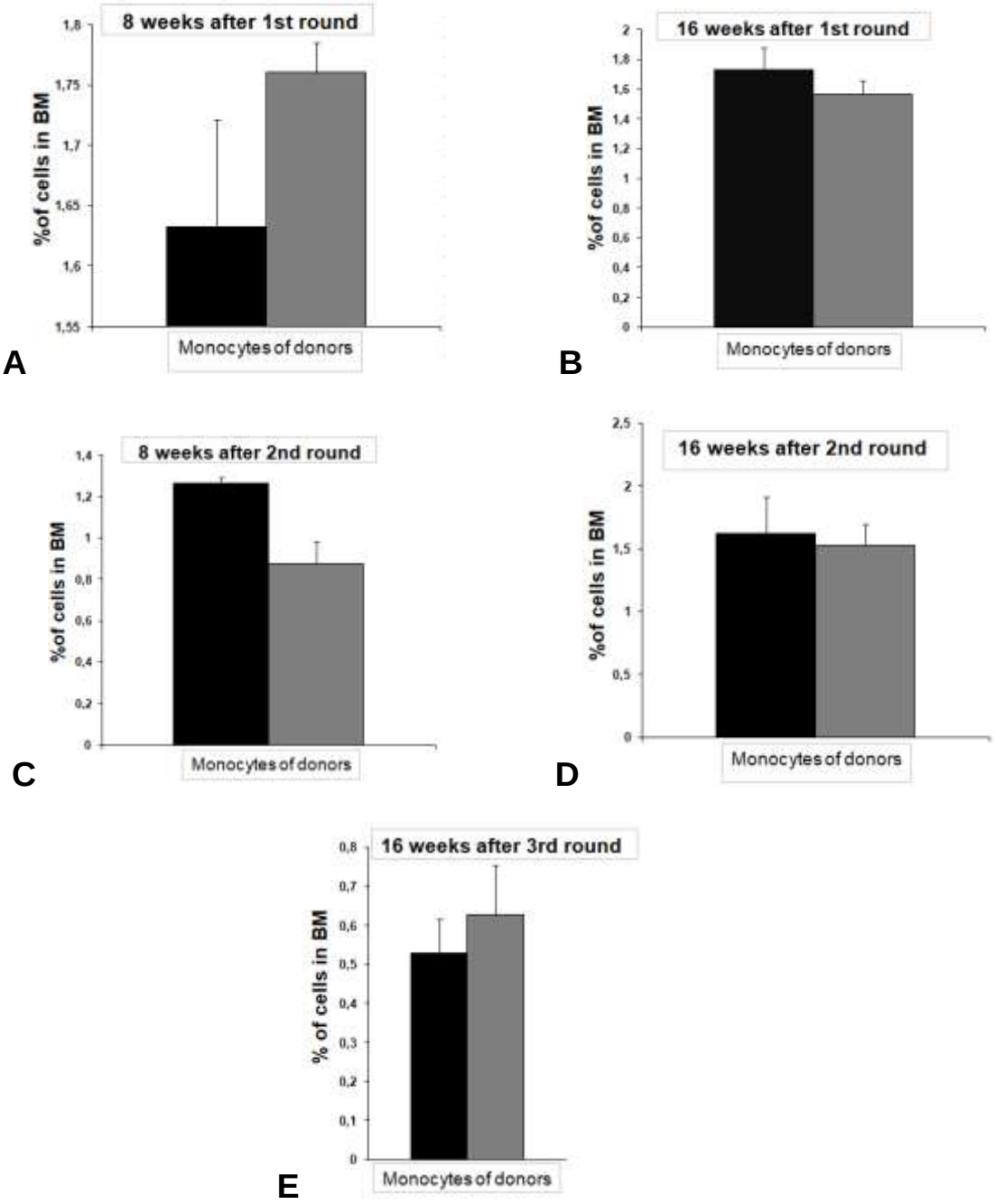


Figure 37. Unimpaired monocyte reconstitution in BM of $CD44^{-/-}$:$OPN^{-/-}$-BM-recipients during 3 rounds of serial transplantation. Relative abundance of donor monocyte cells expressing the pan-hematopoietic surface marker CD45.1 in BM of recipient mice are shown. **(A)** 8 weeks after 1st round; n = 3. **(B)** 16 weeks after 1st round; n = 7. **(C)** 8 weeks after 2nd round; n = 3. **(D)** 16 weeks after 2nd round; n = 7. **(E)** 16 weeks after 3rd round of transplant; controls: n = 10; $CD44^{-/-}$:$OPN^{-/-}$-BM-recipients: n= 7. ■ Black bar: wild-type-BM-recipient mice (controls); ■ dark grey bar: $CD44^{-/-}$:$OPN^{-/-}$-BM-recipient mice. Percentage of cells is referring to the extracted BM of 1 femur per mouse. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM.

3.3.3.5 Diminished erythrocyte reconstitution in BM of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice at 16 weeks after 1st round of transplantation

Erythrocyte values in BM of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice displayed a decreased tendency compared to control levels at 8 weeks after 1st (17,7% vs. 18%) (Fig.40.(A)) and 8 weeks after 2nd (10,5% vs. 13,4%) (Fig.40.(C)) round of transplantation. 16 weeks after 1st round of transplantation erythrocyte numbers in BM of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice were significantly reduced in comparison to those of controls (19,3% vs. 24,6%; $P \leq 0,05$]) (Fig.38.(B)).

A tendency of elevated erythrocyte cell counts in BM of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice relating to wild-types has been observed at 16 weeks after 2nd (14,2% vs. 13,2 %) (Fig.38.(D)) and 16 weeks after 3rd (13,1% vs. 10,5%) round of transplantation (Fig.38. (E)).

■ wild-type-BM-recipient mice (controls); ■ $CD44^{-/-}$:$OPN^{-/-}$-BM-recipient mice

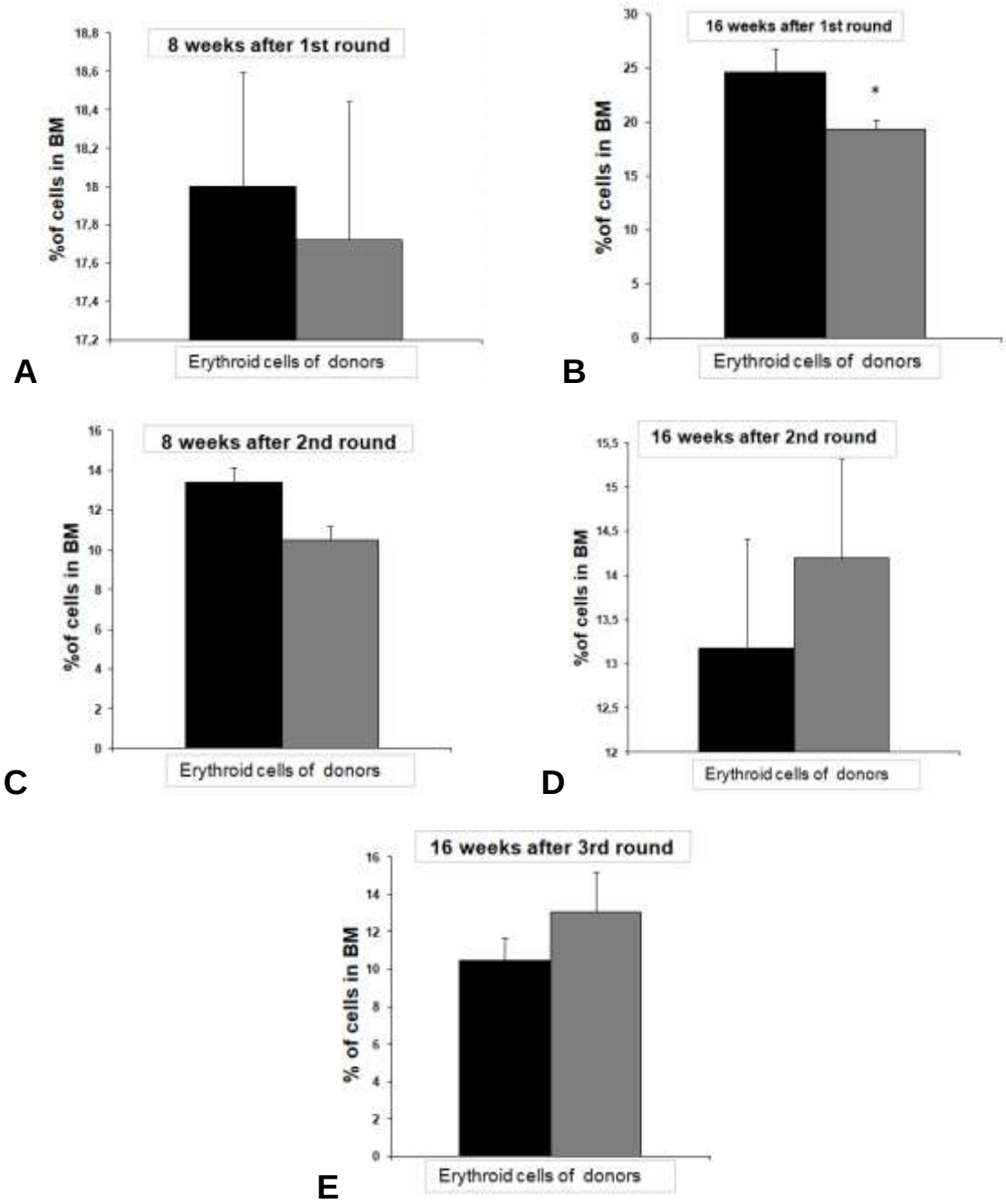


Figure 38. Decreased erythroid cell reconstitution in BM of $CD44^{-/-}$:$OPN^{-/-}$-BM-recipient mice at 16 weeks after 1st round of serial transplantation. Relative abundance of erythrocyte donor cells expressing the pan-hematopoietic surface marker CD45.1 in BM of recipient mice are shown. **(A)** 8 weeks after 1st round; n = 3. **(B)** 16 weeks after 1st round; n = 7. **(C)** 8 weeks after 2nd round; n = 3. **(D)** 16 weeks after 2nd round; n = 7. **(E)** 16 weeks after 3rd round of transplant; controls: n = 10; $CD44^{-/-}$:$OPN^{-/-}$-BM-recipients: n= 7. ■ Black bar: wild-type-BM-recipient mice (controls); ■ dark grey bar: $CD44^{-/-}$:$OPN^{-/-}$-BM-recipient mice. Percentage of cells is referring to the extracted BM of 1 femur per mouse. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM; *$P \leq 0,05$.

3.3.3.6 Diminished reconstitution ability of progenitors in BM of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice at 16 weeks after 3rd round of serial transplantation

8 weeks after 1st round of serial transplantation, LKS and MPP counts in BM of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice were at comparable levels than those of controls (LKS: 0,16% and MPP: 0,11%) (Fig. 39). CLP and CMP progenitor numbers in BM of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice exhibited a significant reduction compared to wild-type-BM-recipient controls. GMP progenitor numbers in BM of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice displayed a tendency of reduction in reference to wild-type-BM-recipients (Fig. 39).

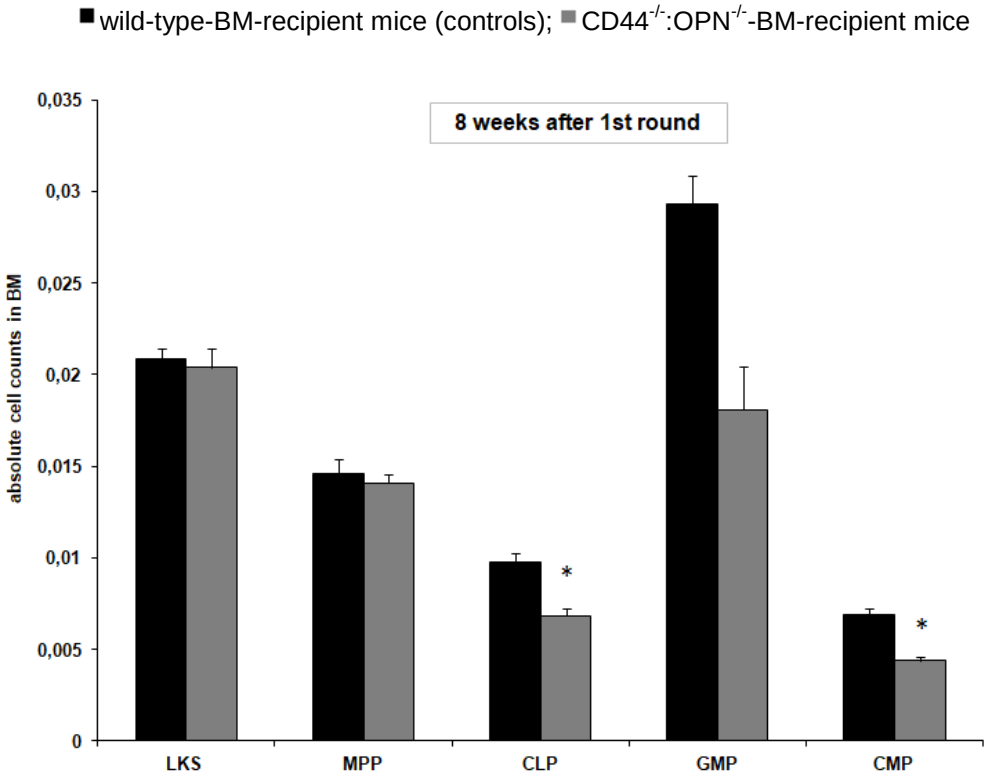


Figure 39. Reduced CLP and CMP progenitor reconstitution in BM of CD44$^{-/-}$:OPN$^{-/-}$-recipient mice at 8 weeks after 1st round of serial transplantation. Relative abundance of progenitor donor cells expressing the pan-hematopoietic surface marker CD45.1 in BM of recipient mice are shown. Lineage negative, c-kit positive and Sca1 positive cells LKS; multipotent progenitors (MPP); common lymphhoid progenitors (CLP); granulocyte-monocyte progenitors GMP; common myeloid progenitors (CMP). ■ Black bar: wild-type-BM-recipient mice (controls); n = 3; ■ dark grey bar: CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice; n = 3. Percentage of cells is referring to the extracted BM of 1 femur per mouse. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM; *$P \leq 0,05$.

16 weeks after 1st round of transplantation CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice showed a significant reduction of lineage negative, c-kit positive and Sca1positive cells (LKS: 0,3%;)- and multipotent progenitors (MPP: 0,2%; $P \leq 0,05$) within the BM relating to controls (LKS: 0,5% ; MPP: 0,4%) (Fig.40.). Common myeloid progenitor levels (CMP) in BM of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice were significantly elevated (0,2%; $P \leq 0,05$) compared to those of wild-type controls (0,1%) (Fig.40). A tendency of higher numbers of common lymphoid progenitors (CLP) in CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice (0,1%) has been observed, while their granulocyte-monocyte progenitors (GMP) (0,1%) exhibited a decreased number relating to controls (0,6%; 0,2%) (Fig.40).

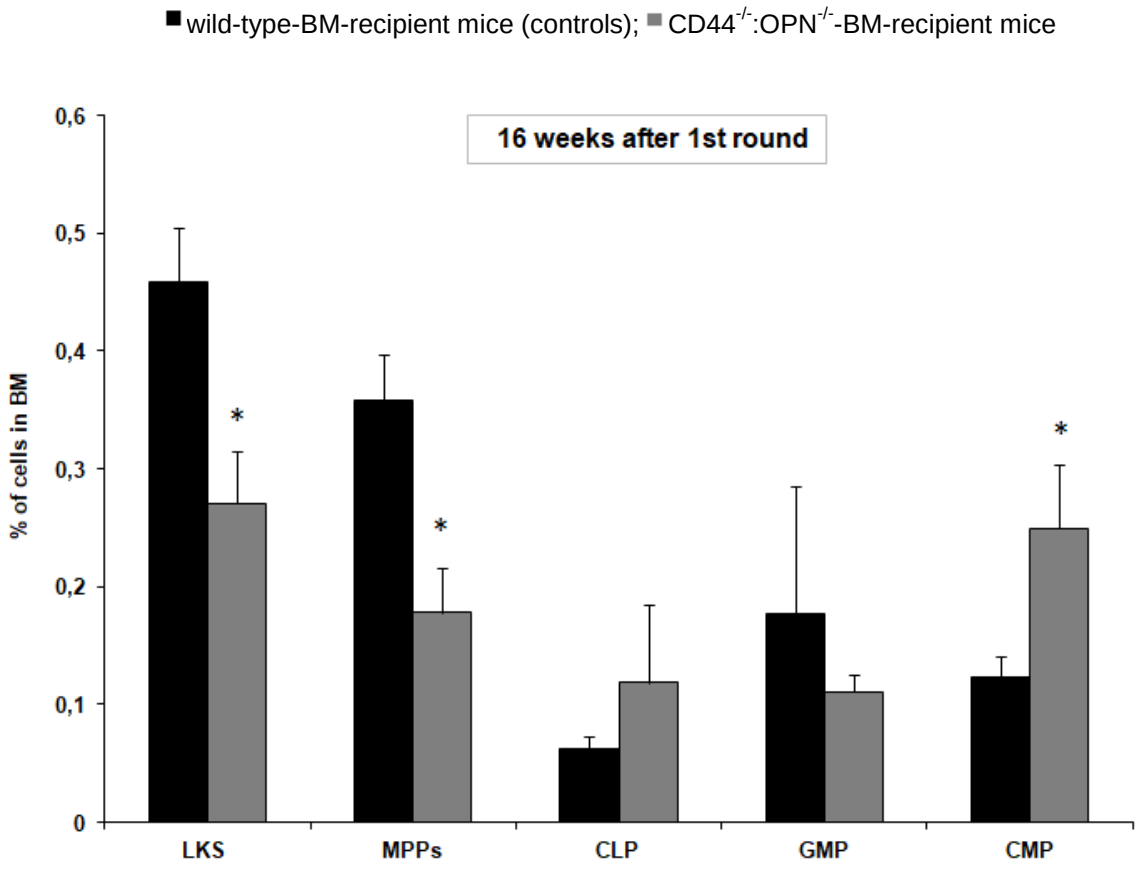


Figure 40. Reduced LKS and MPP- and enhanced CMP reconstitution in BM of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice at 16 weeks after 1st round of serial transplantation. Relative abundance of progenitor donor cells expressing the pan-hematopoietic surface marker CD45.1 in BM of recipient mice are shown. Lineage negative, c-kit positive and Sca1 positive cells (LKS); multipotent progenitors (MPP); common lymphhoid progenitors (CLP); granulocyte-monocyte progenitors GMP; common myeloid progenitors (CMP). ■Black bar: wild-type-BM-recipient mice (controls); n = 7; ■dark grey bar: CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice; n = 7. Percentage of cells is referring to the extracted BM of 1 femur per mouse. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM; *$P \leq 0,05$.

8 weeks after 2nd round of serial transplant no significant differences in LKS, MPP, CLP, GMP and CMP cell populations in BM of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient- and control mice could have been observed (Fig.41). Although, LKS and MPP cells in BM of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice showed a tendency of elevation, while CLPs, GMPs and CMPs exhibited a decreased tendency (Fig.41) in relation to wild-types.

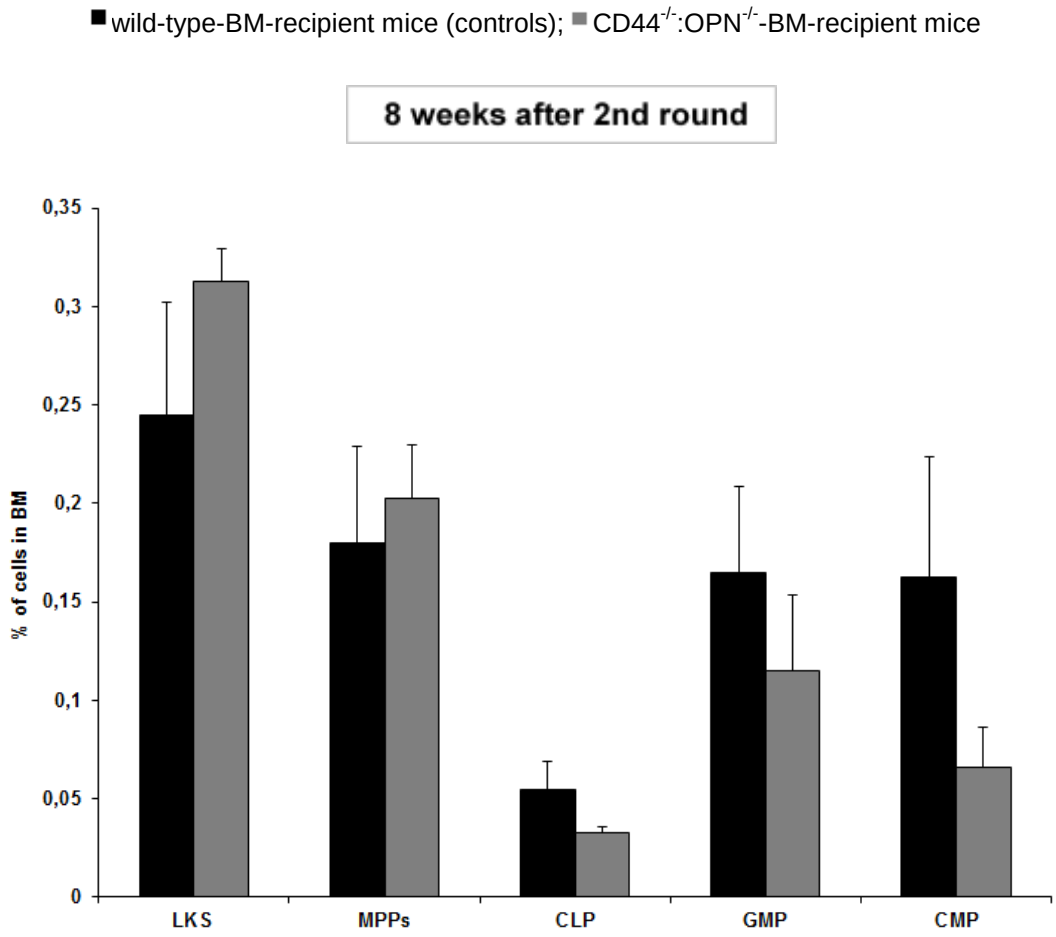


Figure 41. Unimpaired progenitor reconstitution in BM of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice at 8 weeks after 2nd round of serial transplantation. Relative abundance of progenitor donor cells expressing the pan-hematopoietic surface marker CD45.1 in BM of recipient mice are shown. Lineage negative, c-kit positive and Sca1 positive cells (LKS); multipotent progenitors (MPP); common lymphhoid progenitors (CLP); granulocyte-monocyte progenitors GMP; common myeloid progenitors (CMP). ■ Black bar: wild-type-BM-recipient mice (controls); n = 3; ■ dark grey bar: CD44$^{-/-}$:OPN$^{-/-}$-BM- recipient mice; n = 3. Percentage of cells is referring to the extracted BM of 1 femur per mouse. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM.

16 weeks after 2nd round of transplant, CLP numbers in BM of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice were significant lower (0,3%; $P \leq 0,05$) than in wild-type controls (0,5%) (Fig.42). LKS and MPP cell populations in BM were present in comparable numbers in

both recipeint groups (about 0,2% and 0,1%) (Fig.42). GMP (0,3%) and CMP (0,1%) counts in BM of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice displayed a decreased tendency in reference to controls (0,4%; 0,14%)(Fig.42).

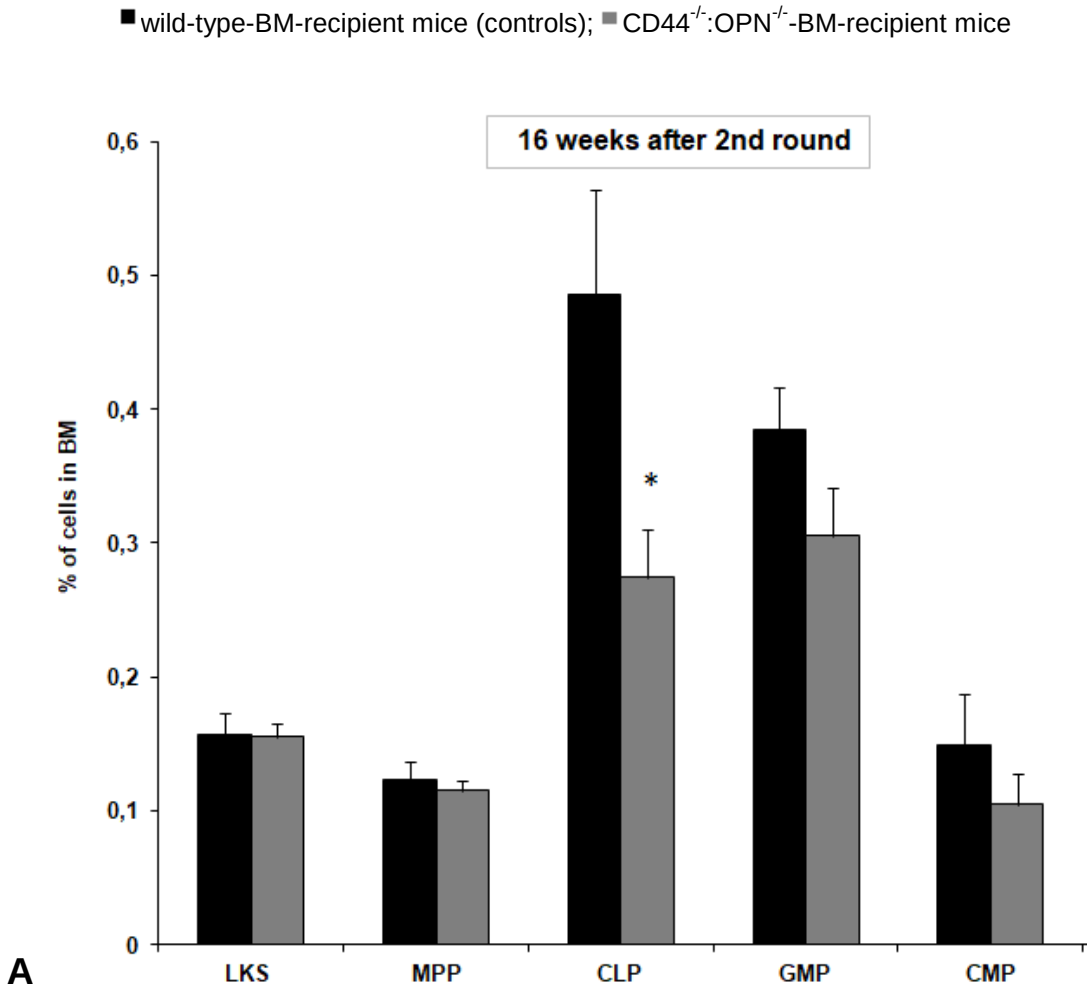


Figure 42. Reduced CLP progenitor reconstitution in BM of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice at 16 weeks after 2nd round of serial transplant. Relative abundance of progenitor donor cells expressing the pan-hematopoietic surface marker CD45.1 in BM of recipient mice are shown. Lineage negative, c-kit positive and Sca1 positive cells (LKS); multipotent progenitors (MPP); common lymphhoid progenitors (CLP); granulocyte-monocyte progenitors GMP; common myeloid progenitors (CMP). ■Black bar: wild-type-BM-recipient mice (controls); n = 7; ■dark grey bar: CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice; n = 7. Percentage of cells is referring to the extracted BM of 1 femur per mouse. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM;*$P \leq 0,05$.

16 weeks after 3rd round of serial transplant, MPP, CLP, GMP and CMP cell populations in BM of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice were significantly lower than in wild-type controls (Fig.43). MPPs were more than 1,4 times ($P \leq 0,05$), CLPs more than 0,6 times ($P \leq 0,05$), GMPs more than 3,5 times ($P \leq 0,0005$) and CMPs more than 0,5 times ($P \leq$

0,05) lower than in controls (Fig.43). LKS cells in BM of both genotypes were at comparable levels (~0,2%) (Fig.43).

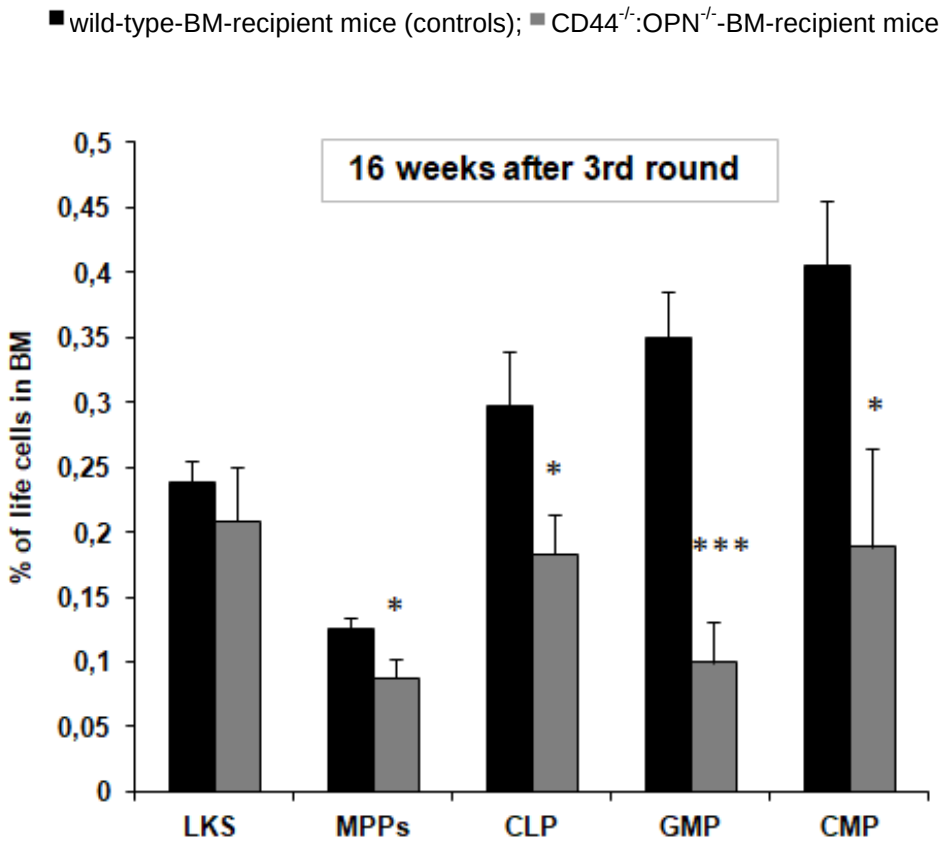


Figure 43. Severely impaired progenitor reconstitution in BM of CD44$^{-/-}$:OPN$^{-/-}$-BM- recipient mice at 16 weeks after 3 rounds of serial transplantation. Relative abundance of progenitor donor cells expressing the pan-hematopoietic surface marker CD45.1 in BM of recipient mice are shown. Lineage negative, c-kit positive and Sca1 positive cells (LKS); multipotent progenitors (MPP); common lymphhoid progenitors (CLP); granulocyte-monocyte progenitors GMP; common myeloid progenitors (CMP). ■ Black bar: wild-type-BM-recipient mice (controls); n = 10; ■ dark grey bar: CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice; n = 7. Percentage of cells is referring to the extracted BM of 1 femur per mouse. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM;**$P \leq 0,05$; **$P \leq 0,005$; ***$P \leq 0,0005$.

3.3.3.7 Diminished reconstitution ability of HSC in BM of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice after 3rd round of serial transplantation

No significant differences in HSC numbers in BM of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice and wild-type controls have been found at 8 weeks after 1st (Fig.44.(A)), 16 weeks after 1st (Fig.44.(B)), 8 weeks after 2nd (Fig.44.(C)) and 16 weeks after 2nd round (Fig.44.(D)) of serial transplantation.

Hematopoietic stem cell levels in BM of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice exhibited an elevated tendency referring to wild-type-BM-recipient mice (controls) at 8 weeks after 1st (0,016% vs. 0,012%; Fig.44.(A)), 8 weeks after 2nd (0,06% vs. 0,04%; Fig.44.(C)) and 16 weeks after 2nd (0,03% vs. 0,02%; round of transplant Fig.44.(D)). 16 weeks after 1st round of transplant HSCs of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice showed a tendency of reduction compared to wild-types (Fig.44.(B)).

A significant reduction of HSC ($P \leq 0,05$) in BM of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice within the serial stem cell transplant setting, was noticable at 16 weeks after 3rd round of transplantation (Fig.44.(E)). In this case HSCs in BM of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice (0,05%) were 0,6 times fewer present than in control mice (0,08%).

■ wild-type-BM-recipient mice (controls); ■ $CD44^{-/-}$:$OPN^{-/-}$-BM-recipient mice

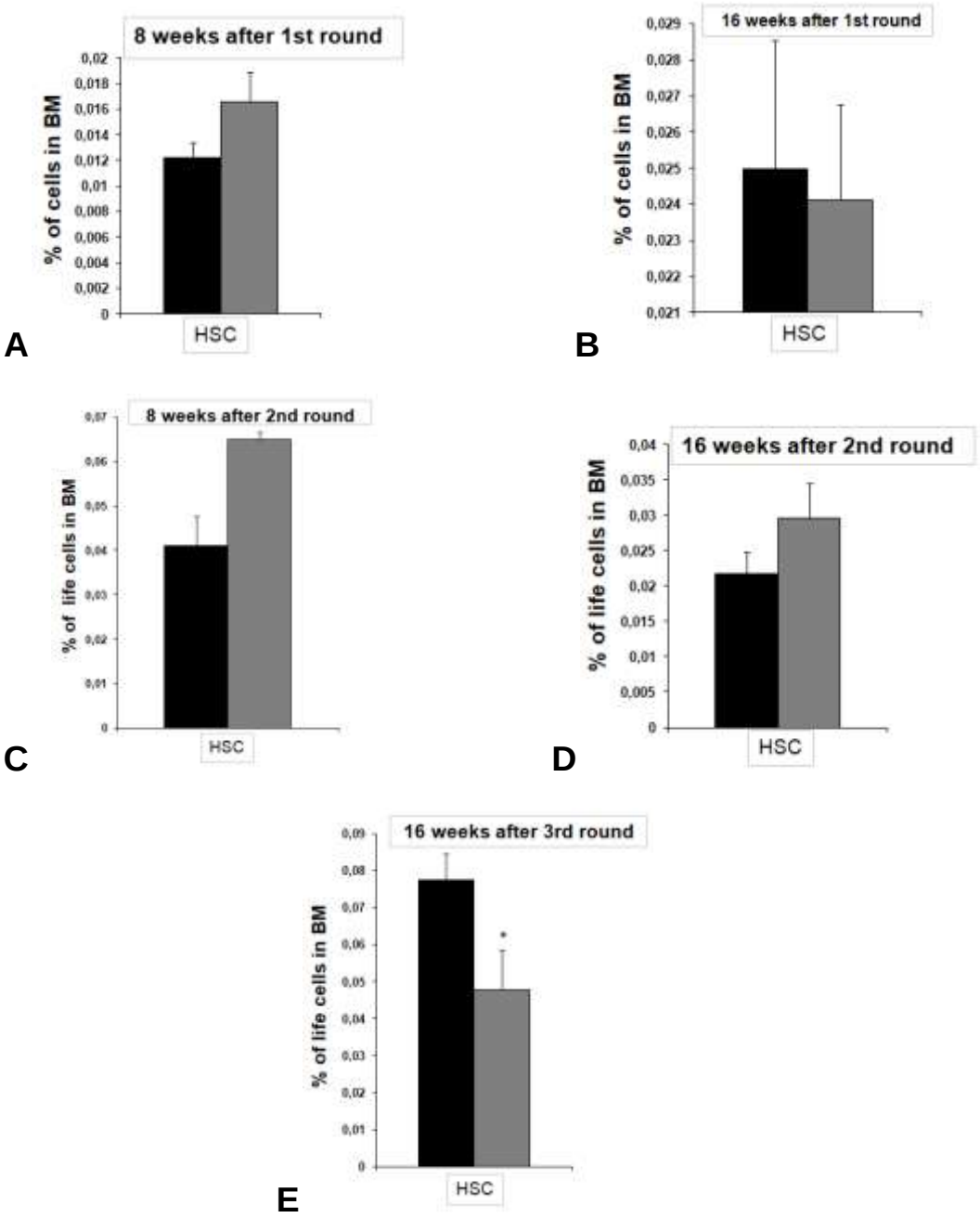


Figure 44. Reduced HSC reconstitution in BM of $CD44^{-/-}$:$OPN^{-/-}$-BM-recipient mice after 3 rounds of serial transplantation. Relative abundance of donor hematopoietic stem cells (HSC) expressing the pan-hematopoietic surface marker CD45.1 in BM of recipient mice are shown. **(A)** 8 weeks after 1st round; n = 3. **(B)** 16 weeks after 1st round; n = 7. **(C)** 8 weeks after 2nd round; n = 3. **(D)** 16 weeks after 2nd round; n = 7. **(E)** 16 weeks after 3rd round of transplant; controls: n = 10; $CD44^{-/-}$:$OPN^{-/-}$-BM- recipients: n= 7. ■ Black bar: wild-type-BM-recipient mice (controls); ■ dark grey bar: $CD44^{-/-}$:$OPN^{-/-}$- BM-recipient mice. Percentage of cells is referring to the extracted BM of 1 femur per mouse. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM; $*P \leq 0{,}05$.

3.3.3.8 MEP numbers in BM of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice decline at 8 weeks after 1st round and 16 weeks after 3rd round of serial transplantation

Megakaryocyte-erythroid progenitor numbers in BM of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice exhibited to be significantly lower (0,2%; $P \leq 0,05$) than in controls (0,3%) at 8 weeks after 1st (Fig.45.(A)) and 16 weeks after 3rd round of transplant (0,1% vs. 0,2%; $P \leq 0,05$) (Fig.45.(E)). A tendency of reduced MEP levels in BM of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice relating to wild-type controls has been found at 8 weeks after 2nd (0,2% vs. 0,4%; Fig.45.(C)) and at 16 weeks after 2nd (0,19% vs. 0,21%; Fig.45.(D)) round of transplantation.

Only one time within the serial transplant setting, MEP in BM of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient showed a minor elevation compared to wild-type conrols. At 16 weeks after 1st round of transplantation CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice exhibited 0,34% of MEP in BM; control levels have been at 0,3% (Fig.45.(B)).

■ wild-type-BM-recipient mice (controls); ■ CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice

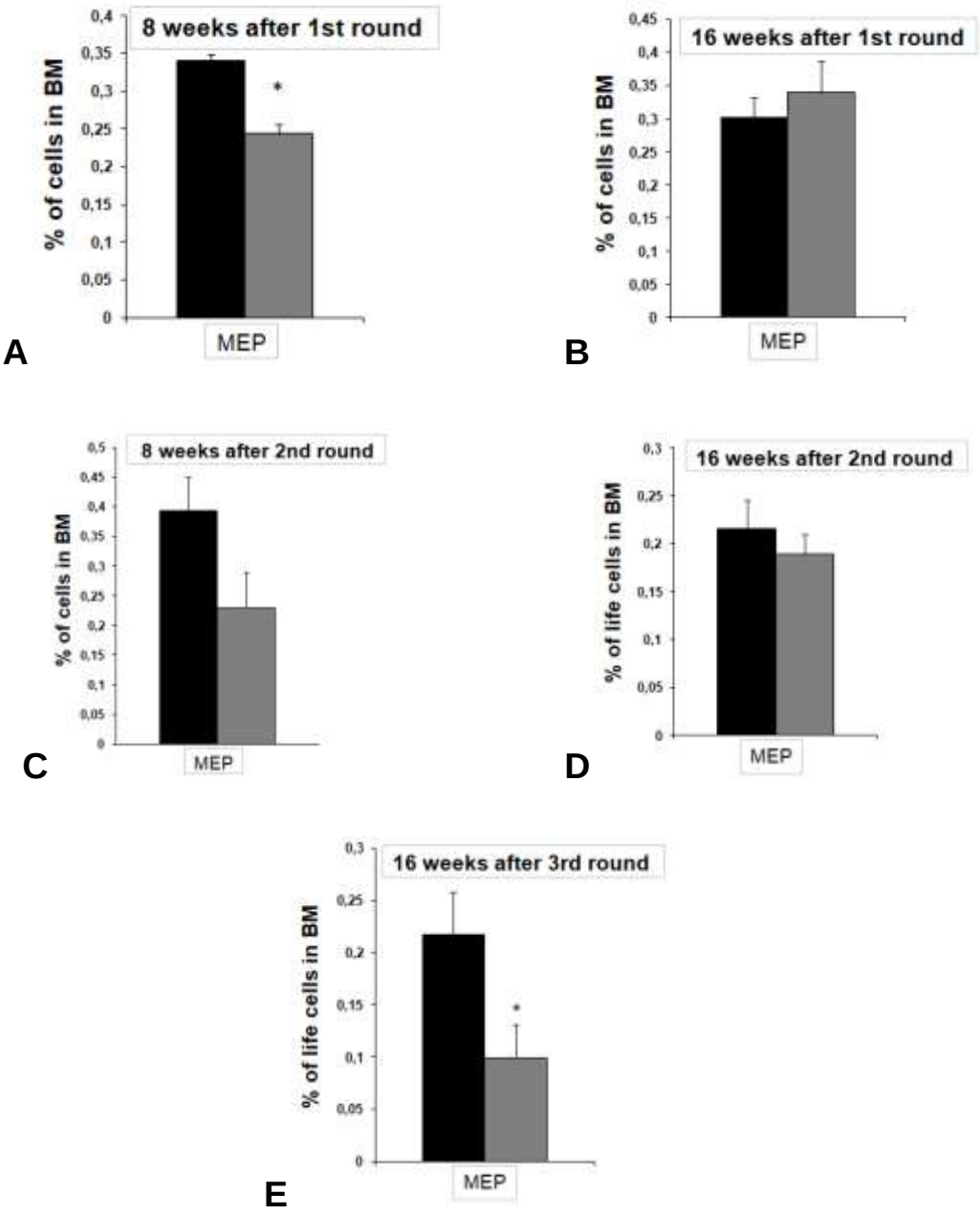


Figure 45. Reduced MEP reconstitution in CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice at 8 weeks after 1st and 16 weeks after 3rd round of serial transplantation. Relative abundance of megakaryocyte-erythroid progenitors (MEP) expressing the pan-hematopoietic surface marker CD45.1 in BM of recipient mice are shown. **(A)** 8 weeks after 1st round; n = 3. **(B)** 16 weeks after 1st round; n = 7. **(C)** 8 weeks after 2nd round; n = 3. **(D)** 16 weeks after 2nd round; n = 7. **(E)** 16 weeks after 3rd round of transplant; controls: n = 10; CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice: n= 7. ■ Black bar: wild-type-BM-recipient mice (controls); ■ dark grey bar: CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice. Percentage of cells is referring to the extracted BM of 1 femur per mouse. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM; *$P \leq 0{,}05$.

3.4 The effect of G-CSF stress on immune response of CD44$^{-/-}$:OPN$^{-/-}$- and OPN$^{-/-}$ mice

The daily intraperitoneal (ip) injection of G-CSF has quickly led to very high levels of white blood cells in the peripheral blood of the referring mice. The measurement with the BC-5000 Vet blood analyzer for some samples was very difficult, even after dilution with sterile PBS. The high content of leukocytes in the extracted peripheral blood of G-CSF treated mice has additionally been difficult to handle in blood staining for further leukocyte subset analyses (B, T, granulocyte and monocyte numbers) on BD Diva FACS measurement. For some samples, complete blood counts have not been possible. For this reason the “n” numbers, which indicates the count of treated animals in each cohort, varies between the figures. Only reliable data have been included into the data matrix calculations. After G-CSF treatment for the duration of 6 days with daily (IP) injections of 0,4 µg/g (body weight per mouse) following results have been observed.

3.4.1 G-CSF reduces thrombocyte levels in peripheral blood of OPN$^{-/-}$- and wild-type mice at 4 and 6 days after treatment

Before treatment, thrombocyte values in peripheral blood exhibited a slightly elevated tendency in CD44$^{-/-}$:OPN$^{-/-}$- (1.113.200 cells/µl) and OPN$^{-/-}$ mice (1.100.100cells/µl) compared to wild-types (986.800 cells/µl) (Fig.46.(A)). Peripheral blood count measurements 3 days after G-CSF treatment, showed no significant differences in thrombocyte levels in treated wild-types (777.100 cells/µl), CD44$^{-/-}$:OPN$^{-/-}$- (1.006.000cells/µl) and OPN$^{-/-}$ mice (874.181 cells/µl) compared to untreated controls (968.500 cells/µl) (Fig.46.(B)).

wild-type control mice; treated wild-type mice; treated CD44$^{-/-}$:OPN$^{-/-}$ mice; treated OPN$^{-/-}$ mice

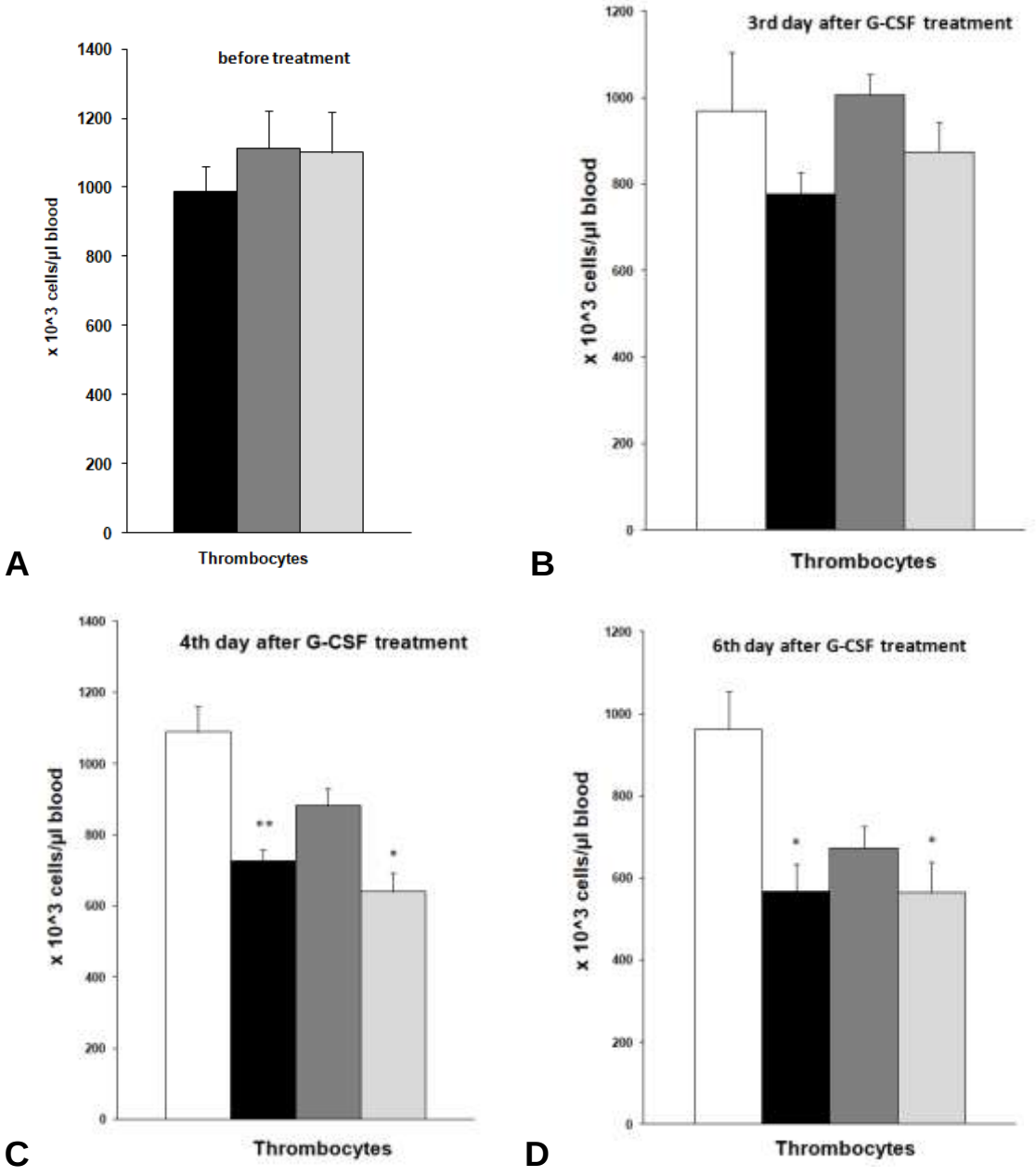


Figure 46. G-CSF reduces thrombocyte levels in peripheral blood of OPN$^{-/-}$ and wild-type mice at 4 and 6 days after treatment. Absolute thrombocyte counts in peripheral blood are shown. **(A)** Before treatment with G-CSF; wild-type: n = 9; CD44$^{-/-}$:OPN$^{-/-}$: n = 9; OPN$^{-/-}$: n = 9. **(B)** 3 days after G-CSF treatment; untreated wild-types (controls): n = 2; treated wild-types: n = 10; treated CD44$^{-/-}$:OPN$^{-/-}$ mice: n = 6; treated OPN$^{-/-}$: n = 11. **(C)** 4 days after G-CSF treatmen; untreated wild-types (controls): n = 2; treated CD44$^{-/-}$:OPN$^{-/-}$ mice: n = 6; OPN$^{-/-}$ mice: n = 7. **(D)** 6 days after G-CSF treatment; untreated wild-type mice (controls): n = 2; treated wild-types: n = 7; treated CD44$^{-/-}$:OPN$^{-/-}$ mice: n = 7; treated OPN$^{-/-}$ mice: n = 7. White bar: wild-type control mice (not treated with G-CSF); Black bar: treated wild-type mice; dark grey bar: treated CD44$^{-/-}$:OPN$^{-/-}$ mice; light grey bar: treated OPN$^{-/-}$ mice. Unpaired, 2-tailed t test was performed to determine significance.Values are the mean ± SEM; *$P \leq 0{,}05$; **$P \leq 0{,}005$.

4 days after Neupogen treatment thrombocyte levels in peripheral blood of treated wild-types (725.571 cells/µl; $P \leq 0{,}005$) and $OPN^{-/-}$ mice (639.857 cells/µl; $P \leq 0{,}05$) were significantly decreased in comparison to untreated controls (1.088.000cells/µl), whereas no significant difference in thrombocyte levels has been found between $CD44^{-/-}$:$OPN^{-/-}$ mice (881.333 cells/µl) and controls (Fig.46.(C)).

After 6 days of treatment, thrombocyte levels in peripheral blood of treated wild-types (565.571 cells/µl; $P \leq 0{,}005$) and $OPN^{-/-}$ mice (563.857 cells/µl; $P \leq 0{,}005$) were significantly decreased in comparison to untreated controls (962.500 cells/µl), whereas no significant difference in thrombocyte levels has been found between $CD44^{-/-}$:$OPN^{-/-}$- (881.333 cells/µl) and control mice (Fig.46.(D)).

3.4.2 G-CSF treatment shows no different impact on leukocyte cell counts in peripheral blood of $CD44^{-/-}$:$OPN^{-/-}$ mice than in wild-types

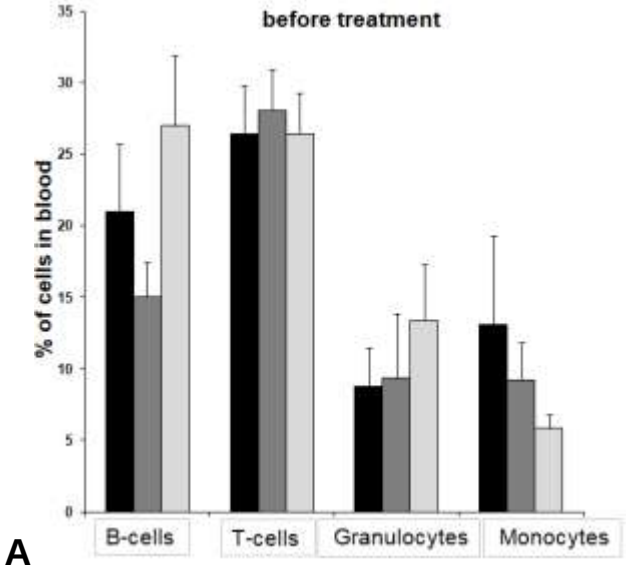
Before G-CSF treatment, no significant differences in B cell, T cell, granulocyte- and monocyte cell counts between WT, $CD44^{-/-}$:$OPN^{-/-}$- and $OPN^{-/-}$ mice have been observed (Fig.47.(A)). A tendency of decreased B cell levels in peripheral blood has been found in $CD44^{-/-}$:$OPN^{-/-}$ mice (15%), whereas B cells of $OPN^{-/-}$ mice seemed to be elevated (27%) referring to wild-types (33%) (Fig. 47.(A)). T cell counts in peripheral blood of all three observed genotypes have been at comparable levels (between 26 till 28 %) before Neupogen treatment (Fig. 47.(A)). Granulocyte counts in the peripheral blood of $CD44^{-/-}$:$OPN^{-/-}$ mice (9%) have been similar to those of wild-type levels (8,7%) before treatment, but $OPN^{-/-}$ mice exhibited an elevated tendency (13%). Before treatment, monocyte levels in peripheral blood of wild-types were highest of all cohorts (13%), slightly reduced in $CD44^{-/-}$:$OPN^{-/-}$- (9%) and lowest in $OPN^{-/-}$ mice (5,8%), without being significant (Fig. 47.(A)).

3 days after treatment B cells in peripheral blood of $OPN^{-/-}$ mice were at comparable numbers (36%) than those of controls (33%) (Fig.47.(B)). B cell levels in peripheral blood of $CD44^{-/-}$:$OPN^{-/-}$ mice were diminished to 22% referring to controls, T cell (~17%)

and granulocyte- counts (~29%) were comparable in peripheral blood of all 3 observed genotypes in reference to untreated controls (Fig.47.(B)). No significant differences in T cell, granulocyte and monocyte levels in peripheral blood of G-CSF treated wild-types, $CD44^{-/-}$:$OPN^{-/-}$- and $OPN^{-/-}$ mice have been found referring to untreated controls (Fig.47.(B)).

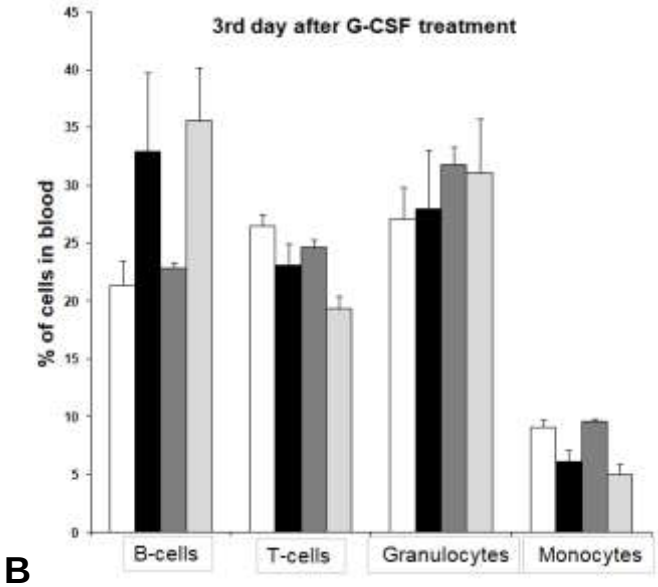
3 days after Neupogen treatment, granulocyte counts in peripheral blood of WT have risen to 27,93% (before treatment, WT had only exhibeted 8,73% granulocytes in peripheral blood), that is an exaggeration of 19,2%. $CD44^{-/-}$:$OPN^{-/-}$ mice's granulocyte levels in peripheral blood have risen about 22,46% (before treat: 9,34%; after: 31,8%) and in $OPN^{-/-}$ mice about 17,7% (before treat: 13,38%; after treat: 31,08%) (compare Fig.47.(A) and (B)).

■ wild-type mice; ■ CD44$^{-/-}$:OPN$^{-/-}$ mice; ■ OPN$^{-/-}$ mice



A

□ wild-type control mice; ■ treated wild-type mice; ■ treated CD44$^{-/-}$:OPN$^{-/-}$ mice; ■ treated OPN$^{-/-}$ mice



B

Figure 47. G-CSF has no impact on blood leukocyte numbers in wild-type, CD44$^{-/-}$:OPN$^{-/-}$- and OPN$^{-/-}$ mice at 3 days after treatment. Relative B cell, T cell, granulocyte and monocyte counts in peripheral blood are shown. **(A)** Before treatment; wild-type mice: n = 12; CD44$^{-/-}$:OPN$^{-/-}$ mice: n = 9; OPN-/-: n = 13. **(B)** 3 days after treatment; untreated wild-type mice: n = 2; treated CD44$^{-/-}$:OPN$^{-/-}$ mice: n = 2; treated OPN$^{-/-}$ mice: n = 6. □ White bar: wild-type control mice (not treated with G-CSF); ■ Black bar: wild-type mice; ■ dark grey bar: CD44$^{-/-}$:OPN$^{-/-}$ mice; ■ light grey bar: OPN$^{-/-}$ mice. Unpaired, 2-tailed t test was performed to determine significance.Values are the mean ± SEM.

4 days after G-CSF treatment B cells and monocytes in peripheral blood of treated wild-types (B cells: 18%; monocytes: 5%), $CD44^{-/-}$:$OPN^{-/-}$- (B cells: 22%; monocytes: 4%) and $OPN^{-/-}$ mice (B cells: 22%; monocytes: 4%) were at comparable levels than those of untreated wild-types (B cells: 22%; monocytes: 8%) (Fig.48.(A)). T cell counts in peripheral blood of treated wild-types (15%; $P \leq 0,05$), $CD44^{-/-}$:$OPN^{-/-}$- (15%; $P \leq 0,05$) and $OPN^{-/-}$ mice (14%; $P \leq 0,05$) were significantly decreased compared to untreated wild-type levels (40%) (Fig.48.(A)). Granulocyte counts in peripheral blood of treated wild-type- (54%), $CD44^{-/-}$:$OPN^{-/-}$- (53%) and $OPN^{-/-}$ mice (54%) exhibited an increased tendency in comparison to those of untreated wild-types (19%)(Fig.48.(A)). G-CSF treatment for a duration of 4 days led to a more than 2,8 fold increase in granulocyte levels in peripheral blood of wild-types, $CD44^{-/-}$:$OPN^{-/-}$- and $OPN^{-/-}$ mice in reference to untreated controls.

wild-type control mice; treated wild-type mice; treated $CD44^{-/-}$:$OPN^{-/-}$ mice; treated $OPN^{-/-}$ mice

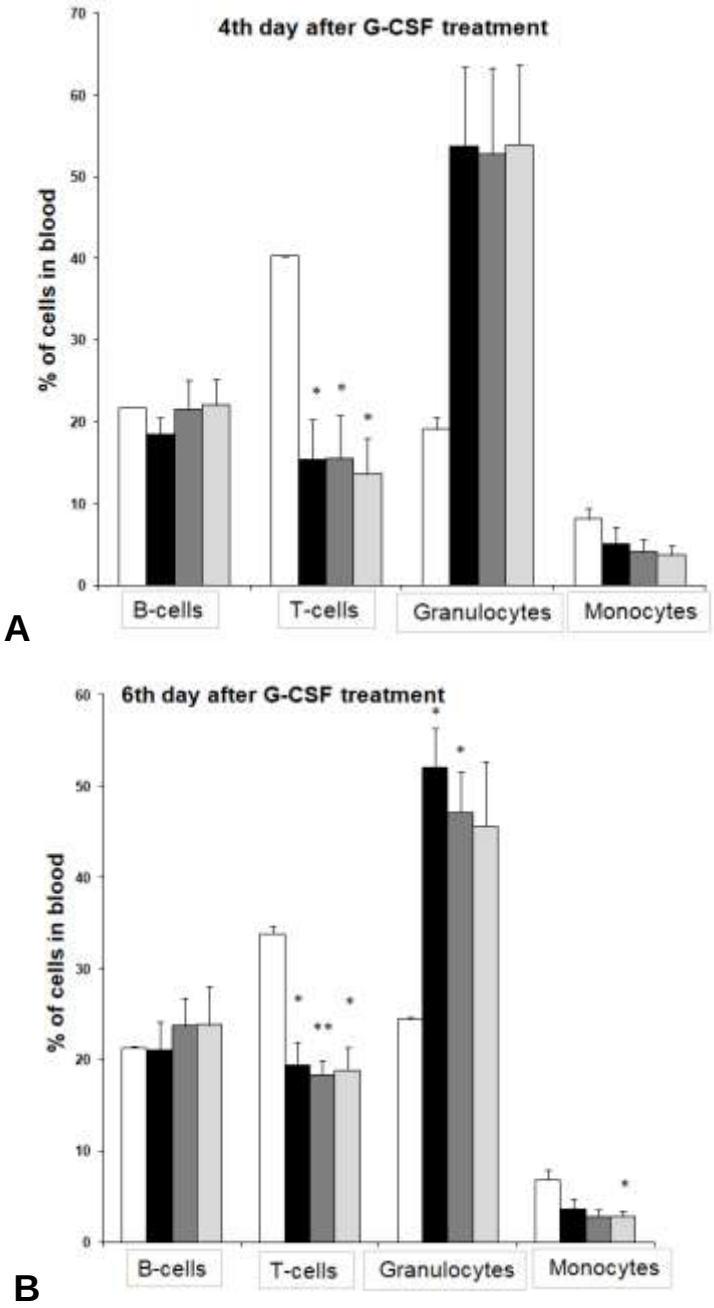


Figure 48. G-CSF treatment lowers blood T cell levels in wild-type-, $CD44^{-/-}$:$OPN^{-/-}$- and $OPN^{-/-}$ mice, reduces blood monocyte levels in $OPN^{-/-}$ mice and elevates blood granulocyte levels in wild-type and $CD44^{-/-}$:$OPN^{-/-}$ mice. Relative B cell, T cell, granulocyte and monocyte cell numbers in peripheral blood are shown. **(A)** 4 days after treat; controls: n = 2; treated wild-types: n = 7; $CD44^{-/-}$:$OPN^{-/-}$ mice: n = 7; $OPN^{-/-}$ mice: n = 7. **(B)** 6 days after treat; controls: n = 2; treated wild-types: n = 7; $CD44^{-/-}$:$OPN^{-/-}$ mice: n = 7; $OPN^{-/-}$ mice: n = 7. White bar: wild-type control mice (not treated with G-CSF);); Black bar: treated wild-type mice; dark grey bar: treated $CD44^{-/-}$:$OPN^{-/-}$ mice; light grey bar: treated $OPN^{-/-}$ mice. Unpaired, 2-tailed t test was performed to determine significance.Values are the mean ± SEM; **P* ≤ 0,05; ***P* ≤ 0,005.

6 days after Neupogen treatment, B cell levels in peripheral blood of treated wild-type- (21%), $CD44^{-/-}$:$OPN^{-/-}$- (24%) and $OPN^{-/-}$ mice (24%) displayed to be within a similar range than those of untreated controls (21%) (Fig.48.(B)). T cell counts in peripheral blood of treated wild-types (19%; $P \leq 0,05$), $CD44^{-/-}$:$OPN^{-/-}$- (18%; $P \leq 0,005$) and $OPN^{-/-}$ mice (19%; $P \leq 0,05$) displayed to be significant declined referring to untreated controls (34%) (Fig.48.(B)). Granulocyte counts in peripheral blood of treated wild-type- (52%; $P \leq 0,05$) and $CD44^{-/-}$:$OPN^{-/-}$ mice (47%; $P \leq 0,05$) showed a significant increase relating to untreated controls (24%), whereas granulocyte levels in OPN mice (46%) displayed an increased tendency, without being significant (Fig.48.(B)). Monocyte levels in peripheral blood of treated wild-types (4%) and $CD44^{-/-}$:$OPN^{-/-}$ mice (3%) did not differ significantly from those of untreted controls (7%) (Fig.48.(B)). Monocyte counts in peripheral blood of $OPN^{-/-}$ mice (2,86%; $P \leq 0,05$) were significantly decreased in reference to untreated wild-type controls (7%)(Fig.50.(B)).

In comparison of granulocyte values before and 6 days after treatment, WT values have risen the most. After treatment, 43,27% more granulocytes in peripheral blood of wild-types have been found compared to pre-treatment state (before treat: 8,73%; after: 52%). Granulocyte levels of $CD44^{-/-}$:$OPN^{-/-}$ mice showed a smaller elevation about 37,79 % (before treat: 9,34%; after treat: 47,13%) and $OPN^{-/-}$ exhibited the smallest granulocyte level elevation to 32,21% (before treat: 13,38%; after treat: 45,59%) in the peripheral blood of all 3 observed genotypes.

3.4.3 G-CSF treatment does not impact erythrocyte numbers in peripheral blood of wild-type, $CD44^{-/-}$:$OPN^{-/-}$- and $OPN^{-/-}$ mice

Erythrocyte levels in peripheral blood of control, $CD44^{-/-}$:$OPN^{-/-}$- and $OPN^{-/-}$ mice exhibited no significant differences before and after G-CSF treatment (compare Fig.49.(A-D)).

Before G-CSF treatment, erythrocyte levels of $OPN^{-/-}$ mice have shown an elevated tendency ($9x10^6$cells/µl blood), while those of $CD44^{-/-}$:$OPN^{-/-}$ mice ($8,8x10^6$ cells/µl) were at similar level than those of wild-types ($8,9x10^6$ cells/µl) (Fig.49.(A)).

3 days after treatment, erythrocyte counts in peripheral blood of controls ($7.6x10^6$ cells/µl) and $OPN^{-/-}$ mice ($7,7x10^6$ cells/µl) exhibited to be similar in range, but $CD44^{-/-}$:$OPN^{-/-}$ mice erythrocyte counts ($7,9x10^6$ cells/µl) showed to be slightly elevated (Fig.49.(B)).

Erythrocyte counts in peripheral blood of wild-types ($8,2x10^6$ cells/µl) and $OPN^{-/-}$ mice ($8,1x10^6$ cells/µl) were at comparable levels at 4 days after Neupogen treatment (Fig.49.(C)). Erythrocyte levels of $CD44^{-/-}$:$OPN^{-/-}$ mice ($7,6x10^6$ cells/µl) exhibited a tendency of being decreased referring to controls.

A tendency of erythrocyte number reduction in peripheral blood of $CD44^{-/-}$:$OPN^{-/-}$ mice ($7,6x10^6$ cells/µl; $P \leq 0,05$)) compared to untreated controls ($8,3x10^6$ cells/µl) occurred at 6 days after G-CSF treatment (Fig.49.(D)). $OPN^{-/-}$ mice`s erythrocyte counts in peripheral blood ($8,1x10^6$ cells/µl) had been similar to those of untreated wild-types (Fig.49.(D)).

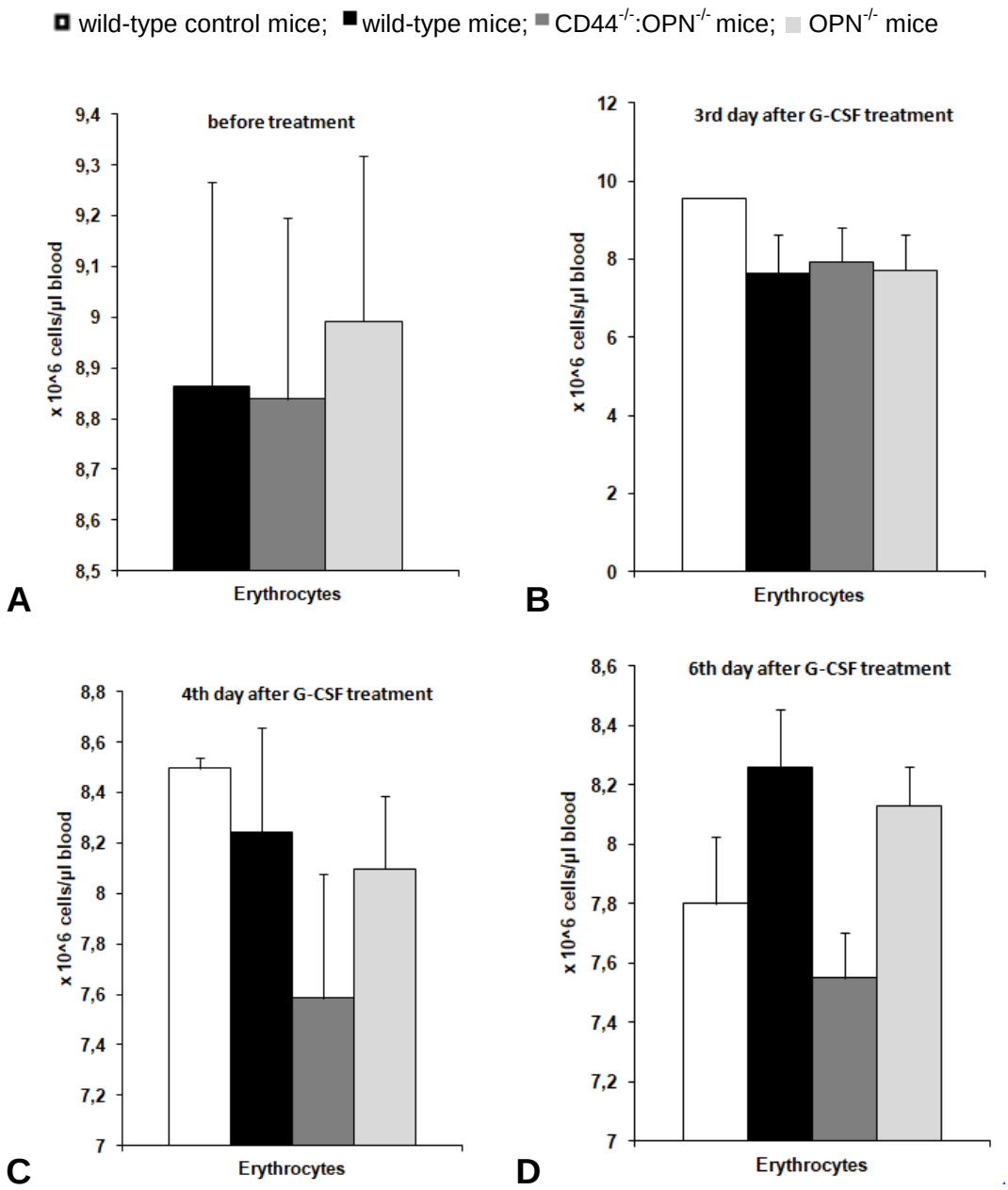


Figure 49. G-CSF treatment does not impact erythrocyte blood levels in CD44$^{-/-}$:OPN$^{-/-}$ and OPN$^{-/-}$ mice. Absolute erythrocyte values in peripheral blood before and during G-CSF treatment are shown. **(A)** before treatment; controls: n = 9; CD44$^{-/-}$:OPN$^{-/-}$ mice: n = 9; OPN$^{-/-}$ mice: n = 9. **(B)** 3 days after treatment; controls: n = 2; treated wild-types: n = 10; treated CD44$^{-/-}$:OPN$^{-/-}$ mice: n = 7; treated OPN$^{-/-}$ mice: n = 11. **(C)** 4 days after treatment; controls: n = 2; treated CD44$^{-/-}$:OPN$^{-/-}$ mice: n = 7; treated OPN$^{-/-}$ mice n = 7. **(D)** 6 days after treatment; controls: n = 2; treated wild-types: n = 7; treated CD44$^{-/-}$:OPN$^{-/-}$ mice n = 7; treated OPN$^{-/-}$ mice: n = 7. White bar: wild-type control mice (not treated with G-CSF); Black bar: wild-type mice; dark grey bar: CD44$^{-/-}$:OPN$^{-/-}$ mice; light grey bar: OPN$^{-/-}$ mice. Unpaired, 2-tailed t test was performed to determine significance.Values are the mean ± SEM.

3.4.4 G-CSF treatment does not impact hemoglobin levels in peripheral blood of wild-type-, CD44$^{-/-}$:OPN$^{-/-}$- and OPN$^{-/-}$ mice

Before G-CSF treatment (Fig.50.(A)) and 4 days after treatment (Fig.50(C)) hemoglobin levels in peripheral blood of wild-types and OPN$^{-/-}$ mice have been at comparable levels, whereas those of CD44$^{-/-}$:OPN$^{-/-}$ mice showed a decreased tendency.

3 days after treatment hemoglobin levels in the peripheral blood of all 3 observed genotypes were at comparable levels (~12,6 µg/µl blood) and did not differ significantly from those of untreated controls (14,4 µg/µl blood) (Fig.50.(B)).

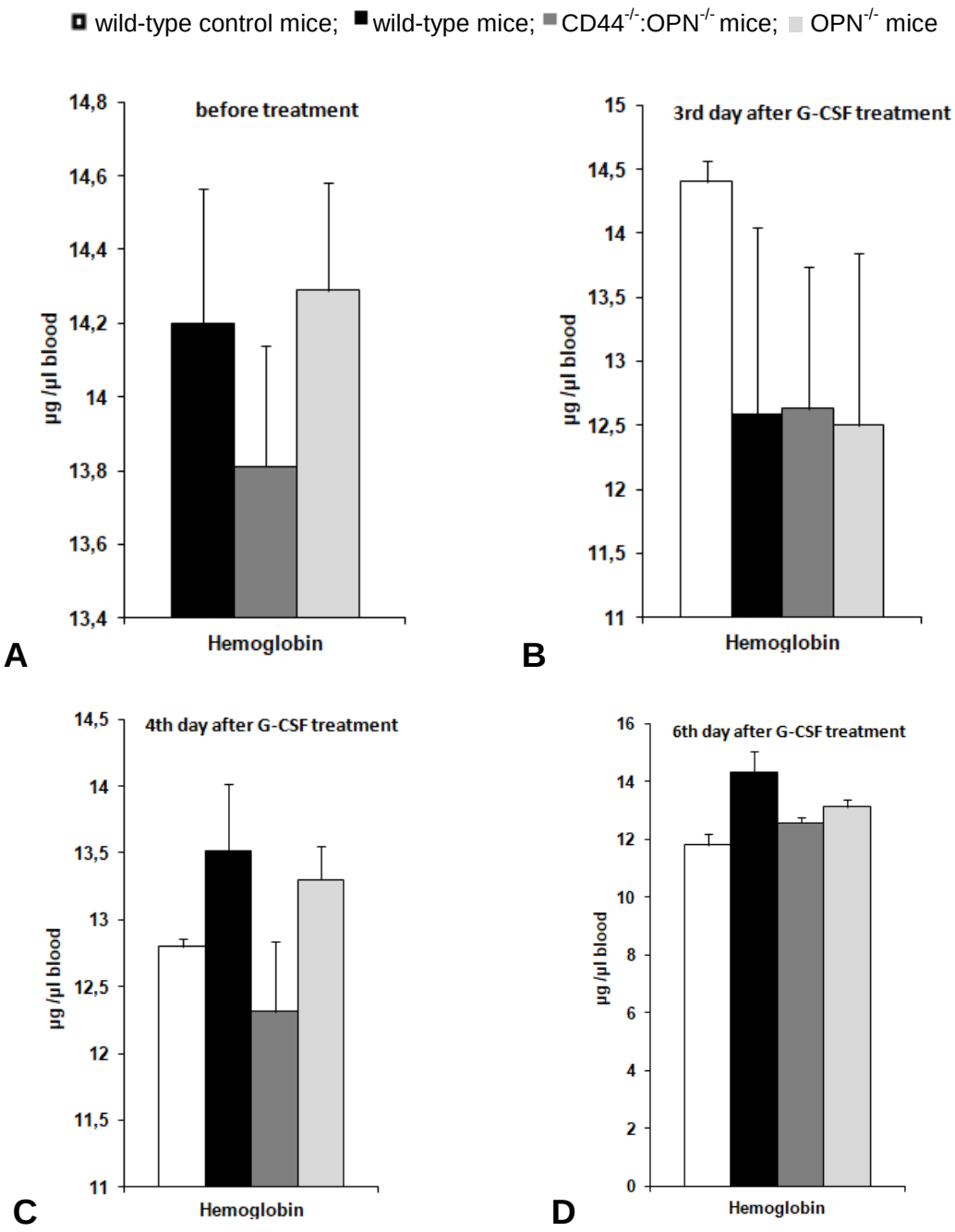


Figure 50. G-CSF treatment does not impact hemoglobin levels in CD44$^{-/-}$:OPN$^{-/-}$- and OPN$^{-/-}$ mice. Absolute hemoglobin levels in peripheral blood before and during G-CSF treatment are shown. **(A)** before treatment; wild-type mice: n = 9; CD44$^{-/-}$:OPN$^{-/-}$ mice: n = 9; OPN$^{-/-}$ mice: n = 9. **(B)** 3 days after treatment; controls: n = 2; treated wild-types: n = 10; CD44$^{-/-}$:OPN$^{-/-}$ mice n = 7; treated OPN$^{-/-}$ mice: n = 11. **(C)** 4 days after treatment; controls: n = 2; treated wild-types: n = 7; treated CD44$^{-/-}$:OPN$^{-/-}$ mice: n = 7; treated OPN$^{-/-}$ mice: n = 7. **(D)** 6 days after treatment; controls: n = 2; treated wild-types: n = 7; treated CD44$^{-/-}$:OPN$^{-/-}$ mice: n = 7; OPN$^{-/-}$ mice: n = 6. White bar: wild-type control mice (not treated with G-CSF); Black bar: wild-type mice; dark grey bar: CD44$^{-/-}$:OPN$^{-/-}$ mice; light grey bar: OPN$^{-/-}$ mice. Unpaired, 2-tailed t test was performed to determine significance.Values are the mean ± SEM.

After 6 days of treatment, hemoglobin levels in peripheral blood of treated wild-type mice showed a slightly increased tendency (14,3 µg/µl) in comparison to untreated controls (11,8 µg/µl), whereas those of treated $CD44^{-/-}$:$OPN^{-/-}$- (12,6 µg/µl) and $OPN^{-/-}$mice (13,1 µg/µl blood) were very similar to hemoglobin levels of untreated controls (11,5 µg/µl blood) (Fig.50.(D)).

3.4.5 G-CSF treatment elevates B and T cell levels in BM of wild-type-, $CD44^{-/-}$:$OPN^{-/-}$- and $OPN^{-/-}$ mice

6 days after Neupogen treatment, B cell-, T cell, granulocyte- and monocyte levels in bone marrow of treated wild-types (B cells: 25%; *P* ≤0,005); T cells: 20% (*P* ≤0,005)), $CD44^{-/-}$:$OPN^{-/-}$ mice (B cells: 28% (*P* ≤ 0,0005); T cells: 18% (*P* ≤ 0,0005)) and $OPN^{-/-}$ mice (B cells: 29%; *P* ≤ 0,005); T cells: 21%; *P* ≤ 0,005)) were significantly elevated compared to those of untreated controls (B cells: 6%; T cells: 1%) (Fig.51.(A)).

No differences in granulocyte and monocyte levels in BM of treated wild-type (granulocytes: 43%; monocytes: 2%), $CD44^{-/-}$:$OPN^{-/-}$ mice (granulocytes: 44%; monocytes: 2%), $OPN^{-/-}$ mice (granulocytes: 39%; monocytes: 2%) and untreated controls (granulocytes: 43%; monocytes: 2%) was observable after six days of G-CSF treatment (Fig.51.(A)).

3.4.6 G-CSF treatment does not alter LKS, HPC, HSC and MPP numbers in BM of $CD44^{-/-}$:$OPN^{-/-}$ mice

LKS (Lin^- $cKit^+$ $Sca1^+$) and HPC (myeloid restricted progenitor cells being Lin^-, $cKit^+$ and $Sca1^-$) in BM of treated wild-type-, $CD44^{-/-}$:$OPN^{-/-}$- and $OPN^{-/-}$ mice did not display to be significantly differing in numbers in comparison to those of untreated controls (Fig.51.(B)).

▒ wild-type mice (controls); ■ treated wild-type mice; ■ treated CD44$^{-/-}$:OPN$^{-/-}$ mice; ■ treated OPN$^{-/-}$ mice

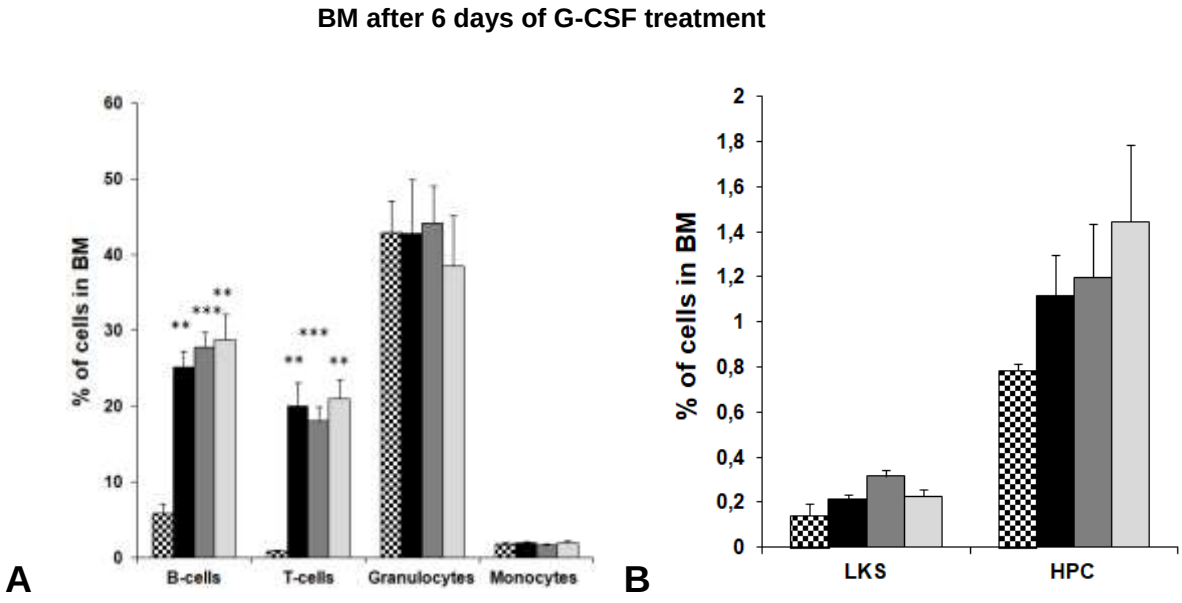


Figure 51. G-CSF treatment elevates B and T cell levels in BM of wild-type-, CD44$^{-/-}$:OPN$^{-/-}$- and OPN$^{-/-}$ mice. (**A**) B cell, T cell, granulocyte and monocyte values in BM after 6 days of G-CSF treatment; untreated controls: n = 4; treated wild-types: n = 5; treated CD44$^{-/-}$:OPN$^{-/-}$ mice: n = 5; treated OPN$^{-/-}$ mice: n = 5. (**B**) LKS (Lin^{-} cKit^{+} Sca1^{+}) progenitors and and myeloid restricted hematpoietic progenitor cells (HPC [Lin^{-}, cKit^{+} and Sca1^{-}]) in BM after 6 days of G-CSF treatment; controls: n = 3; treated wild-types: n = 10; treated CD44$^{-/-}$:OPN$^{-/-}$ mice: n = 5; treated OPN$^{-/-}$ mice: n = 9. ▒ Tiled bar: untreated wild-type mice (controls); ■ Black bar: treated wild-type mice; ■ dark grey bar: treated CD44$^{-/-}$:OPN$^{-/-}$ mice; ■ light grey bar: treated OPN$^{-/-}$ mice. Percentage of cells in BM is referring to the extracted BM of 2 femurs, 2 tibiae and 2 fibulae per mouse. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM; ***P* ≤ 0,005; ****P* ≤ 0,0005.

Hematopoietic stem cell numbers in BM of treated wild-type- (0,006%), CD44$^{-/-}$:OPN$^{-/-}$- (0,005%) and OPN$^{-/-}$ mice (0,008%) showed a tendency of decrease in reference to untreated controls (0,03%), without being significant (Fig.52.(A)).

No significant differences in multipotent progenitor levels in BM of treated wild-type (0,15%), CD44$^{-/-}$:OPN$^{-/-}$- (0,17%) and OPN$^{-/-}$ mice (0,16%) in comparison to untreated controls (0,1%) has been found after 6 days of daily G-CSF treatment (Fig.52.(B)).

▒ wild-type mice (controls); ■ treated wild-type mice; ■ treated CD44$^{-/-}$:OPN$^{-/-}$ mice; ■ treated OPN$^{-/-}$ mice.

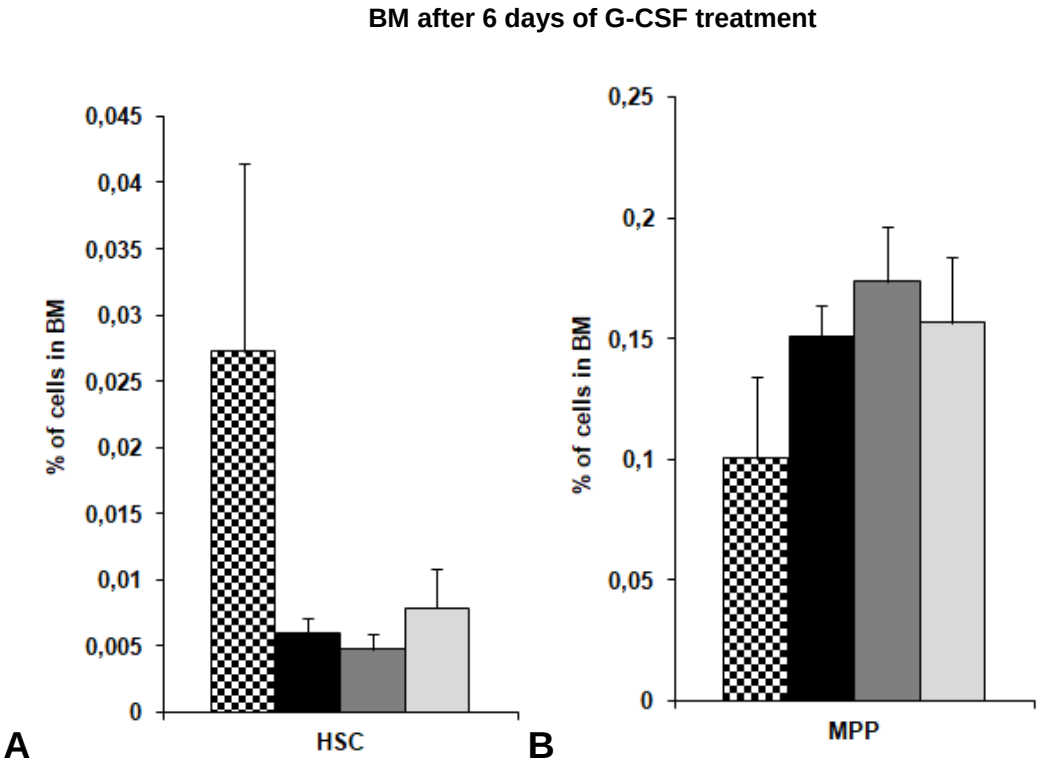


Figure 52. G-CSF treatment shows a tendency of HSC number reduction in BM of wild-type-, CD44$^{-/-}$:OPN$^{-/-}$- and OPN$^{-/-}$ mice while MPP numbers are unaltered. (A) Hematopoietic stem cells (HSC) in BM after 6 days of G-CSF treatment; untreated controls: n = 3; treated wild-types: n = 9; treated CD44$^{-/-}$:OPN$^{-/-}$ mice: n = 5; treated OPN$^{-/-}$ mice: n = 9**. (B)** Multipotent progenitor cells (MPP) in BM after 6 days of G-CSF treatment; untreated controls: n = 3; treated wild-types: n = 10; treated CD44$^{-/-}$:OPN$^{-/-}$ mice: n = 5; treated OPN$^{-/-}$ mice: n = 9. ▒ Tiled bar: untreated wild-type mice (controls); ■ Black bar: wild-type mice; ■ dark grey bar: CD44$^{-/-}$:OPN$^{-/-}$ mice; ■ light grey bar: OPN$^{-/-}$ mice. Percentage of cells in BM is referring to the extracted BM of 2 femurs, 2 tibiae and 2 fibulae per mouse. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM.

3.5 The effect of Poly(I:C) stress on immune response of $CD44^{-/-}$:$OPN^{-/-}$ mice

3.5.1 Peripheral blood results

3.5.1.1 Poly(I:C) stress reduces thrombocyte levels in wild-type-, $CD44^{-/-}$:$OPN^{-/-}$-, $CD44^{+/-}$:$OPN^{+/-}$- and $OPN^{-/-}$ mice in comparable amounts and does not impact erythrocyte counts

Before treatment, thrombocyte counts in peripheral blood of wild-type- ($1037x10^3$ cells/µl), $CD44^{-/-}$:$OPN^{-/-}$- ($1084x10^3$ cells/µl), $CD44^{+/-}$:$OPN^{+/-}$- ($1125x10^3$ cells/µl) and $OPN^{-/-}$ mice ($1110x10^3$ cells/µl) showed no significant differences (Fig.53.(A)).

After 24h hours of Poly(I:C) treatment, thrombocyte levels in peripheral blood of all 4 observed genotypes have been decreased (wild-types: $712x10^3$ cells/µl; $P \leq 0,05$); $CD44^{-/-}$:$OPN^{-/-}$ mice: $745x10^3$ cells/µl; $CD44^{+/-}$:$OPN^{+/-}$ mice: $793x10^3$ cells/µl; $OPN^{-/-}$mice: $790x10^3$ cells/µl) in comparison to those of untreated controls ($1037x10^3$ cells/µl), but only in treated wild-types the decrease of thrombocytes has been significant (Fig.53.(B)).

untreated wild-type mice. ■ wild-type mice after treatment; □ $CD44^{-/-}$:$OPN^{-/-}$ mice (A and C: untreated; B and D: treated) ■ $CD44^{+/-}$:$OPN^{+/-}$ mice (A and C: untreated; B and D: treated); ■ $OPN^{-/-}$ mice (A and C: untreated; B and D: treated)

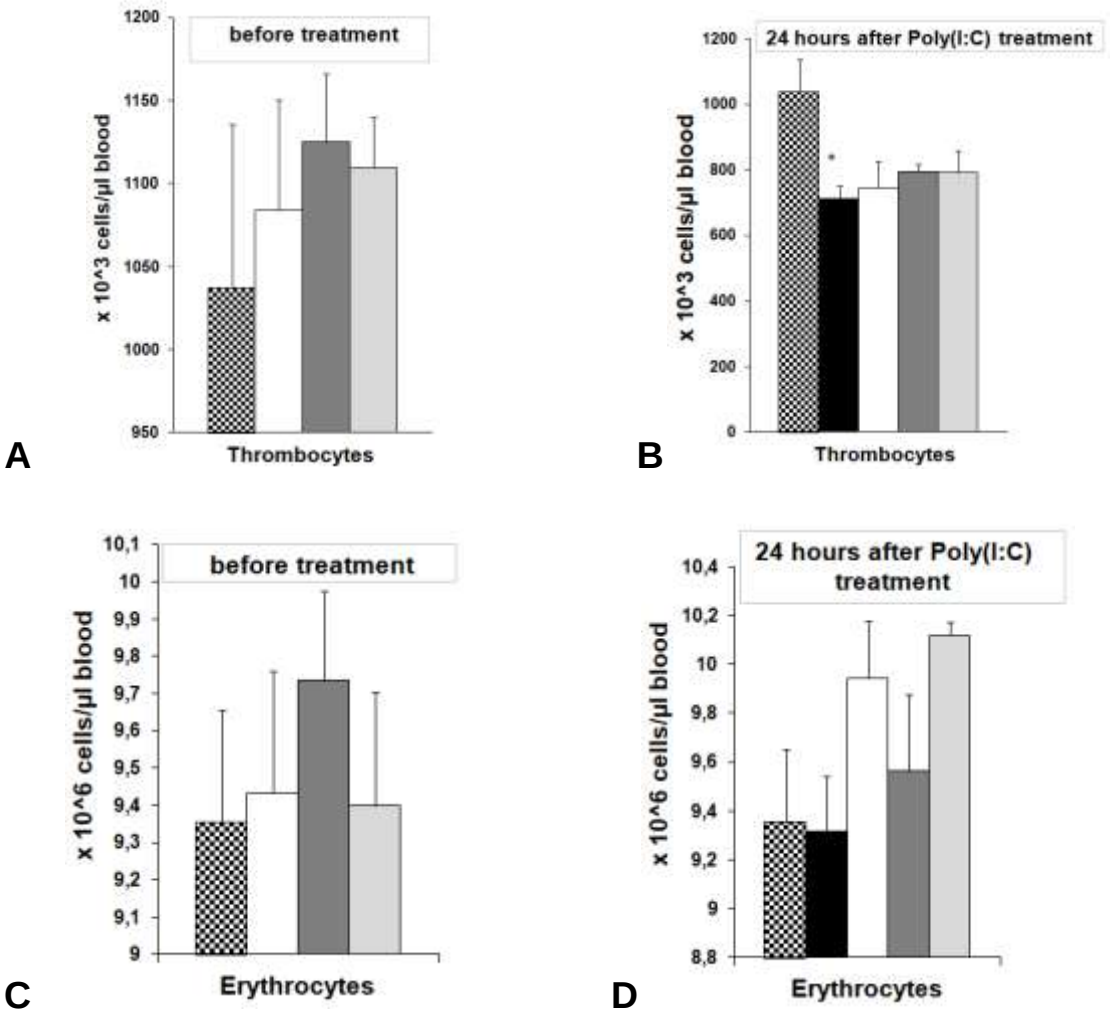


Figure 53. Poly(I:C) stress does not impact thrombocyte and erythrocyte numbers in peripheral blood of $CD44^{-/-}$:$OPN^{-/-}$-, $CD44^{+/-}$:$OPN^{+/-}$- and $OPN^{-/-}$ mice. (A) Thrombocyte cell counts in peripheral blood before Poly(I:C) treatment; wild-type mice: n = 6; $CD44^{-/-}$:$OPN^{-/-}$ mice: n = 7; $CD44^{+/-}$:$OPN^{+/-}$ mice: n = 7; $OPN^{-/-}$ mice: n = 7. **(B)** Thrombocyte cell counts in peripheral blood 24 hours after Poly(I:C) treatment; controls: n = 6; treated wild-type mice: n = 5; treated $CD44^{-/-}$:$OPN^{-/-}$ mice: n = 5; treated $CD44^{+/-}$:$OPN^{+/-}$ mice: n = 5; treated $OPN^{-/-}$ mice: n = 4. **(C)** Erythrocyte cell counts in peripheral blood before Poly(I:C) treatment; wild-type mice: n = 6; $CD44^{-/-}$:$OPN^{-/-}$ mice: n = 7; $CD44^{+/-}$:$OPN^{+/-}$ mice: n = 7; $OPN^{-/-}$ mice: n = 7. **(D)** Erythrocyte cell counts in peripheral blood 24 hours after Poly(I:C) treatment; controls: n = 6; treated wild-type mice: n = 5; treated $CD44^{-/-}$:$OPN^{-/-}$ mice: n = 5; treated $CD44^{+/-}$:$OPN^{+/-}$ mice: n = 5; treated $OPN^{-/-}$ mice: n = 4. ▩ Tiled bar: untreated wild-type mice; ■ Black bar: wild-type mice after treatment; □ white bar: $CD44^{-/-}$:$OPN^{-/-}$ mice; ■ dark grey bar: $CD44^{+/-}$:$OPN^{+/-}$ mice; ■ light grey bar: $OPN^{-/-}$ mice. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM; *$P \leq 0,05$.

Before treatment, no differences in erythrocyte values in peripheral blood of wild-type- (9,4x10^6 cells/µl), CD44$^{-/-}$:OPN$^{-/-}$- (9,4x10^6 cells/µl), CD44$^{+/-}$:OPN$^{+/-}$- (9,7x10^6 cells/µl) and OPN$^{-/-}$ mice (9,4x10^6 cells/µl) were observable (Fig.53.(C)).

24 hours after Poly(I:C) treatment, erythrocyte levels in peripheral blood of CD44$^{-/-}$:OPN$^{-/-}$- (9,9x10^6 cells/µl) and OPN$^{-/-}$ mice (10,1x10^6 cells/µl) exhibited a tendency of elevation in comparison to untreated controls (9,4x10^6 cells/µl) (Fig.55.(D)). Erythrocyte levels in peripheral blood of treated wild-type (9,3x10^6 cells/µl) and CD44$^{+/-}$:OPN$^{+/-}$ (9,6x10^6 cells/µl) displayed to be at comparable levels than those of untreated controls (9,4x10^6 cells/µl) (Fig.53.(D)).

3.5.1.2 Leukocyte subsets in peripheral blood of CD44$^{-/-}$:OPN$^{-/-}$ mice react with different intensity in case of Poly(I:C) stress than leukocytes of wild-types

Before Poly (I:C) treatment, no significant differences between B cell and monocyte-counts of all 4 observed genotypes have been detected in the peripheral blood (Fig.54.(A)). Wild-type`s (41%), CD44$^{-/-}$:OPN$^{-/-}$ mice`s (41%), CD44$^{+/-}$:OPN$^{+/-}$ mice`s (38%) and OPN$^{-/-}$ mice`s (43%) B cells counts in peripheral blood were at comparable levels (Fig.54.(A)). T cell counts in peripheral blood of CD44$^{-/-}$:OPN$^{-/-}$- (29%; $P \leq 0,05$) and OPN$^{-/-}$ mice (32%; $P \leq 0,05$) were significantly higher than in controls (25%) (Fig.54.(A)). CD44$^{+/-}$:OPN$^{+/-}$ mice`s T cell levels (29%) were in similar range to those of CD44$^{-/-}$:OPN$^{-/-}$ mice (29%) (Fig.54.(A)). Granulocytes in peripheral blood of CD44$^{-/-}$:OPN$^{-/-}$ mice (12%; $P \leq 0,05$) have been found to be significantly lower than in wild-types (18%), whereas those of CD44$^{+/-}$:OPN$^{+/-}$- (16%) and OPN$^{-/-}$ mice (14%) were at comparable levels than in wild-types (18%) (Fig.54.(A)). Before Poly (I:C) treatment, monocyte levels of all 4 observed genotypes have been resemblant at ~8% (Fig.54.(A)).

▩ untreated wild-type mice. ■ wild-type mice after treatment; □ $CD44^{-/-}$:$OPN^{-/-}$ mice (A untreated; B: treated); ■ $CD44^{+/-}$:$OPN^{+/-}$ mice (A: untreated; B: treated); ■ $OPN^{-/-}$ mice (A: untreated; B: treated)

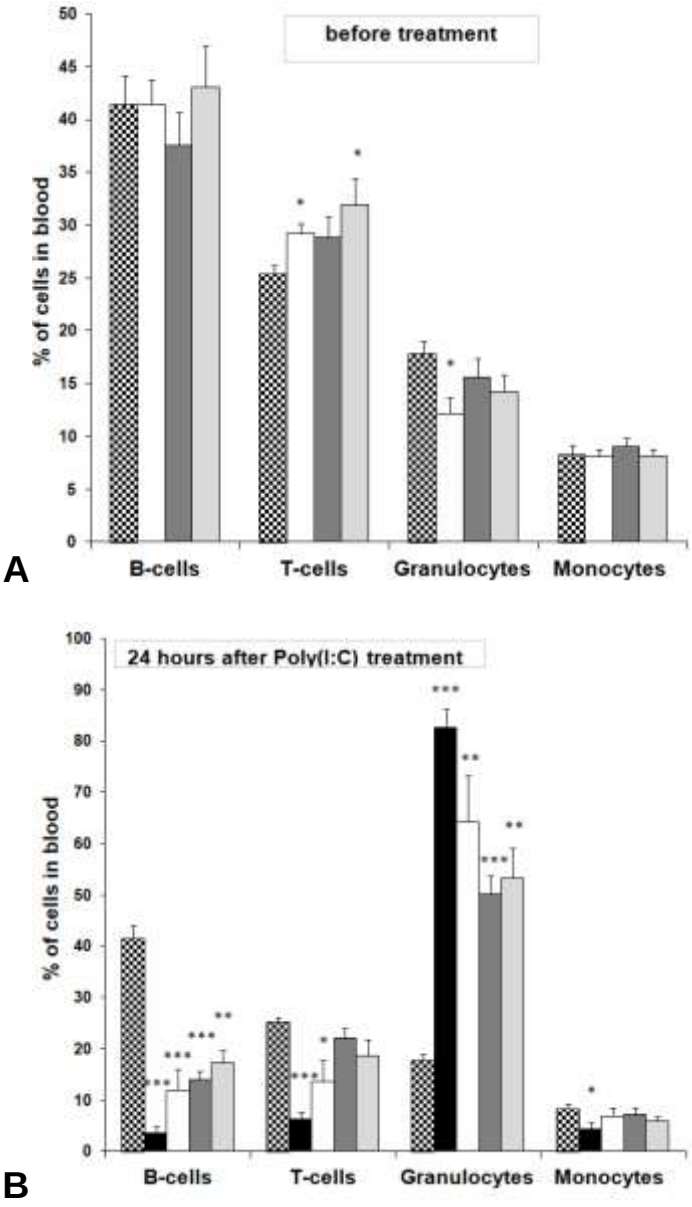


Figure 54. Poly(I:C) stress impacts B, T and granulocyte numbers in peripheral blood of $CD44^{-/-}$:$OPN^{-/-}$ mice with different intesity than in wild-type mice. B, T, granulocyte and monocyte numbers in peripheral blood of wild-type, $CD44^{-/-}$:$OPN^{-/-}$-, $CD44^{+/-}$:$OPN^{+/-}$ and $OPN^{-/-}$ mice before and 24 hours after Poly(I:C) treatment are shown. (**A)** Before Poly(IC) treatment; untreated wild-types: n = 6; untreated $CD44^{-/-}$:$OPN^{-/-}$ mice: n = 7; untreated $CD44^{+/-}$:$OPN^{+/-}$ mice: n = 7; untreated $OPN^{-/-}$ mice: n = 7. **(B)** 24 hours after Poly(IC) treatment; untreated wild-types (controls): n = 6; treated wild-types: n = 6; treated $CD44^{-/-}$:$OPN^{-/-}$ mice: n = 5; treated $CD44^{+/-}$:$OPN^{+/-}$ mice: n = 5; treated $OPN^{-/-}$ mice: n = 4. ▩ Tiled bar: untreated wild-type mice. ▩ Tiled bar: untreated wild-type mice; ■ Black bar: wild-type mice after treatment; □ white bar: $CD44^{-/-}$:$OPN^{-/-}$ mice; ■ dark grey bar: $CD44^{+/-}$:$OPN^{+/-}$ mice; ■ light grey bar: $OPN^{-/-}$ mice. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM; * $P \leq 0,05$; ** $P \leq 0,005$; *** $P \leq 0,0005$.

24 hours after Poly(I:C) treatment, B cell levels in peripheral blood of all 4 observed genotypes have been reduced significantly compared to wild-type values before treatment (Fig.54.(B)). Before treatment, 41% of cells in the peripheral blood of wild-types have been B cells; after treatment, B cells values decreased to 3,7% ($P \leq 0,0005$) in wild-types, to 12% in $CD44^{-/-}$:$OPN^{-/-}$ mice ($P \leq 0,0005$), to 14% in $CD44^{+/-}$:$OPN^{+/-}$ mice ($P \leq 0,0005$) and to 17,3% in $OPN^{-/-}$ mice ($P \leq 0,005$) (Fig.54.(B)). T cell counts in peripheral blood of wild-types (6,5%; $P \leq 0,0005$) and $CD44^{-/-}$:$OPN^{-/-}$ mice (13,6%; $P \leq 0,05$) were significantly decreased after Poly(I:C) treatment, those of $CD44^{+/-}$:$OPN^{+/-}$ mice (22,1%) and $OPN^{-/-}$ mice (18,6%) were comparable to wild-type levels (25,4%) before treatment (Fig.54.(B)).

Granulocyte counts in peripheral blood of all 4 treated genotypes have shown a significant elevation referring to wild-types before treatment (Fig.54.(B)). Wild-type granulocyte numbers in peripheral blood after treatment have shown to be 4,6 times higher (82,7%; $P \leq 0,0005$), $CD44^{-/-}$:$OPN^{-/-}$ mice`s being 3,6 times higher (64,2%, $P \leq 0,005$)), $CD44^{+/-}$:$OPN^{+/-}$ mice's 2,8 times higher (50,12%, $P \leq 0,0005$) and $OPN^{-/-}$mice`s 2 times higher (53,3%, $P \leq 0,005$) than prior treatment values of wild-types (17,8%) (Fig.54.(B)).

Only monocyte counts in peripheral blood of of Poly(I:C) treated wild-type mice have significant decreased (4,5%; $P \leq 0,05$); those of $CD44^{-/-}$:$OPN^{-/-}$- (6,9%), $CD44^{+/-}$:$OPN^{+/}$- (7,2%) and $OPN^{-/-}$ mice (6%) have shown similar levels than untreated wild-types (8,3%) (Fig.54.(B)).

3.5.1.3 Poly(I:C) stress has no impact on hemoglobin levels in peripheral blood of $CD44^{-/-}$:$OPN^{-/-}$ mice

Prior to Poly (I:C) and after treatment no significant differences in hemoglobin levels in peripheral blood of wild-type-, $CD44^{+/-}$:$OPN^{+/}$-, $CD44^{-/-}$:$OPN^{-/-}$- and $OPN^{-/-}$ mice have been observed (Fig.55.(A and B)). Before treatment hemoglobin levels in peripheral blood of the four observed genotypes ranged between 14,9 and 15,3 µg/µl (Fig.55.(A)).

After Poly(I:C) treatment wild-type`s (14,9µg/µl), $CD44^{-/-}$:$OPN^{-/-}$ mice`s (15,3 µg/µl), $CD44^{+/-}$:$OPN^{+/-}$ mice`s (14,8µg/µl) and $OPN^{-/-}$ mice`s (15,5 µg/µl) hemoglobin levels in peripheral blood were at comparable levels than those of untreated controls (15,3 µg/µl) (Fig.57.(B)).

untreated wild-type mice. ■ wild-type mice after treatment; □ $CD44^{-/-}$:$OPN^{-/-}$ mice (A untreated; B: treated); ■ $CD44^{+/-}$:$OPN^{+/-}$ mice (A: untreated; B: treated); ■ $OPN^{-/-}$ mice (A: untreated; B: treated)

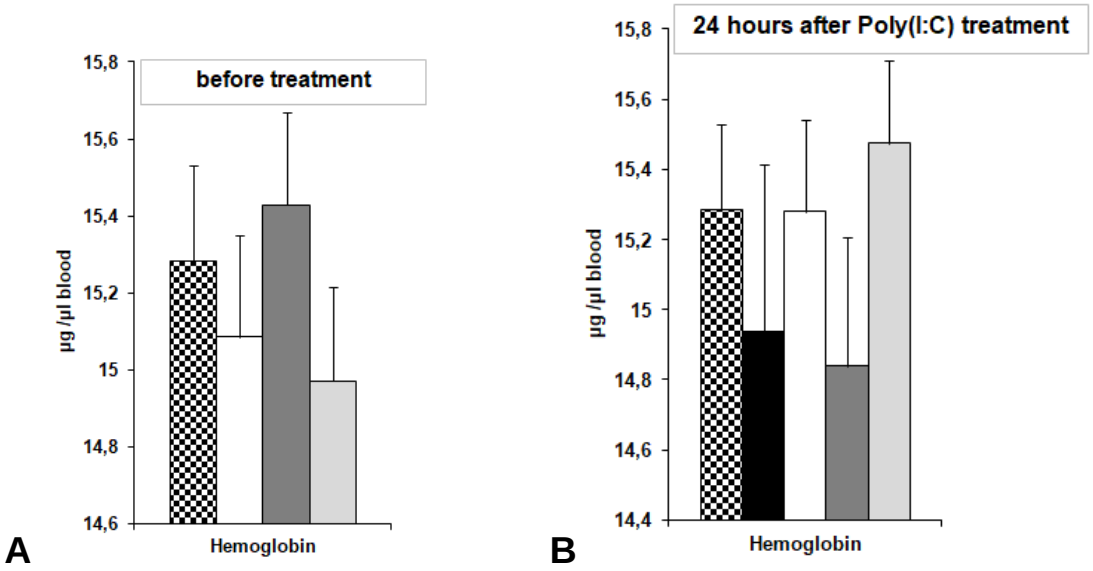


Figure 55. Poly(I:C) treatment does not impair hemoglobin levels in peripheral blood of $CD44^{-/-}$:$OPN^{-/-}$-, $CD44^{+/-}$:$OPN^{+/-}$- and $OPN^{-/-}$ mice. Hemoglobin values in peripheral blood are shown. **(A)** Before Poly: IC treatment; untreated wild-types: n = 6; untreated $CD44^{-/-}$:$OPN^{-/-}$ mice: n = 7; untreated $CD44^{+/-}$:$OPN^{+/-}$ mice: n = 7; untreated $OPN^{-/-}$ mice: n = 7. **(B)** 24 hours after Poly(I:C) treatment; untreated wild-types (controls): n = 6; treated wild-types: n = 6; treated $CD44^{-/-}$:$OPN^{-/-}$ mice n = 5; treated $CD44^{+/-}$:$OPN^{+/-}$ mice: n = 5; treated OPN-/-: n = 4. Tiled bar: untreated wild-type mice; ■ Black bar: wild-type mice after treatment; □ white bar: $CD44^{-/-}$:$OPN^{-/-}$ mice; ■ dark grey bar: $CD44^{+/-}$:$OPN^{+/-}$ mice; ■ light grey bar: $OPN^{-/-}$ mice. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM.

3.5.2 BM results

In mock mice no significant differences of granulocyte numbers in BM between the 4 observed genotypes have been found (wild-type: 58%; $CD44^{-/-}$:$OPN^{-/-}$: 53%; $CD44^{+/-}$:$OPN^{+/-}$: 55%; $OPN^{-/-}$: 57%) (Fig.56.(A)).

3.5.2.1 Poly(I:C) treatment increases T cell numbers in BM of wild-type-, $CD44^{-/-}$:$OPN^{-/-}$-, $CD44^{+/-}$:$OPN^{+/-}$- and $OPN^{-/-}$ mice

After 24 hours of Poly(I:C) treatment granulocyte levels in BM of all 4 observed genotypes (WT: 46%; $CD44^{-/-}$:$OPN^{-/-}$: 46%; $CD44^{+/-}$:$OPN^{+/-}$: 50%; $OPN^{-/-}$: 49%) have been resemblant, while only those of wild-types have shown a significant reduction referring to mock wild-type values (Fig.56.(B)).

Erythroid lineage cell- (WT: 11%; $CD44^{-/-}$:$OPN^{-/-}$: 12%; $CD44^{+/-}$:$OPN^{+/-}$: 13%, $OPN^{-/-}$: 12%) and B cell (WT: 7%; $CD44^{-/-}$:$OPN^{-/-}$: 9%; $CD44^{+/-}$:$OPN^{+/-}$: 7%, $OPN^{-/-}$: 7%) counts in BM of mock mice have each been at similar levels (Fig.56.C)).

After Poly(I:C) treatment no significant differences in erythroid lineage cell numbers in BM of treated wild-type (14%), $CD44^{-/-}$:$OPN^{-/-}$ mice (9%), $CD44^{+/-}$:$OPN^{+/-}$ mice (12%) and $OPN^{-/-}$ mice (10%) in comparison to mock controls (11%) have been found (Fig.56.(D)). 24 hours after treatment no significant differences of B cell counts in BM of treated wild-type-, $CD44^{-/-}$:$OPN^{-/-}$- (11%), $CD44^{+/-}$:$OPN^{+/-}$- (7%) and $OPN^{-/-}$ mice (10%) referring to controls (10%) have been found (Fig.56.(D)).

untreated wild-type mice. ■ wild-type mice after treatment; □ $CD44^{-/-}$:$OPN^{-/-}$ mice (A and C: untreated; B and D: treated) ■ $CD44^{+/-}$:$OPN^{+/-}$ mice (A and C: untreated; B and D: treated); ■ $OPN^{-/-}$ mice (A and C: untreated; B and D: treated)

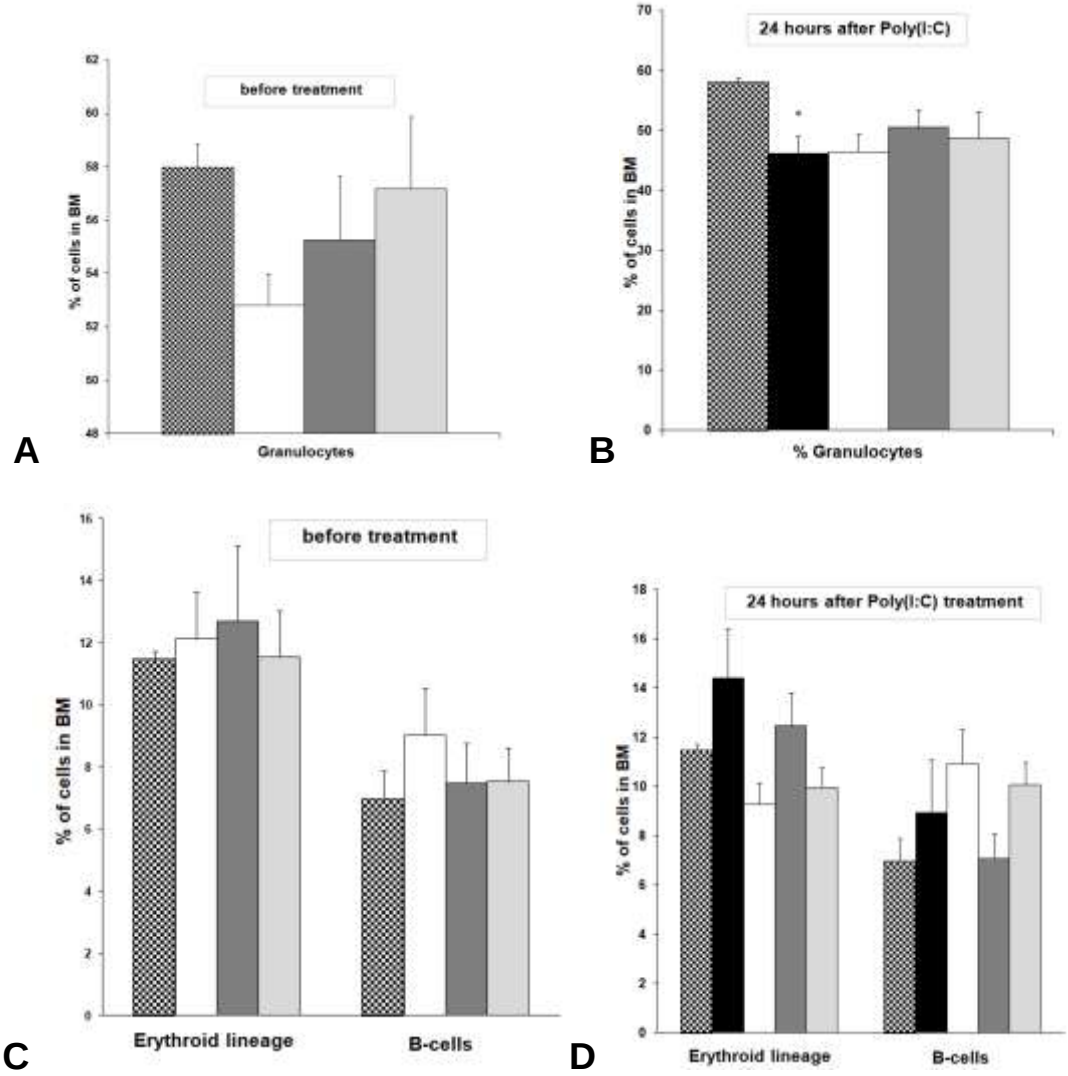


Figure 56. Poly(I:C) treatment has no impact on granulocyte, erythroid lineage and B cell numbers in BM of $CD44^{-/-}$:$OPN^{-/-}$-, $CD44^{+/-}$:$OPN^{+/-}$- and $OPN^{-/-}$ mice. Granulocyte, erythroid lineage and B cell numbers in BM of control mice and Poly(I:C) treated mice are shown**. (A)** Granulocyte counts in BM of mock mice (controls, being treated with sterile PBS); wild-types: n = 3; $CD44^{-/-}$:$OPN^{-/-}$ mice: n = 3; $CD44^{+/-}$:$OPN^{+/-}$ mice: n = 3; $OPN^{-/-}$ mice: n = 3. **(B)** Granulocyte counts in BM 24 hours after Poly(I:C) treatment; controls: n = 3; treated wild-types: n = 5; treated $CD44^{-/-}$:$OPN^{-/-}$ mice: n = 5; treated $OPN^{-/-}$ mice: n = 5; treated $OPN^{-/-}$ mice: n = 4. **(C)** Erythroid lineage- and B cells counts in BM of mock mice (controls, being treated with sterile PBS); wild-types: n = 3; $CD44^{-/-}$:$OPN^{-/-}$ mice: n = 3; $CD44^{+/-}$:$OPN^{+/-}$ mice: n = 3; $OPN^{-/-}$ mice: n = 3. **(D)** Erythroid lineage- and B cell counts in BM 24 hours after Poly(I:C) treatment; controls: n = 3; treated wild-types: n = 5; treated $CD44^{-/-}$:$OPN^{-/-}$ mice: n = 5; treated $CD44^{+/-}$:$OPN^{+/-}$ mice: n = 5; treated $OPN^{-/-}$ mice: n = 4. ▩ Tiled bar: untreated wild-type mice; ■ Black bar: wild-type mice after treatment; □ white bar: $CD44^{-/-}$:$OPN^{-/-}$ mice; ■ dark grey bar: $CD44^{+/-}$:$OPN^{+/-}$ mice; ■ light grey bar: $OPN^{-/-}$ mice. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM; * $P \leq 0{,}05$.

In mock mice no significant differences in T- and monocyte- values in BM have been found (Fig.57.(A)). T cell counts in BM of mock wild-type (2%), $CD44^{-/-}$:$OPN^{-/-}$- (2%), $CD44^{+/-}$:$OPN^{+/-}$- (2,4%) and $OPN^{-/-}$ mice (2,3%) were very similar (Fig.57.(A)). Monocyte numbers in BM of wild-types (1,8%) and $CD44^{-/-}$:$OPN^{-/-}$ mice (2%) were resemblant in mock mice, while those of $CD44^{+/-}$:$OPN^{+/-}$- (3,9%) and $OPN^{-/-}$ mice (2,9%) exhibited a slightly increased tendency (Fig.57.(A)).

24 hours after Poly(I:C) treatment a significant elevation of T cells in BM of treated wild-types (5,4%; $P \leq 0,05$), $CD44^{-/-}$:$OPN^{-/-}$- (5,9%; $P \leq 0,05$), $CD44^{+/-}$:$OPN^{+/-}$- (6,4%; $P \leq 0,05$) and $OPN^{-/-}$ mice (5,5%; $P \leq 0,05$) referring to mock controls (2%) was observable (Fig.57.(B)). Monocyte levels in BM of $OPN^{-/-}$ mice (1,9%) were analogous to those of controls (1,8%); those of treated wild-types (2,2%) and of $CD44^{-/-}$:$OPN^{-/-}$ mice (2,7%) were slightly elevated, whereas $CD44^{+/-}$:$OPN^{+/-}$ mice`s (1,4%) showed a slightly decreased tendency (Fig.57.(B)).

untreated wild-type mice. ■ wild-type mice after treatment; □ $CD44^{-/-}$:$OPN^{-/-}$ mice (A untreated; B: treated); ■ $CD44^{+/-}$:$OPN^{+/-}$ mice (A: untreated; B: treated); ■ $OPN^{-/-}$ mice (A: untreated; B: treated)

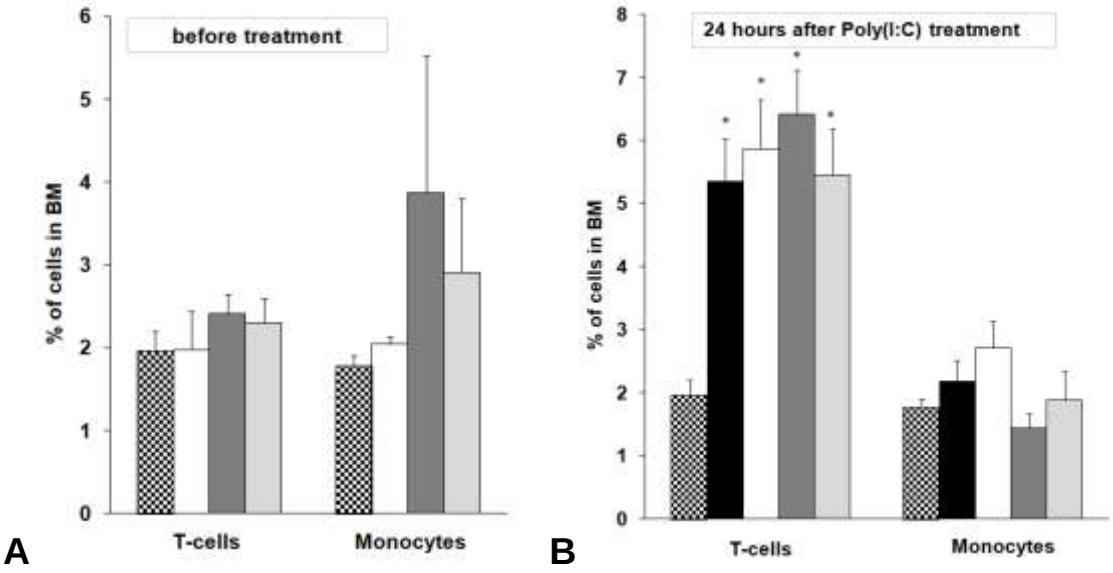


Figure 57. Poly(I:C) treatment elevates T cell values in BM of wild-type-, $CD44^{-/-}$:$OPN^{-/-}$-, $CD44^{+/-}$:$OPN^{+/-}$- and $OPN^{-/-}$ mice. T cell and monocyte numbers in BM of control mice and Poly(I:C) treated mice are shown. **(A)** T cell and monocyte cell counts in BM of mock mice (controls, being treated with sterile PBS); wild-types: n = 3; $CD44^{-/-}$:$OPN^{-/-}$ mice: n = 3; $CD44^{+/-}$:$OPN^{+/-}$ mice: n = 3; $OPN^{-/-}$ mice: n = 3. **(B)** T cell and monocyte cell counts in BM 24 hours after Poly(I:C) treatment; controls: n = 3; treated wild-types: n = 5; treated $CD44^{-/-}$:$OPN^{-/-}$ mice: n = 5; treated $CD44^{+/-}$:$OPN^{+/-}$ mice: n = 5; treated $OPN^{-/-}$ mice: n = 4. ▩ Tiled bar: untreated wild-type mice; ■ Black bar: wild-type mice after treatment; □ white bar: $CD44^{-/-}$:$OPN^{-/-}$ mice; ■ dark grey bar: $CD44^{+/-}$:$OPN^{+/-}$ mice; ■ light grey bar: $OPN^{-/-}$ mice. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM; * $P \leq 0,05$.

3.5.2.2 Poly(I:C) stress increases HSC- and MPP numbers in BM of $OPN^{-/-}$ mice

Mock mice of all 4 genotypes exhibited no significant differences in LKS values in BM (Fig.58.(A)). $OPN^{-/-}$ mice (0,42%) displayed a comparable amount of LKS cells in BM relating to wild-types (0,37%); $CD44^{-/-}$:$OPN^{-/-}$ mice (0,54%) and $CD44^{+/-}$:$OPN^{+/-}$ mice (0,63%) showed a tendency of elevation compared to wild-types (Fig.58.(A)).

After Poly(I:C) treatment, LKS levels in BM of wild-type- (2,33%; $P \leq 0,05$), $CD44^{+/-}$:$OPN^{+/-}$- (2,53%; $P \leq 0,05$) and $OPN^{-/-}$ mice (2,76%; $P \leq 0,005$) displayed a significant increase referring to controls (0,37%) (Fig.58.(B)). LKS level in BM of

Poly(I:C) treated $CD44^{-/-}$:$OPN^{-/-}$ mice (2,86%) was highest of all 4 observed genotypes, but not being significant (Fig.58.(B)).

untreated wild-type mice. ■ wild-type mice after treatment; □ $CD44^{-/-}$:$OPN^{-/-}$ mice (A and C: untreated; B and D: treated) ■ $CD44^{+/-}$:$OPN^{+/-}$ mice (A and C: untreated; B and D: treated); ■ $OPN^{-/-}$ mice (A and C: untreated; B and D: treated)

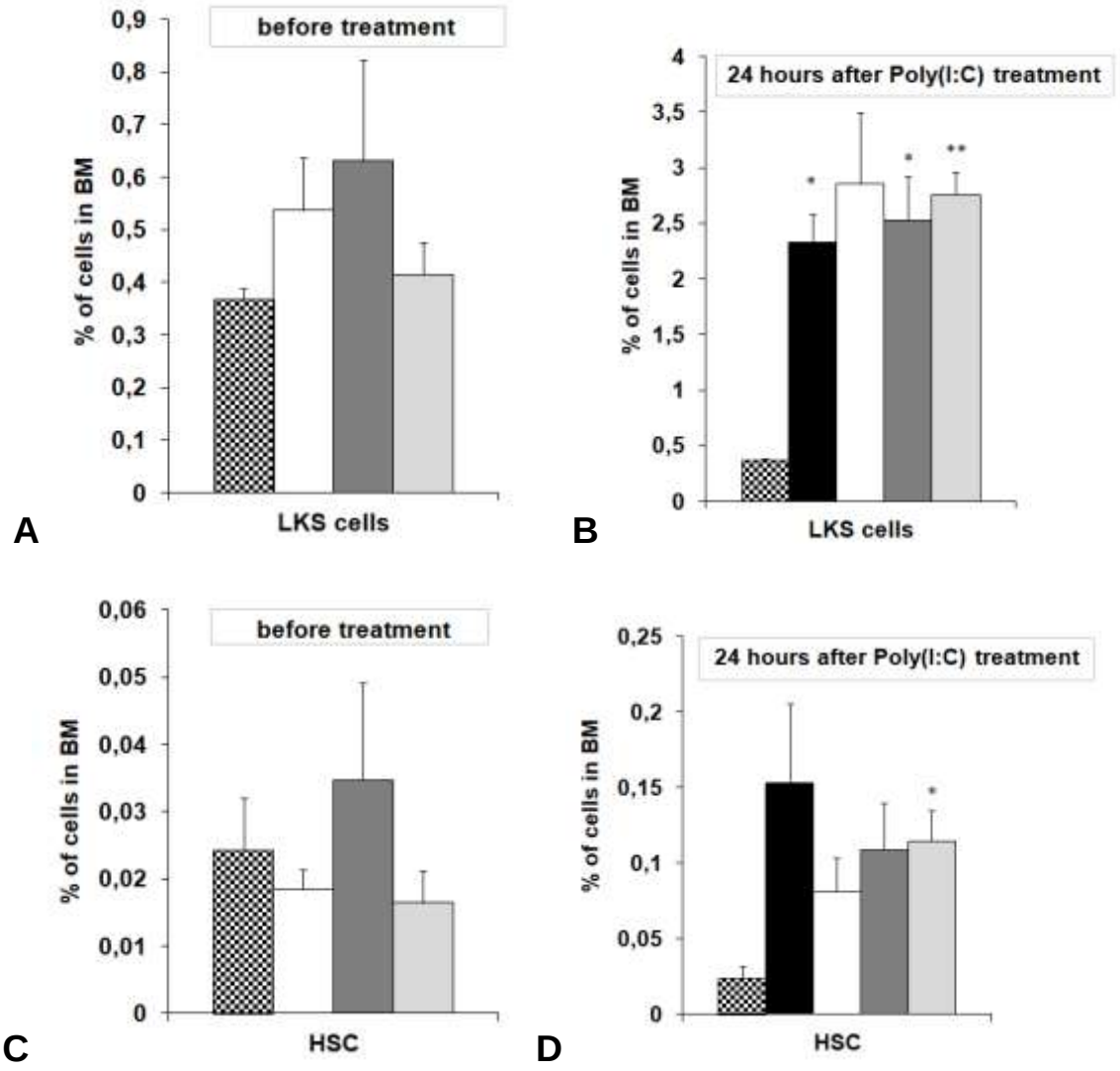


Figure 58. Poly(I:C) treatment elevates HSC numbers in BM of $OPN^{-/-}$ mice. Lin^-, $cKit^+$ and $Sca1^+$ (LKS) and hematopoietic stem cell (HSC) numbers in BM of mock mice and Poly(I:C) treated mice are shown. (**A)** LKS numbers in BM of mock mice (controls, being treated with sterile PBS); wild-types: n = 3; $CD44^{-/-}$:$OPN^{-/-}$ mice: n = 3; $CD44^{+/-}$:$OPN^{+/-}$ mice: n = 3; $OPN^{-/-}$ mice: n = 3. **(B)** LKS cell counts in BM 24 hours after Poly(I:C) treatment; controls: n = 3; treated wild-types: n = 5; treated $CD44^{-/-}$:$OPN^{-/-}$ mice: n = 5; treated $CD44^{+/-}$:$OPN^{+/-}$ mice: n = 5; treated $OPN^{-/-}$ mice: n = 4. **(C)** HSC: hematopoietic stem cells in BM of mock mice (controls, being treated with sterile PBS); wild-types: n = 3; $CD44^{-/-}$:$OPN^{-/-}$ mice: n = 3; $CD44^{+/-}$:$OPN^{+/-}$ mice: n = 3; $OPN^{-/-}$ mice: n = 3. **(D)** HSC in BM 24 hours after Poly(I:C) treatment; controls: n = 3; treated wild-types: n = 5; treated $CD44^{-/-}$:$OPN^{-/-}$ mice: n = 5; treated $CD44^{+/-}$:$OPN^{+/-}$ mice: n = 5; treated $OPN^{-/-}$ mice: n = 4. ▩ Tiled bar: untreated wild-type mice; ■ Black bar: wild-type mice after treatment; □ white bar: $CD44^{-/-}$:$OPN^{-/-}$ mice; ■ dark grey bar: $CD44^{+/-}$:$OPN^{+/-}$ mice; ■ light grey bar: $OPN^{-/-}$ mice. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM; **P* ≤ 0,05; ***P* ≤ 0,005.

HSCs in BM of mock $CD44^{+/-}$:$OPN^{+/-}$ mice (0,035%) showed a tendency of being increased relating to wild-types (0,024%), whereas those of $CD44^{-/-}$:$OPN^{-/-}$- (0,018%) and $OPN^{-/-}$ mice (0,017%) exhibited a similar level than mock wild-type mice (Fig.58.(C)).

After Poly(I:C) treatment HSC numbers were highest in BM of treated wild-types (0,153%) without being significant (Fig.58.(D)). HSCs in BM of $CD44^{-/-}$:$OPN^{-/-}$- (0,081%) and $CD44^{+/-}$:$OPN^{+/-}$ mice (0,109%) were also elevated in comparison to mock mice, but only $OPN^{-/-}$ mice`s HSC levels (0,115%) in a significant way ($P \leq 0,05$) (Fig.58.(D)).

No significant differnces in MPP values in BM of mock wild-type (0,17%), $CD44^{-/-}$:$OPN^{-/-}$- (0,3%), $CD44^{+/-}$:$OPN^{+/-}$- and $OPN^{-/-}$ mice (0,26%) were found, although a tendency of increased MPP values in BM of $CD44^{-/-}$:$OPN^{-/-}$-, $CD44^{+/-}$:$OPN^{+/-}$- and $OPN^{-/-}$ mice in reference to those of wild-types has been observed (Fig.59.(A)).

After Poly(I:C) treatment MPP values in BM of treated wild-type- (0,89%), $CD44^{-/-}$:$OPN^{-/-}$- (1,41%) , $CD44^{+/-}$:$OPN^{+/-}$- (1,19%; $P \leq 0,05$) and $OPN^{-/-}$ mice (1,5%; $P \leq 0,0005$) were elevated referring to mock controls (0,17%) (Fig.59.(B)).

untreated wild-type mice. ■ wild-type mice after treatment; □ $CD44^{-/-}$:$OPN^{-/-}$ mice (A untreated; B: treated); ■ $CD44^{+/-}$:$OPN^{+/-}$ mice (A: untreated; B: treated); ■ $OPN^{-/-}$ mice (A: untreated; B: treated)

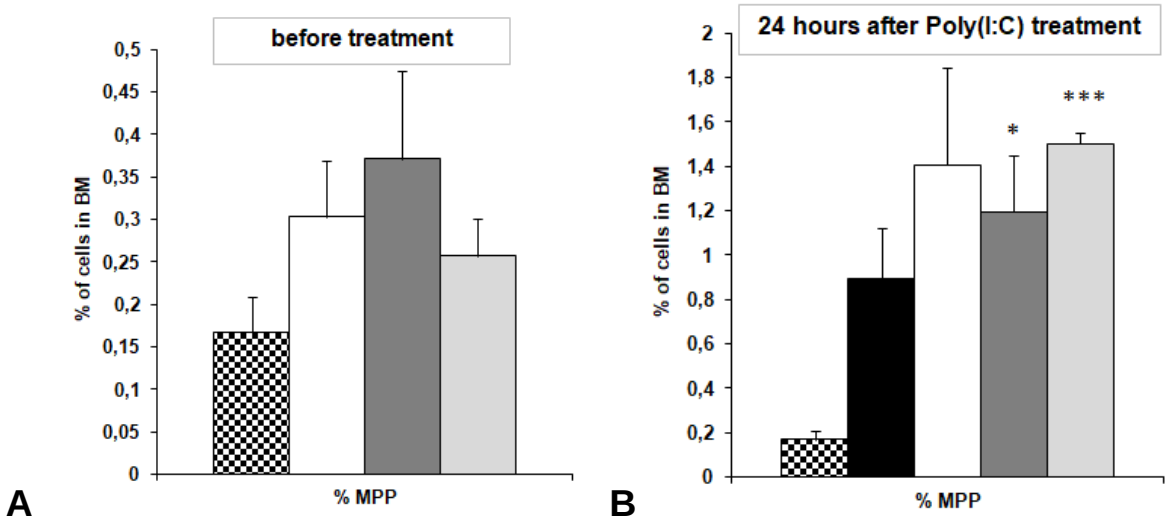


Figure 59. Poly(I:C) treatment elevates multipotent progenitors in BM of $CD44^{+/-}$:$OPN^{+/-}$- and $OPN^{-/-}$ mice. Multipotent progenitor (MPP) numbers in BM of mock and Poly(I:C) treated mice are shown. **(A)** MPP counts in BM of mock mice (controls, being treated with sterile PBS); wild-types: n = 3; $CD44^{-/-}$:$OPN^{-/-}$ mice: n = 3; $CD44^{+/-}$:$OPN^{+/-}$ mice: n = 3; $OPN^{-/-}$ mice: n = 3. **(B)** MPP counts in BM 24 hours after Poly(I:C) treatment; controls: n = 3; treated wild-types: n = 5; treated $CD44^{-/-}$:$OPN^{-/-}$ mice: n = 5; treated $CD44^{+/-}$:$OPN^{+/-}$ mice: n = 5; treated $OPN^{-/-}$ mice: n = 4. ▩ Tiled bar: untreated wild-type mice; ■ Black bar: wild-type mice after treatment; □ white bar: $CD44^{-/-}$:$OPN^{-/-}$ mice; ■ dark grey bar: $CD44^{+/-}$:$OPN^{+/-}$ mice; ■ light grey bar: $OPN^{-/-}$ mice. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM; *$P \leq 0,05$; ***$P \leq 0,0005$.

3.5.2.3 Poly(I:C)stress increases HSC proliferation in BM of $OPN^{-/-}$mice and MPP proliferation in BM of $CD44^{-/-}$:$OPN^{-/-}$ mice

In the mock mouse cohort proliferation of HSCs in BM of $CD44^{-/-}$:$OPN^{-/-}$- (10,7%), $CD44^{+/-}$:$OPN^{+/-}$- (12,2%) and $OPN^{-/-}$ mice (9,2%) showed a tendency of elevation in comparison to mock wild-types (6,5%) (Fig.60.(A)).

After Poly(I:C) treatment proliferation of HSCs in BM of wild-type- (11,8%), $CD44^{-/-}$:$OPN^{-/-}$- (14,5%), $CD44^{+/-}$:$OPN^{+/-}$- (13,5%) and $OPN^{-/-}$ mice (16,6%; $P \leq 0,05$) have shown to be elevated relating to controls (6,5%) (Fig.60.(B)).

untreated wild-type mice. ■ wild-type mice after treatment; □ $CD44^{-/-}$:$OPN^{-/-}$ mice (A and C: untreated; B and D: treated) ■ $CD44^{+/-}$:$OPN^{+/-}$ mice (A and C: untreated; B and D: treated); ■ $OPN^{-/-}$ mice (A and C: untreated; B and D: treated)

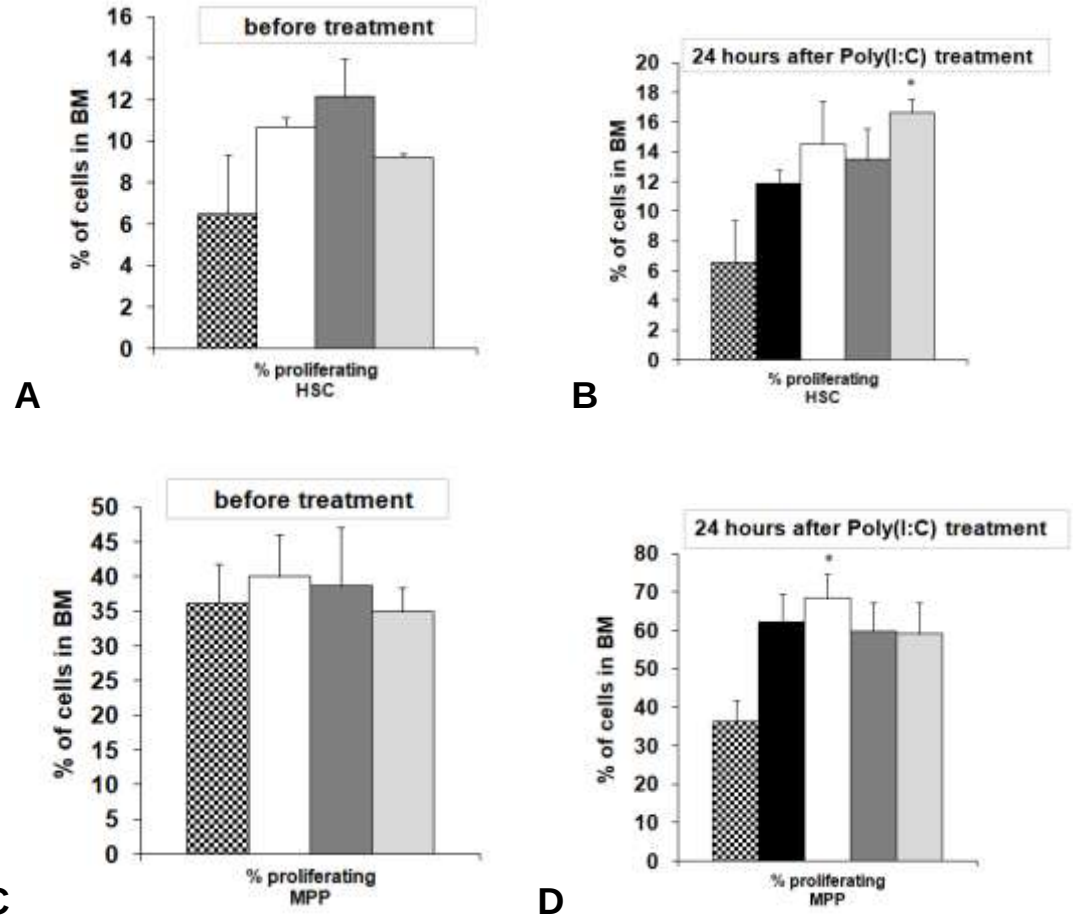


Figure 60. Poly(I:C) treatment increases HSC proliferation in BM of $OPN^{-/-}$ and MPP proliferation in BM of $CD44^{-/-}$:$OPN^{-/-}$ mice. Percentage of proliferating hematopoietic stem cells (HSC) and multipotent progenitors (MPP) in BM of mock and Poly(I:C) treated mice are shown. **(A)** Proliferating HSC in BM of mock mice (controls, being treated with sterile PBS); wild-types: n = 3; $CD44^{-/-}$:$OPN^{-/-}$ mice: n = 3; $CD44^{+/-}$:$OPN^{+/-}$ mice: n = 3; $OPN^{-/-}$ mice: n = 3. **(B)** Proliferating HSC in BM of treated mice; 24 hours after Poly(I:C) treatment; controls: n = 3; treated wild-types: n = 5; treated $CD44^{-/-}$:$OPN^{-/-}$ mice: n = 5; treated $CD44^{+/-}$:$OPN^{+/-}$ mice: n = 5; treated $OPN^{-/-}$ mice: n = 4. **(C)** Proliferating MPP: Multipotent progenitor cell counts in BM of mock mice (controls, being treated with sterile PBS); wild-types: n = 3; $CD44^{-/-}$:$OPN^{-/-}$ mice: n = 3; $CD44^{+/-}$:$OPN^{+/-}$ mice: n = 3; $OPN^{-/-}$ mice: n = 3. **(D)** Proliferating MPP: Multipotent progenitor cell counts in BM of treated mice; 24 hours after Poly(I:C) treatment; controls: n = 3; treated wild-types: n = 5; treated $CD44^{-/-}$:$OPN^{-/-}$ mice: n = 5; treated $CD44^{+/-}$:$OPN^{+/-}$ mice: n = 5; treated $OPN^{-/-}$ mice: n = 4. ▩ Tiled bar: untreated wild-type mice; ■ Black bar: wild-type mice after treatment; □ white bar: $CD44^{-/-}$:$OPN^{-/-}$ mice; ■ dark grey bar: $CD44^{+/-}$:$OPN^{+/-}$ mice; ■ light grey bar: $OPN^{-/-}$ mice. Unpaired, 2-tailed t test was performed to determine significance. Values are the mean ± SEM; $*P \leq 0{,}05$.

In BM of the mock cohort proliferation of MPPs in wild-type (36,2%), $CD44^{-/-}$:$OPN^{-/-}$- (40%), $CD44^{+/-}$:$OPN^{+/-}$- (38,6%) and $OPN^{-/-}$ mice (34,9%) was resemblant (Fig.60.(C)).

24 hours after Poly(I:C) treatment proliferation of MPPs in BM of treated wild-types (62,2%), $CD44^{-/-}$:$OPN^{-/-}$ mice (68,4%; $P \leq 0,05$)), $CD44^{+/-}$:$OPN^{+/-}$- (59,8%) and $OPN^{-/-}$ mice (59,2%) showed to be exaggerated in comparison to proliferation rate of MPPs in mock wiltype-mice (36,2%); but only in $CD44^{-/-}$:$OPN^{-/-}$ mice in a significant way (Fig.60.(D)).

4. Discussion

Continuous observation of blood cell- and BM- populations in $CD44^{-/-}$:$OPN^{-/-}$-, $CD44^{+/-}$:$OPN^{+/-}$- and $OPN^{-/-}$ mice in comparison to wild-types during their lifespan have shown, that significant differences between the genotypes exist under steady-state and stress-state conditions.

4.1 Impact of $CD44^{-/-}$:$OPN^{-/-}$ double-deficiency on thrombocytes

4.1.1 Little is known about thrombocyte function in relation to CD44 and OPN dependence

CD44 has been described as being required for the signalling of macrophage migration inhibitory factor (MIF) (Merk et al., 2012; Sanchez-Niño et al., 2013), an anti-apoptotic pro-inflammatory cytokine expressed in and released from blood platelets (Strüßmann et al., 2013). But the exact functional role of CD44 in thrombocytes has not been described so far (Liu et al., 2016).

OPN contains a protease-hypersensitive site, which is located between the integrin- and CD44-binding domains (Liersch et al., 2012). This means OPN is potentially sensitive for protease-activated cytokines like thrombin, which plays a major role in thrombocyte activation in case of injury (Ware, 2004). Furthermore, OPN binds to platelets and lymphocytes via the integrin $\alpha_V\beta_3$ (Bennett et al., 1997) and participates in the regulation of platelet and lymphocyte adhesion (Helluin et al., 2000). Pang et al., 2019 assumed that the adhesion of platelets to OPN requires agonist stimulation and named thrombin as the most potent physiologic platelet agonist, which mediates cleavage of OPN and enhances cell adhesion.

A study (by Wang et al., 2017) showed that in $OPN^{-/-}$ mice the recruitment of mesenchymal stem cells (MSCs) to the skin wound was significantly inhibited, and the expressions of CD44 and its receptor E-selectin were significantly decreased in the lesions of $OPN^{-/-}$ mice compared to wild-type mice. The investigators suggested that OPN may regulate the migration of MSCs through its interactions with CD44 during skin wound recovery (Wang et al., 2017).

4.1.2 CD44$^{-/-}$:OPN$^{-/-}$ double-deficiency is associated with an increase in thrombocytes in peripheral blood during aging

The results of this PhD thesis show that thrombocyte values in the peripheral blood of CD44$^{-/-}$:OPN$^{-/-}$ mice were significantly increased in several age cohorts (9, 15 and 18-21) in comparison to wild type controls under steady-state conditions. Additionally, 18-21 months old OPN$^{-/-}$ mice exhibited significantly elevated thrombocyte counts in peripheral blood in reference to wild-types at that age cohort.

Interestingly, prior steady-state analyses of our working group did not find significant differences in thrombocyte counts in peripheral blood of aging OPN$^{-/-}$ mice compared to wild-types (Li et al., 2018). To definitely clarify, if OPN deficiency impacts thrombocyte numbers in peripheral blood of old mice, experiments with more individuals in the 18 and 21 months old mouse cohorts would be desirable, but hard to implement, because minor mice reach this progressed age.

Under steady-state conditions no significant differences of megakaryocyte/erythroid progenitors (MEP) in BM of CD44$^{-/-}$:OPN$^{-/-}$-, OPN$^{-/-}$ and wild-type mice have been found. The circumstance that thrombocyte levels in the peripheral blood of CD44$^{-/-}$:OPN$^{-/-}$ mice exhibited a continuous elevation with aging, while MEP numbers in BM of young and old CD44$^{-/-}$:OPN$^{-/-}$ mice were similar to those of wild-types, shows, that risen thrombocyte levels in peripheral blood are not related to an potentially altered MEP pool within the BM compartement.

General function of thrombocytes is blood coagulation in case of injury (Tomaiuolo et al., 2017). They adhere on injured blood vessels and sorrounding tissue and participate in wound healing processes (Tomaiuolo et al., 2017). Therefore, the elevated thrombocyte counts in peripheral blood of aging CD44$^{-/-}$:OPN$^{-/-}$ mice indicate that double-deficiency in both genes might have an impact on thrombocyte activation and adhesion capability. A reduction of thrombocyte activation and their diminished adherence ability could explain the elevated thrombocyte counts in peripheral blood of old CD44$^{-/-}$:OPN$^{-/-}$ mice. This elevation of thrombocytes in peripheral blood implies a potential loss of adequate thrombocyte function in relation to CD44$^{-/-}$:OPN$^{-/-}$ double-deficiency, which leads to

prolonged persistence of thrombocytes in the peripheral blood. The finding that wound healing processes in CD44$^{-/-}$:OPN$^{-/-}$ mice took longer than in widltypes underlines the theory of a diminished thrombocyte function in those mice.

The aging process of CD44$^{-/-}$:OPN$^{-/-}$ mice seems to have an impact on blood thrombocyte levels, because thrombocyte levels were firstly elevated in the 9 months cohort; and the amount of thrombocyte elevation further progressed in older cohorts. The result that younger CD44$^{-/-}$:OPN$^{-/-}$ mice, in contrast to old mice of this genotype, did not exhibit blood thrombocyte elevation, opens the possibility that thrombocytes of young mice express additional receptor and activator proteins, which might be similar in function to CD44 and OPN and could participate in thrombocyte coagulation processes. Those additional proteins could compensate the CD44$^{-/-}$:OPN$^{-/-}$ double-deficiency in young mice and may support thrombocyte function, which would explain the similar blood thrombocyte levels in young CD44$^{-/-}$:OPN$^{-/-}$- and wild-type mice. An aging related loss of those potentially additional proteins could erase the thrombocyte-function-saving-effect, which would lead to elevated thrombocyte levels in the peripheral blood of old CD44$^{-/-}$:OPN$^{-/-}$ mice, which could explain the steady-state results for thrombocytes of old CD44$^{-/-}$:OPN$^{-/-}$ mice within this thesis.

4.1.3 CD44$^{-/-}$:OPN$^{-/-}$ double-deficiency reduces thrombocyte reconstitution in serial transplantation

The serial stem cell transplant experiments (stress-state) of this thesis, which have been performed with young CD44$^{-/-}$:OPN$^{-/-}$-BM-donors and wild-type-BM-donors as controls, have shown, that the replenishment of thrombocyte levels in peripheral blood was disturbed in the absence of CD44 and OPN. 16 weeks after the 1st round, 4 weeks after 3rd round, 12 weeks after 3rd round and 16 weeks after 3rd round of transplant thrombocyte numbers in peripheral blood of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipients were significantly lower that in wild-type controls.

Surprisingly, 16 weeks after 2nd round of transplantation thrombocyte numbers in peripheral blood of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipients were significantly elevated in reference

to wild-type-BM-recipients. This is the only observable point of time in the serial transplantation experiment, in which blood thrombocyte levels in $CD44^{-/-}$:$OPN^{-/-}$-BM-recipients have been elevated in comparison to wild-type-BM-recipients. It is possible, that the $CD44^{-/-}$:$OPN^{-/-}$-BM-recipients might have had an infection at that time point, which could explain the temporary elevated thrombocyte levels.

4 weeks after 3rd round of serial transplantation the reconstitution potential of blood thrombocytes, leukocytes and erythrocytes in $CD44^{-/-}$:$OPN^{-/-}$-BM-recipients was severely diminshed, resulting in thrombocytopenia and leucopenia in the referring mice which led to the premature death of 3 mice (of which 2 mice had to be killed for relief because of neurological abnormalies), while all 10 wild-type-BM-recipents survived.

Summarizigly, the serial stem cell transplantation experiment results of this PhD thesis show, that young HSCs which are double-deficient in CD44 and OPN fail to adequatly reconstitute thrombocytes, leukocytes and erythrocytes after 3 rounds while HSCs which posess both genes are able to replenish those blood cell populations in the referring recipient mice.

A former investigation of a serial stem cell transplantation, in which old $OPN^{-/-}$-BM-donor-cells have been transferred into young wild-type recipient mice showed inferior thrombocyte and erythrocyte reconstitution in the recipient mice, leading to the death of 50% of $OPN^{-/-}$ recipient mice, while all wild-type-BM recipient mice survived until 7 months after transplantation (Li et al., 2018).

The results of this PhD thesis show that young HSCs which are double-deficient in CD44 and OPN show severely impaired hematopoietic reconstitution abilitiy under serial transplantational stress.

4.1.4 Reduced reconstitution potential of megakaryocyte/erythroid progenitors in CD44$^{-/-}$:OPN$^{-/-}$ mice in serial transplantation

In healthy adult hematopoiesis MEP progenitors give rise to megakaryocytes, which are the producing cells of thrombocytes (Pang et al., 2005). It is assumed that erythroid and megakaryocyte lineages share a common MEP (Kanz et al., 1982; Nakahata et al., 1982; Akashi et al., 2000), but the regulating signalling processes are still mostly unravelled (Pang et al., 2005). CD44 is present on murine megakaryocytes and peripheral blood platelets (Koshiishi et al., 1994). Entire megakaryocytes have been found to be able to traverse the marrow-blood barrier, but the signalling pathway and detailed processes are still not fully understood (Tavassoli and Aoki, 1981).

The results of this PhD thesis show that not only thrombocyte replensihment in peripheral blood was significantly disturbed in the absence of CD44 and OPN under serial transplantation stress, but additionally, MEP numbers in BM of CD44$^{-/-}$:OPN$^{-/-}$ mice have declined significantly at 8 weeks after 1st round and 16 weeks after 3rd round of serial transplantation. Declining numbers of thrombocytes in the peripheral blood and decreased MEP-numbers within the BM of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice indicate that the differentiation potential of MEPs, which derived out of CD44$^{-/-}$:OPN$^{-/-}$-BM-donor-cells into megakaryocytes, was deminished. The reduced number of MEPs within die BM of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipients can be one cause for a decrease of thrombocytes within the peripheral blood of the referring mice.
Furthermore, it might be possible, that MEPs, which have derived from CD44$^{-/-}$:OPN$^{-/-}$-BM-donors were lacking an anchoring function, which disables them to successfully stick to their specific BM niche. This could have lead to an inhibited differentiation potential of MEPs into megakaryocytes, because they would not have been in the cytokine niche stimulating environment and would have probably caused less production of thrombocytes. Another reason for reduced thrombocyte counts in CD44$^{-/-}$:OPN$^{-/-}$-BM-recipients could be, that the lack of both genes has an impact on thrombocyte or megakaryocyte migration (or both) from the BM to the peripheral blood and eventually, vice versa.

4.1.5 G-CSF treatment does not impact thrombocyte levels in peripheral blood of CD44$^{-/-}$:OPN$^{-/-}$ mice, but lowers blood thrombocyte counts in treated wild-types and OPN$^{-/-}$ mice

Interestingly, G-CSF treatment significantly lowered thrombocyte counts in peripheral blood of wild-type- and OPN$^{-/-}$ mice at 4 and 6 days after treamtment, while blood thrombocyte levels of CD44$^{-/-}$:OPN$^{-/-}$ mice only exhibited a slightly reduced tendency of thrombocyte levels in peripheral blood compared to untreated wild-types.

Wu et al., 2020 investigated the G-CSF effect on platelets in peripheral blood of stem cell donors and recognized a reduced thrombocyte count with increased density of platelet surface antigens, a lower platelet count and a smaller platelet size in comparison to untreated controls. Therefore, I hypothize that CD44$^{-/-}$:OPN$^{-/-}$ mice might have a reduced G-CSF-receptor densitiy on their platelet surface than those of wild-type- and OPN$^{-/-}$ mice, which would explain that CD44$^{-/-}$:OPN$^{-/-}$ mice`s thrombocyte levels were not severely impacted by G-CSF treatment. A minor G-CSF-receptor-density on thrombocytes of CD44$^{-/-}$:OPN$^{-/-}$ mice would make them less sensitive vor the thrombocyte-number-reduction-effect of G-CSF.

4.1.6 Poly(I:C) stress does not impact thrombocyte levels in peripheral blood of CD44$^{-/-}$:OPN$^{-/-}$ mice

Double-deficiency of CD44 and OPN as well as single-deficiency of OPN and double-heterozygosity of both genes have no impact on thrombocyte levels in the peripheral blood referring to Poly(I:C) stress. Thrombocytes in peripheral blood of wild-types were significantly lower after Poly(I:C) treatment. Blood thrombocyte levels in CD44$^{-/-}$:OPN$^{-/-}$-, CD44$^{+/-}$:OPN$^{+/-}$ and OPN$^{-/-}$ mice showed a reduced tendency after Poly(I:C) treatment in comparison to controls, which was comparable to thrombocyte levels of treated wild-types without being significant. Therefore, Poly(I:C) is not involed in thrombocyte function referring to CD44$^{-/-}$:OPN$^{-/-}$ double-deficiency.

4.2 Impact of CD44$^{-/-}$:OPN$^{-/-}$ double-deficiency on erythrocytes

4.2.1 Erythrocyte numbers in peripheral blood of CD44$^{-/-}$:OPN$^{-/-}$ mice affected under steady- and stress-state conditions

The steady-state results of this PhD thesis displayed significantly elevated erythrocyte levels in peripheral blood of 3 months old OPN$^{-/-}$ mice and 15 months old CD44$^{-/-}$:OPN$^{-/-}$ mice. Former results of our working group did not find blood erythrocyte level differences between wild-type- and OPN$^{-/-}$ mice under steady-state conditions (Li et al., 2018). Reasons for differing results of this PhD thesis and former results of our working group referring to erythrocyte numbers in blood of young OPN$^{-/-}$ mice are hard to evaluate. It might be possible that the elevated erythrocyte levels in peripheral blood of OPN$^{-/-}$ mice in the 3 months age cohort of this thesis are due to the chosen time point for analysis, because former working group results only focused on 2, 6, 12, 18 and 24 months old OPN$^{-/-}$ mouse cohorts.

Interestingly, 9 months old CD44$^{-/-}$:OPN$^{-/-}$ mice exhibited significantly reduced hemoglobin levels in peripheral blood, while erythrocyte numbers were at similar levels than those of wild-types. Reduced hemoglobin levels in erythrocytes, which are unaffected in numers could be a sign of reduced oxygen and carbondioxide binding capability in 9 months old CD44$^{-/-}$:OPN$^{-/-}$ mice. A deficiency of CD44 and OPN might have an effect on erythrocyte function, which could have an impact on oxygen supply of organs and carbon dioxyde exchange transport to the lungs.

In untreated aging mice no differences in MEP (which are progenitors of erythrocytes and megakaryocytes) numbers in the BM of young and old CD44$^{-/-}$:OPN$^{-/-}$-, OPN$^{-/-}$- and wild-type mice have been found. Under stress-state conditions of the serial stem cell transplantation erythrocyte numbers in the peripheral blood of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice have been significantly reduced compared to wild-types at 12 weeks after 2nd, 16 weeks after 2nd and 16 weeks after 3rd round of transplant. Erythrocyte numbers in BM of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipients were significantly reduced at 16 weeks after 1st round. Additionally, hemoglobin levels in peripheral blood of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipients were significatly reduced at 12 weeks after 2nd round and 16 weeks after 2nd

round of serial transplantation. These results show that serial transplantational stress leads to a diminished reconstitution ability of red blood cells in $CD44^{-/-}$:$OPN^{-/-}$ double-deficient hematopoietic stem cells. This finding is comparable to former results of our working group, which identified a severely impaired reconstitution ability of red blood cells in a serial transplantation with old $OPN^{-/-}$-BM-donor-cells (Li et al., 2018). Summarizingly, old HCSs, which are deficient in OPN and young HSCs, which are double-deficient in CD44 and OPN, are impaired in their reconstitution ability of red blood cells under serial transplantational stress.

G-CSF treatment and Poly(I:C) stress did not affect erythrocyte and hemoglobin levels in peripheral blood of $CD44^{-/-}$:$OPN^{-/-}$ mice. Furthermore, 24 hours after Poly(I:C) treatment, the amount of erythroid lineage cells in BM of $CD44^{-/-}$:$OPN^{-/-}$ mice were at comparable levels than in treated wild-types. Therefore, it is assumable that $CD44^{-/-}$:$OPN^{-/-}$ deficient erythrocytes are not differentially affected by viral or bacterial infections than erythrocytes, which posess fully functional CD44 and OPN genes.

4.3 Impact of $CD44^{-/-}$:$OPN^{-/-}$ double-deficiency on leukocytes

4.3.1 Role of CD44 and OPN in leukocyte function

The role of OPN expression in T cells and its role in T-cell development is poorly understood, though some main principles have been described so far (Shinohara et al., 2005). OPN gene expression in activated T cells has been reported to be regulated by T-bet, a transcription factor that controls $CD4^+$ T helper (Th1) cell lineage commitment (Shinohara et al., 2005). Furthermore, examinations have found OPN to be produced by macrophages and by activated T-lymphocytes in vivo (Patarca et al., 1989; Miyazaki et al., 1990; Craig and Denhardt, 1991) and it has been found to affect B cell and macrophage function (Lampe et al., 1991). OPN is assumed to act as a cytokine for T cells, as well as a chemoattractant for macrophages (Weber and Cantor, 1996). Furthermore CD44 is involved in T-precursor trafficking to the thymus and lymph nodes (O'Neill, 1989). These findings lead to the question, how CD44 and OPN functions and their implication in cell signalling processes can be compensated in $CD44^{-/-}$:$OPN^{-/-}$ mice.

Hypothetically, it seems plausible to assume, that other receptors and protein-ligand complexes, which are similar to those of CD44 and OPN, take over the functions of both genes in $CD44^{-/-}$:$OPN^{-/-}$ mice.

4.3.2 $CD44^{-/-}$:$OPN^{-/-}$ double-deficiency affects blood leukocyte numbers under steady- and stress-state conditions

Steady-state analyses of $CD44^{-/-}$:$OPN^{-/-}$ mice showed elevated T-and B cell levels in the peripheral blood of young and old cohorts with an emphasis on the older age. Three months old $CD44^{-/-}$:$OPN^{-/-}$- and $OPN^{-/-}$ mice exhibited significantly elevated T cell-, granulocyte- and monocyte levels in their peripheral blood in comparison to wild-type controls. Referring to $OPN^{-/-}$ mice this result is very interesting, because prior studies of $OPN^{-/-}$ mice of our working group did not find elevated leukocyte levels in peripheral blood of 2 and 6 months old $OPN^{-/-}$ mice under steady-state conditions (Li el al., 2018). The results of this thesis indicate that besides $CD44^{-/-}$:$OPN^{-/-}$ double-deficiency, $OPN^{-/-}$ deficiency has an impact on leukocyte numbers in the peripheral blood of young mice and of old ones.

Interestingly, B- and T cell- levels in peripheral blood of $CD44^{-/-}$:$OPN^{-/-}$ mice were elevated in comparison to those of wild-types in all age cohort (except for the 3 months cohort, in which B cell numbers in peripheral blood of $CD44^{-/-}$:$OPN^{-/-}$ mice were similar to those of wild-types), whereas single-deficiency of OPN mainly seemed to lead to blood leukocyte elevation (especially B and T cells) in young and old mouse cohorts but not in middle aged ones. A reason for elevated leukocyte levels in the peripheral blood under $CD44^{-/-}$:$OPN^{-/-}$ double-deficiency could be an impaired leukocyte function. The deficiency of OPN (being a T cell activating gene (Ashkar et al., 2000)) could lead to a function loss of T helper cells, cytotoxic T cells or regulatory T cells. If some of these T cell subsets can not be sucessfuly activated, T cell response in case of infection would be impaired. Additionally, the absence of CD44, which is involved in T-precursor trafficking to the thymus and lymph nodes (O'Neill, 1989), might lead to T cells, which could be impaired in their pathogen-recognizing-capability. The circumstance that CD44 is known to be involved in B cell activation (Rachmilewitz and Tykocinski, 1998) could be one cause for elevated B cell values in the peripheral blood of $CD44^{-/-}$:$OPN^{-/-}$ mice in several age

cohorts. If B cells cannot successfully be activated, they would be impaired in their immune response capability, which might be indicated in the elevated B-cell blood levels.

Hypothetically, $CD44^{-/-}$:$OPN^{-/-}$ deficiency might influence the interaction between T-helper- and B cells.

4.3.3 $CD44^{-/-}$:$OPN^{-/-}$ double-deficiency does not impact B and T cell values in BM but reduces granulocyte numbers under steady-state conditions

In the BM department of aging mice (steady-state) B- and T cell levels did not differ in $CD44^{-/-}$:$OPN^{-/-}$- and $OPN^{-/-}$ mice referring to wild-type values; neither in the young cohort (6 months aged) nor in the old (12-21 months aged) cohort. Furthermore, common lymphoid progenitors (CLP) (which can differentiate into B, T and natural killer (NK) cells) (Kondo et al., 2003), did not differ in numbers in BM of young and old $CD44^{-/-}$:$OPN^{-/-}$ mice in comparison to those of wild-types. These circumstances underline, that elevated B- and T cells numbers in the peripheral blood of aging $CD44^{-/-}$:$OPN^{-/-}$ mice were not based on altered numbers of B and T-cell pools within the BM compartment, but were most probably the result of an impaired B and T cell functionality. Granulocytes in BM of old $CD44^{-/-}$:$OPN^{-/-}$ mice (18-21 months old) were significantly reduced compared to wild-type granulocyte values. Peripheral blood granulocyte numbers in 3 months old and 15 months aged $CD44^{-/-}$:$OPN^{-/-}$ mice were significantly elevated compared to those of controls. These results might indicate that granulocytes have a tendency of enhanced migration into the peripheral blood in a $CD44^{-/-}$:$OPN^{-/-}$ deficient BM environment, especially in older mice. Furthermore, granulocytes in the peripheral blood of $CD44^{-/-}$:$OPN^{-/-}$ mice could be impaired in their function.

4.3.4 Serial transplantation stress impairs leukocyte reconstitution of $CD44^{-/-}$:$OPN^{-/-}$ double-deficient hematopoietic stem cells

In the stress-state setting of a serial transplantation, in which BM of young $CD44^{-/-}$:$OPN^{-/-}$-BM-donors has been transplanted into lateral tail veins of irradiated wild-type-recipient mice, leukocyte reconstitution was seriously impaired. Four weeks after

2nd round of transplant B cells in peripheral blood of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipients were significantly reduced compared to those of wild-types. 8 weeks after 2nd round of transplant B-, T cells, granulocytes and monocytes in the peripheral blood of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipients were significantly decreased referring to control values.
B and T cell values in peripheral blood seemed to be stronger impacted by serial translational stress, because they were the first blood cell populations, which were significantly lowered after transplantation and did not adapt to wild-type-BM-recipient values, whereas granulocyte and monocytes blood values temporarily recovered in numbers (16 weeks after 2nd round of transplant) after being significantly decreased 8 weeks after 2nd round of transplant in comparison to wild-type control values. Interestingly, B cell numbers in BM of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipients were significantly elevated 8 weeks after 1st round (CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient B cell value: 20%; WT-BM-recipient B cell value: 15%) and 16 weeks after 3rd round (CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient B cell value: 17%; WT-BM-recipient B cell value: 5,6%) of serial transplantation. While 6 and 12 weeks after 1st round of serial transplant B cell values in peripheral blood of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipients showed a tendency of elevation in comparison to those of WT-BM-recipients, peripheral blood B cell values in CD44$^{-/-}$:OPN$^{-/-}$-BM-recipients were significantly decreased at 16 weeks after 3rd round of transplant. The result of significantly elevated B cell BM levels in CD44$^{-/-}$:OPN$^{-/-}$-BM-recipients at 16 weeks after 3rd round of serial transplant, but the very small number of B cells in peripheral blood at that time, indicate, that CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient B cells might be severely impaired in their maturation processes. Serial transplantation stress could affect a development disturbance in CD44$^{-/-}$:OPN$^{-/-}$ deficient B cells, which alters their maturation processes in the BM compartment. It might be possible, that the lack of CD44 and OPN in B cells alters their interaction potential with the BM niche and impairs their migration skill into the peripheral blood.

4.3.5 G-CSF stress response of leukocytes in CD44$^{-/-}$:OPN$^{-/-}$ mice unaltered compared to controls

Over the duration of 6 days of G-CSF treatment leukocyte mobilization did not differ between treated wild-type-, CD44$^{-/-}$:OPN$^{-/-}$- and OPN$^{-/-}$ mice in comparison to untreated

controls. 4 and 6 days after treatment T cell levels in peripheral blood of wild-type-, $CD44^{-/-}$:$OPN^{-/-}$- and $OPN^{-/-}$ mice were significantly lowered compared to untreated controls. This result seems to be surprising, because G-CSF mobilization has been found to increase the frequency of T cells in peripheral blood of healthy human donors (Zhao et al., 2017). The decrease of T cells in peripheral blood of wild-type-, $CD44^{-/-}$:$OPN^{-/-}$- and $OPN^{-/-}$ mice at 4 and 6 days of treatment is most likely not an effect of G-CSF stimulation, but a result of unnatural elevation of T-cell levels in the peripheral blood of the control mice. T cell values in the peripheral blood of untreated control mice rose from 25% before treatment to 40% after the 4^{th} day of treatment, which might indicate that they had an infection.

6 days after G-CSF treatment, granulocyte numbers in peripheral blood of treated wild-type and $CD44^{-/-}$:$OPN^{-/-}$ mice were significantly elevated in comparison to untreated wild-type values. $OPN^{-/-}$ granulocyte numbers in peripheral blood also showed an elevated tendency referring to untreated controls. These results were expected, because the main role of G-CSF is granulocyte stimulation and the enhancement of granulocyte release into the peripheral blood (Deotare et al., 2015).

Because granulocyte levels in peripheral blood of G-CSF treated wild-type-, $CD44^{-/-}$:$OPN^{-/-}$- and $OPN^{-/-}$ mice as well as B and T cell values in BM of the three genotypes were elevated at comparable amounts in reference to untreated controls, CD44 and OPN do not seem to be involved in G-CSF-dependent signaling processes of leukocytes.

4.3.6 Poly(I:C) treatment impacts $CD44^{-/-}$:$OPN^{-/-}$ double-deficient leukocytes

24 hours after Poly(I:C) treatment B cell numbers in peripheral blood of wild-types were significantly decreased from 41% (before treatment) to 4% (after treatment), while those of $CD44^{-/-}$:$OPN^{-/-}$ mice were lowered from 41% to 12%, those of $CD44^{+/-}$:$OPN^{+/-}$ mice from 38% to 14% and $OPN^{-/-}$ mice values from 43% to 17%. Poly(I:C) seems to have a stronger reducing effect on B cell numbers in peripheral blood of wild-types than in $CD44^{-/-}$:$OPN^{-/-}$-, $CD44^{+/-}$:$OPN^{+/-}$- and $OPN^{-/-}$ mice.

T cell numbers in peripheral blood of Poly(I:C) treated wild-types and $CD44^{-/-}$:$OPN^{-/-}$mice were significantly reduced in reference to control mice 24 hours after treatment, while blood T cell values of het- and $OPN^{-/-}$ mice were at comparable levels than those of controls. Granulocyte numbers in peripheral blood of Poly(I:C) treated wild-type mice (before treatment: 18%; after treatment: 83%) were significantly stronger elevated than those of $CD44^{-/-}$:$OPN^{-/-}$- (before treatment: 12%; after treatment: 64%), $CD44^{+/-}$:$OPN^{+/-}$- (before treatment: 16%; after treatment: 50%) and $OPN^{-/-}$ mice (before treatment: 14 %; after treatment: 53%) 24 hours after treatment. Monocytes in the peripheral blood of wild-types decreased from 8,3% before treatment, to 4,5% after Poly(I:C) treatment, whereas those of $CD44^{-/-}$:$OPN^{-/-}$-, $CD44^{+/-}$:$OPN^{+/-}$- and $OPN^{-/-}$ mice were comparable with monocyte levels of control mice.
In BM of Poly(I:C) treated mice T cell numbers in wild-type-, $CD44^{-/-}$:$OPN^{-/-}$-, $CD44^{+/-}$:$OPN^{+/-}$- and $OPN^{-/-}$ mice were significantly elevated in reference to control values. No significant differences in BM counts of granulocytes, B cells and monocytes in $CD44^{-/-}$:$OPN^{-/-}$-, $CD44^{+/-}$:$OPN^{+/-}$- and $OPN^{-/-}$ mice were found in comparison to control values.

Summarizing, $CD44^{-/-}$:$OPN^{-/-}$ double-deficiency seems to impact leukocyte blood levels in case of infection. A stronger reduction in B and T cell levels in peripheral blood of Poly(I:C) treated wild-types than in $CD44^{-/-}$:$OPN^{-/-}$ mice could indicate, that B and T cells were successfully activated in wild-types and have already been emigrated out of the blood into the infected tissue (the Poly(I:C) injection site). $CD44^{-/-}$:$OPN^{-/-}$ deficient leukocytes might have an impaired functionality. Causative for an altered leukocyte response in $CD44^{-/-}$:$OPN^{-/-}$ mice could be a disturbance in B and/or T cell receptor-binding-affinity as well as functional problems in B and T cell activation as well as an interaction between T-helper and B cells.

4.4 Impact of $CD44^{-/-}$:$OPN^{-/-}$ double-deficiency on HSCs and progenitors

4.4.1 Unimpaired progenitor- and HSC numbers in aging $CD44^{-/-}$:$OPN^{-/-}$ mice

$CD44^{-/-}$:$OPN^{-/-}$ double-deficiency does not alter HSC numbers in BM of young and old mice in comparison to wild-type mice under steady-state conditions. This result is surprising, because both genes, CD44 and osteopontin, have been described to be negative regulators of HSCs (Cao et al., 2016; Stier et al., 2005), so their combined absence hypothetically should have led to higher HSC levels in $CD44^{-/-}$:$OPN^{-/-}$ mice than in $OPN^{-/-}$ mice. The finding, that the lack of OPN, which is present in high concentrations in the hematopoietic niche (Stier et al., 2005), has no impact on HSC abundance within the BM of young $CD44^{-/-}$:$OPN^{-/-}$-, $OPN^{-/-}$- and $CD44^{+/-}$:$OPN^{+/-}$ mice seems to be surprising, because OPN and CD44 have individually been described to be negative regulators of the HSC pool within the BM (Stier et al., 2005; Cao et al., 2016).
Interestingly, $OPN^{-/-}$ deficiency enhanced HSC proliferation in BM of young mice and showed a tendency of enhanced MPP proliferation in old mice. The aging process in $OPN^{-/-}$ mice seems to impact HSC and MPP proliferation, which might be related to age-depending changes in the BM microenvironment. But the exact mechansims of the interdependence of OPN and the BM microenvironment during aging remain unclear and need further investigation.

It has been speculated, that OPN regulates cell adhesion and dynamics at bone surfaces (Reinholt et al., 1990; McKee and Nanci, 1995; McKee and Nanci, 1996b; Rittling et al., 1998) and mediates tissue adhesion and cohesion between organic and inorganic phases (Mckee and Nanci, 1996a). The bones and teeth of animals, which lack the OPN gene have been found to be morphologically normal at the level of light and electron microscopy in young mice (Rittling et al., 1998). Therefore, I would recon that the aging process of OPN deficient mice leads to changes in the bone marrow surface and its adhesion/interaction capacity with HSCs. Furthermore, in vivo imaging studies of the bone marrow in the calvarium reveal, that specific sinusoidal domains,

which express CXCL12 and E-selectin, are targeted for homing and engraftment of normal and leukemic HSCs (Sipkins et al., 2005; Boulais and Frenette, 2015). In $OPN^{-/-}$ mice, the expressions of CD44 and its receptor E-selectin were significantly decreased in lesions of a wound healing study, having the effect of less migrating MSCs to sites of wound-healing (Wang et al., 2017). Factual, $CD44^{-/-}$:$OPN^{-/-}$ mice, which have been observed within this thesis, displayed impaired wound healing capacity compared to wild-type mice. Therefore it is assumable, that the migration potential of MSCs to sites of wound-healing is impaired in $CD44^{-/-}$:$OPN^{-/-}$ mice. Furthermore, the migration ability of HSCs in $CD44^{-/-}$:$OPN^{-/-}$ mice is potentially impaired. For further evaluations of this hypothesis, migration essays of $CD44^{-/-}$:$OPN^{-/-}$-deficient-HSCs would be highly desirable.

4.4.2 $CD44^{-/-}$:$OPN^{-/-}$ double-deficiency affects serial reconstitution ability of young HSCs

During serial transplantation donor-HSC-cells migrate from peripheral blood into the BM microenvironment in which they anchor before proliferation (Caocci et al., 2017). After successfully anchorage of HSC in BM they start to proliferate and replenish blood cells (Caocci et al., 2017).

The serial transplantation experiments of this thesis, which were performed with young $CD44^{-/-}$:$OPN^{-/-}$-BM-donors into irradiated wild-type recipient mice, revealed an impaired reconstitution ability of $CD44^{-/-}$:$OPN^{-/-}$ deficient HSCs. Already 8 weeks after 1st round of transplant, CLP and CMP cells in BM of $CD44^{-/-}$:$OPN^{-/-}$-BM-recipients were significantly diminished in comparison to those of wild-type-BM-recipients. 8 weeks after 2nd round of serial transplant leukocyte reconstitution in $CD44^{-/-}$:$OPN^{-/-}$-BM-recipients was severely impaired, resulting in reduced B, T granulocyte and monocytes blood cell numbers, which were only about half the values seen in wild-type-BM-recipients.

Engraftment of $CD44^{-/-}$:$OPN^{-/-}$-donor-HSCs in BM of wild-type recipient mice did not seem to be impaired in comparison to wild-type-donor-HSCs, because 8weeks after1st, 8 weeks after 2nd and 16 weeks after 2nd round of transplant HSC numbers in BM of $CD44^{-/-}$:$OPN^{-/-}$-BM-recipients were at comparable levels than those of wild-type-BM-recipients. 16 weeks after 3rd round of transplant HSC-, MPP-, CLP-, GMP- and CMP

cell numbers in BM of CD44$^{-/-}$:OPN$^{-/-}$-BM-recipients were significantly reduced in comparison to control values. Furthermore the blood cell supply with leukocytes, erythrocytes and thrombocytes was severely diminished in CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice, marking hematopoietic failure, which resulted in the death of 3 CD44$^{-/-}$:OPN$^{-/-}$-BM-recipient mice, while all 10 wild-type-BM-recipient mice survived. These data indicate that CD44$^{-/-}$:OPN$^{-/-}$ double-deficiency in young HSCs leads to an impaired reconstitution of lymphoid and myeloid blood cell populations under serial transplantation stress.

4.4.3 G-CSF does not affect CD44$^{-/-}$:OPN$^{-/-}$ double-deficient HSCs and MPPs numbers in BM

After 6 days of daily G-CSF treatment HSC numbers in BM of wild-type-, CD44$^{-/-}$:OPN$^{-/-}$- and OPN$^{-/-}$ mice exhibited a comparable tendency of decrease in reference to controls, whereas MPPs in BM of those mice showed a tendency of elevation in comparison to control levels. HSCs and MPPs in BM of CD44$^{-/-}$:OPN$^{-/-}$- and OPN$^{-/-}$ mice did not react differently to G-CSF stress than wild-types.
Therefore, CD44$^{-/-}$:OPN$^{-/-}$ double-deficient HSCs and MPPs are not specifically impaired in immune response reactions under G-CSF stress. Summarizing, G-CSF stress has not altered HSC or MPP abundance within the BM of CD44$^{-/-}$:OPN$^{-/-}$- and OPN$^{-/-}$ mice in reference to controls and is therefore not suspected to be a direct effector of the progenitor populations in a CD44 and OPN lacking bone marrow environment.

4.4.4 Poly(I:C) stress increases HSC proliferation in BM of OPN$^{-/-}$ mice and MPP proliferation in BM of CD44$^{-/-}$:OPN$^{-/-}$ mice

Referring to stress-state experiments Poly(I:C) treatment elevates HSC numbers in BM of 6 months old CD44$^{-/-}$:OPN$^{-/-}$- and OPN$^{-/-}$ mice. But the increase of HSCs in BM after Poly(I:C) stress is less pronounced in CD44$^{-/-}$:OPN$^{-/-}$- and OPN$^{-/-}$ mice than in wild-types. Furthermore, Poly(I:C) increases HSC proliferation in BM of OPN$^{-/-}$ mice and MPP proliferation in BM of CD44$^{-/-}$:OPN$^{-/-}$ mice significantly; and in both cases it has been the highest elevation compared to all other observed genotypes within this experimental setting. Interestingly, Poly(I:C) treatment leads to a significant ($P \leq 0{,}0005$) increase of

MPP values in BM of $OPN^{-/-}$ mice, which is the highest of all observed genotypes in treatment. Summarizing, the absence of OPN elevates HSC proliferation and the combined absence of CD44 and OPN increases MPP proliferation in BM in case of viral infection (which has been simulated by application of Poly(I:C) in the referring mice) in reference to controls. Because recent studies have indicated that HSCs proliferate in response to systemic infection and replenish effector immune cells (Harrison and Lerner, 1991; Baldridge et al., 2011), the increased proliferation of HSCs in the BM of $OPN^{-/-}$- and the increase of MPP proliferation in the BM of $CD44^{-/-}$:$OPN^{-/-}$ mice clearly shows, that the immune response of those mice is altered in comparison to wild-types.

5. Conclusion

The experimental results of this thesis underline a severe impact of $CD44^{-/-}$:$OPN^{-/-}$ double-deficiency on hematopoiesis under steady- and stress-state experiments in the observed mouse-model.

The introductory questions can be answered as following:

1. What influence has $CD44^{-/-}$:$OPN^{-/-}$ deficiency on the size of the stem cell pool, on proliferation and differentiation capacity of HSCs?

Interestingly, $CD44^{-/-}$:$OPN^{-/-}$ deficiency does not alter HSC, CLP, GMP, CMP and MEP numbers in BM of young and old mice in comparison to wild-type mice under steady-state conditions. But LKS numbers in BM of old $CD44^{-/-}$:$OPN^{-/-}$ mice are reduced in comparison to wild-type mice. $OPN^{-/-}$ deficiency enhanced HSC proliferation in BM of young mice and showed a tendency of enhanced MPP proliferation in old mice. $OPN^{-/-}$ deficiency seems to impact HSC and MPP proliferation in BM, which might be related to structural changes in the BM microenvironment of the referring mice. What specifically causes this effect remains unclear and needs further investigation. It has been speculated, that OPN regulates cell adhesion and dynamics at bone surfaces (Reinholt et al., 1990; McKee and Nanci, 1995; McKee and Nanci, 1996b; Rittling et al., 1998) and mediates tissue adhesion/cohesion between organic and inorganic phases (Mckee and Nanci, 1996a). Therefore, I would recon that $OPN^{-/-}$ deficiency impacts the

adhesion/interaction capacity of HSCs and MPPs with the BM surface. Furthermore, in vivo imaging studies of the BM in the calvarium revealed, that specific sinusoidal domains, which express CXCL12 and E-selectin, are targeted for homing and engraftment of normal and leukemic HSCs (Sipkins et al., 2005; Boulais and Frenette, 2015). In $OPN^{-/-}$ mice, the expressions of CD44 and its receptor E-selectin were significantly decreased in lesions of a wound healing study, having the effect of less migrating MSCs to sites of wound-healing (Wang et al., 2017). Observations of $CD44^{-/-}$:$OPN^{-/-}$ mice, which were no part of the experiments within this thesis, but happened by chance, showed an impairment in their wound-healing processes in comparison to wild-type mice. This finding could be a hint, that MSC in $CD44^{-/-}$:$OPN^{-/-}$ mice were indeed negatively impacted in their wound-healing capacity. But further experiments would be needed to clarify this assumption. While HSC, MPP and progenitor numbers in BM of young and old $CD44^{-/-}$:$OPN^{-/-}$ mice were unaltered, thrombocyte-, leukocyte- (especially B- and T cells) and erythrocyte levels in the peripheral blood of aging $CD44^{-/-}$:$OPN^{-/-}$ mice were elevated referring to wild-type controls. Hematopoiesis in $CD44^{-/-}$:$OPN^{-/-}$ mice is clearly altered in comparison to wild-type mice. Reasons for elevated blood thrombocyte, leukocyte and erythrocyte levels in $CD44^{-/-}$:$OPN^{-/-}$ mice could be enhanced proliferation of committed precursors into myeloid and lymphoid blood cells, functional defects of $CD44^{-/-}$:$OPN^{-/-}$ deficient erythrocytes, leukocytes and thrombocytes or impaired degradation of those blood cell populations.

2. Is the elimination of $CD44^{-/-}$:$OPN^{-/-}$ in HSCs in reference to wild-types more severe referring to hematopoiesis than $OPN^{-/-}$ deficiency?

In Reference to wild-type mice $CD44^{-/-}$ and $OPN^{-/-}$ double-deficiency seems to have a stronger impact on blood leukocyte levels than $OPN^{-/-}$ single-deficiency, because B- and T cell levels in peripheral blood of $CD44^{-/-}$:$OPN^{-/-}$ mice were elevated in the majority of age cohorts, whereas single-deficiency of $OPN^{-/-}$ mainly seemed to lead to blood leukocyte elevation (especially B and T cells) in young and old mouse cohorts but not in middle aged ones. The results of this PhD thesis show that thrombocyte values in the peripheral blood of $CD44^{-/-}$:$OPN^{-/-}$ mice were significantly increased in several age

cohorts (9, 15 and 18-21) in comparison to wild type controls under steady-state conditions. Granulocyte numbers in BM of old $CD44^{-/-}$:$OPN^{-/-}$ mice (18-21 months old) were significantly reduced compared to wild-type values. Peripheral blood granulocyte numbers in 3 months old and 15 months aged $CD44^{-/-}$:$OPN^{-/-}$ mice were significantly elevated compared to those of controls. These results might indicate that granulocytes have a tendency of enhanced migration into the peripheral blood in a $CD44^{-/-}$:$OPN^{-/-}$-double-deficient BM environment, especially in older mice. Furthermore, granulocytes in the peripheral blood of $CD44^{-/-}$:$OPN^{-/-}$ mice could be impaired in their function. The double-deficiency of CD44 and OPN shows a stronger impact on peripheral blood composition in aging mice than the single deficiency of OPN in reference to wild-type mice.

3. How does induced stress impact the functioning of HSCs in $CD44^{-/-}$:$OPN^{-/-}$ deficient mice?

The serial transplantation experiment results of this thesis show, that $CD44^{-/-}$:$OPN^{-/-}$ deficient HSCs are impaired in their reconstitution ability of thrombocytes, erythrocytes and leukocytes in the peripheral blood in comparison to HSCs, which posess both genes. After 3 rounds of serial transplantation 3 out of 10 $CD44^{-/-}$:$OPN^{-/-}$-BM-recipient mice died, while all 10 out of 10 wild-type-BM-recipient mice (controls) have survived.

G-CSF treatment did not show a different impact on HSC- and MPP numbers in BM of $CD44^{-/-}$:$OPN^{-/-}$ mice than in wild-type controls. Interestingly, G-CSF response of thrombocytes in peripheral blood of $CD44^{-/-}$:$OPN^{-/-}$ mice was altered in comparison to wild-type and $OPN^{-/-}$ mice. Therefore, thrombocyte function in $CD44^{-/-}$:$OPN^{-/-}$ mice might be impaired in case of bacterial infection.

Poly(I:C)-stress elevates HSC numbers in BM of $OPN^{-/-}$ mice and MPP numbers in BM of $CD44^{+/-}$:$OPN^{+/-}$- and $OPN^{-/-}$ mice. Furthermore, Poly(I:C) treatment increases HSC proliferation in BM of $OPN^{-/-}$ and MPP proliferation in BM of $CD44^{-/-}$:$OPN^{-/-}$ mice. In addition, Poly(I:C) treatment impacts $CD44^{-/-}$:$OPN^{-/-}$ double-deficient leukocytes numbers in the peripheral blood. A stronger reduction in B and T cell levels in peripheral

blood of Poly(I:C) treated wild-types than in $CD44^{-/-}$:$OPN^{-/-}$ mice could indicate, that B and T cells were successfully activated in wild-types and have already been emigrated out of the blood into the infected tissue (the Poly(I:C) injection site). $CD44^{-/-}$:$OPN^{-/-}$ deficient leukocytes might have an impaired functionality. Causative for an altered leukocyte response in $CD44^{-/-}$:$OPN^{-/-}$ mice could be a disturbance in B and/or T cell receptor-binding-affinity as well as functional problems in B and T cell activation as well as an interaction between T-helper and B cells. Summarizing, $CD44^{-/-}$:$OPN^{-/-}$ mice seem to be impaired in their immune response capability in case of a simulated viral infection.

Conclusional, CD44 and OPN seem to be important players in hematopoietic processes. OPN, being a protein ligand of CD44, has been found to be an inductor of chemotaxis, whereas hyaluronate has been found to restrict migration of cells in case of binding to the CD44 receptor (Weber et al., 1996). Weber et al., 1996 stated that the different responses of cells after CD44 ligation by either osteopontin or hyaluronate may account for the independent effects of CD44 on cell migration and growth and could be an important factor in tumor cells to promote metastasis formation. Therefore I would speculate that OPN in combination with CD44 are important for anchoring of cells (e.g. B-, T- precursor cells, HSCs and MPPs) within the BM on the one hand and on migration processes of those cells on the other hand. This interdependency of the motility enhancing effect of OPN and the demobilizing effect of hyaluronate on the CD44 receptor could act like a “motility-switch”, which leads to either the agglomeration of a specific cell population within the BM, or the migration out of the BM niche into the peripheral blood. Furthermore, there might be a turnover point, in which critical rising or lowering OPN-levels within the microenvironment affect B cells and T cells in their binding affinity to other cells (e.g. pathogens, different antigens or T-helper-cells). It is even imaginable that different variants of CD44 being expressed in varying densities in different blood cell populations react very specific to certain cytokines.

Referring to the goal of this thesis, which has been aimed to unravel the importance of the genetic impact of CD44 and OPN on hematopoietic processes, it can be described

as being extremely crucial. Additional studies, which investigate the signalling pathways that have an impact on CD44 and OPN function in hematopoiesis are highly desirable.

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