Analysis of the Impact of Bruton Tyrosine Kinase on Myeloid Cell Development and Function

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Table of Contents

Zusammenfassung **1**

Summary **3**

1 Introduction **5**

1.1 Bruton Tyrosine Kinase 5

1.1.1 Tec Family of Kinases 6

1.1.2 Activation of Btk 7

1.1.3 Downstream Effectors of Btk 8

1.1.4 Function of Btk in Hematopoietic Cells 10

1.2 Hematopoiesis 12

1.2.1 Hematopoietic Stem Cells 12

1.2.2 Lineage-Committed Progenitors 15

1.2.3 Regulation of Hematopoiesis 16

1.3 Neutrophils 25

1.3.1 Differentiation of Neutrophils 25

1.3.2 Immune Functions of Neutrophils 28

1.4 Aims of the Study 36

2 Material and Methods **37**

2.1 Mice 37

2.1.1 Mice Strains 37

2.1.2 Isolation of Genomic DNA from Mouse Tail Tips 37

2.1.3 Genotyping 37

2.2 Isolation of Hematopoietic Cells from Mice 38

2.2.1 Sorting of Hematopoietic Progenitor and Stem Cells 38

2.2.2 Purification of $CD11b^+$ Myeloid Cells 39

2.2.3 Preparation of Erythroid Cell-Depleted Bone Marrow Cells 39

2.2.4 Isolation of Murine Bone Marrow PMN 40

2.3 Cell Culture 40

2.3.1 General Cell Culture Procedures 40

2.3.2 Cultivation of Myeloid Cells 41

2.3.3 Cultivation of Bone Marrow-Derived PMN 41

2.3.4 Colony Forming Assay 41

2.4 Neutrophil Function Assays 42

2.4.1 Migration Assay 42
2.4.2 Transendothelial Migration Assay 42
2.4.3 Immune Complex-Induced Degranulation 43
2.4.4 Annexin-V Apoptosis Assay 44
2.4.5 Reverse Passive Arthus Reaction in Skin 44
2.5 Generation of Bone Marrow Chimeras 45
2.6 Flow Cytometry 46
2.6.1 Analysis of Murine Spleen 46
2.6.2 Analysis of Murine Bone Marrow 46
2.6.3 Analysis of Murine Blood 46
2.6.4 BrdU Incorporation 47
2.6.5 Antibodies for Flow Cytometry 47
2.7 Microscopy 47
2.7.1 Cytospin Preparation and Pappenheim Staining 47
2.7.2 Immunofluorescence 48
2.7.3 Toluidin Staining of Mast Cells 49
2.7.4 Transmission Electron Microscopy 49
2.8 Immunoblot (Western Blot) 49
2.8.1 Antibodies for Immunoblotting 50
2.8.2 Buffers and Solutions for Immunoblotting 51
2.9 Quantitative RT-PCR 52
2.9.1 Primer for Quantitative RT-PCR 53
2.10 Reagents and Chemicals 54
2.10.1 Chemicals 54
2.10.2 Kits 55
2.10.3 Cell Culture 55
2.10.4 Antibodies for Flow Cytometry 56
2.10.5 Other Reagents 57
2.10.6 Plastic Ware 58
2.11 Laboratory Equipment 59
2.12 Software 60

3 Results 61

3.1 Mouse Models for Btk Deficiency 61
3.2 Btk Function in Hematopoiesis 61

3.2.1 Analysis of Myeloid Cell Populations in Btk-Mutant Mice 61
3.2.2 Analysis of Hematopoietic Stem Cell and Progenitor Populations in the Context of Btk Deficiency 65
3.2.3 Analysis of Myeloid Development in Btk-Deficient Mice 68
3.2.4 Btk Function in Terminal Differentiation of Neutrophils 73
3.3 Immune Functions of Btk-Deficient Neutrophils 78
3.3.1 Function of Neutrophils Lacking Btk in the Acute Inflammatory Response 78
3.3.2 Analysis of Immune Function of Btk-deficient Neutrophils *in vitro* 81
3.4 Molecular Mechanisms Provoking Impaired Differentiation and Function of Btk-Deficient Neutrophils 85
3.5 Cell-Autonomous Effects of Btk in Neutrophils 91
3.6 Influence of Cytokine Treatment on Granulopoiesis in Btk-Deficient Mice 100

4 Discussion 106
4.1 Btk Function in Myeloid Cells 106
4.2 Altered Myelopoiesis in Btk-Deficient Mice 108
4.3 Btk-Dependent Activation of C/EBPα 112
4.4 Impaired Immune Function of Btk-Deficient Neutrophils 115
4.5 Neutrophil Function of XLA Patients 117
4.6 Conclusions 118

5 Abbreviations 120
6 References 126
Acknowledgements 143
Erklärung 144
Curriculum Vitae 145

Zusammenfassung

Die essenzielle Funktion der Bruton'schen Tyrosine Kinase (Btk) in der Signaltransduktion des B-Zellrezeptors, sowie in der B-Zellentwicklung ist bereits gut beschrieben worden. Jedoch konnte bislang in Zellen der myeloiden Linie und hämatopoetischen Stammzellen, in denen Btk ebenfalls expremiert ist, lediglich gezeigt werden, dass die Btk im Signalweg der Toll-like Rezeptoren (TLR) oder des Fcε-Rezeptor aktiv ist. Ob allerdings Btk in diesen Signalkaskaden eine essenzielle Funktion ausübt und in wieweit sie für die myeloide Zellentwicklung notwendig ist, wird aber kontrovers diskutiert.

In der vorliegenden Arbeit konnte nun gezeigt werden, dass die Btk für die korrekte hämatopoetische Differenzierung von Granulozyten und Monozyten im Knochenmark notwendig ist. Wir konnten zeigen, dass sich aus dem Granulozyten-Makrophagen-Vorläufer präferenziell Granulozyten entwickeln zu Ungunsten von Monozyten und undifferenzierten Zellen, wenn aus dem Knochemark von Btk-defizienten Mäusen isolierte Granulozyten-Makrophagen-Vorläufer in Gegenwart des granulocyte-macrophage colony-stimulating factor (GM-CSF) oder von TLR-Liganden kultiviert werden. Diese erhöhte Granulopoese konnte auch im Knochenmark Btk-defizienter Mäuse nachgewiesen werden. Die gesteigerte Granulopoese war jedoch mit einem signifikanten Defekt in der terminalen Reifung der Btk-defizienten Granulozyten verbunden. Dieser unreife Phänotyp war assoziert mit einer reduzierte Anzahl von Granula und einer verringerte Expression von Granulaproteinen (Myeloperoxidase, neutrophile Elastase, Lactoferrin und Gelatinase). Außerdem zeigte die funktionelle Analyse der neutrophilen Granulozyten in der Arthus-Reaktion eine deutliche Beeinträchtigung der Btk-defizienten Granulozyten in der Gewebeeinwanderung zum Ort der Immunkomplexablagerung. Die Untersuchung des molekularen Mechanismus ergab eine deutlich verminderte Phosphorylierung der Phosphatidylinositol-3-Kinase sowie von Akt und der Glycogensynthase-Kinase 3β in Btk-defizienten myeloiden Zellen nach Stimulation mit GM-CSF, wobei hier ebenfalls nachgewiesen wurde, dass Btk in wildtypischen myeloiden Zellen nach Stimulation mit GM-CSF aktiviert wird. Zusätzlich konnten wir zeigen, dass die Expression der linienbestimmenden Trankriptionsfaktoren CAAT-enhancer binding protein α (C/EBPα) und PU.1 in Btk-defizienten Knochenmarkszellen reduziert ist.

Zusammenfassend konnten wir die essenzielle Funktion der Btk für die Linienentscheidung zur Entwicklung von Granulozyten oder Monozyten während der Hämatopoese aufzeigen, die wahrscheinlich auf die verringerte Aktivierung der C/EBPα-abhängigen Transkription zurück geführt werden kann. Des Weiteren konnten wir zeigen, dass die veränderte Granulopoese zu einer ineffizienten Reifung der neutrophilen Granulozyten führt, welche die Immunfunktion dieser Zellen stark beeinträchtigt.

Summary

The need of Bruton tyrosine kinase (Btk) for B cell receptor signaling as well as B cell development is well acknowledged. However, Btk is also expressed in myeloid cells as well as hematopoietic stem cells, where Btk is involved in Toll-like receptor (TLR) activation as well as in Fcε receptor signaling. Nevertheless, the demand for Btk in these signaling pathways and therefore for proper development as well as function of myeloid cells is controversially discussed.

In the present study, it could be demonstrated that Btk is necessary for the proper hematopoietic differentiation of granulocytes and monocytes downstream of the granulocyte-macrophage progenitor (GMP) in the bone marrow. We could show that GMP isolated from the bone marrow of Btk-deficient mice preferentially developed into granulocytes at the expense of monocytes or undifferentiated cells when cultivated in the presence of granulocyte-monocyte-colony stimulating factor (GM-CSF) or TLR-ligands as differentiation cues. In addition, the enhanced granulopoiesis could be confirmed by the evaluation of the bone marrow compartment of Btk-mutant mice. With respect to the terminal differentiation of granulocytes in the bone marrow we also found a substantial maturation defect in granulocytes obtained from Btk-deficient mice, although the granulopoiesis was enhanced in these mice. This immature phenotype of granulocytes was associated with an inefficient development of granules as well as a reduced expression of granule proteins, like myeloperoxidase, neutrophil elastase, lactoferrin or gelatinase. Moreover, analyzes of neutrophil function in the reverse passive Arthus reaction demonstrated a significant impairment of Btk-deficient neutrophils in tissue migration at the site of immune complex deposition. The examination of the molecular mechanism revealed a decreased phosphorylation of the regulatory subunit p85 of the phosphatidylinositol-3-kinase, Akt and glycogen synthase kinase-3β in Btk-deficient myeloid cells after GM-CSF engagement, where Btk is activated in wild type cells. In addition, we could show that the expression of the lineage-determining transcription factors CAAT-enhancer binding protein α (C/EBPα) and PU.1 is diminished in Btk-deficient bone marrow cells.

Together, we could demonstrate the need for Btk in granulocyte versus monocytes lineage-decision during hematopoietic differentiation, which is probably due to the activation of C/EBPα-dependent transcription. Moreover, the altered

granulopoiesis is accompanied by an inefficient maturation of neutrophils that also prevents their actual immune function.

1 Introduction

The defense of a complex organism against invading pathogens is essential for survival and accomplished by the immune system. This system consists of an extraordinarily diverse inventory of specialized molecules and cells that continuously patrol the blood and tissue for invaders, and protect the epithelial surfaces, which are under constant assault from infectious agents in the environment. The leading roles in this system are played by lymphocytes mediating adaptive immunity as well as myeloid cells conferring innate immunity. For a fine tuned orchestration of such a multicomponent system a proper communication on the inter- and intracellular level is required. Lack of only one component in the immune system can give rise to severe immunodeficiency phenotypes.

1.1 Bruton Tyrosine Kinase

Mutations within the gene coding for Bruton tyrosine kinase (BTK) were initially identified in 1993 as the cause of X-linked agammaglobulinemia (XLA) [1,2], a severe immunodeficiency that is only found in males and has been described first by Ogden C. Bruton in 1952 [3]. XLA is characterized by profoundly decreased or absent immunoglobulins (Ig) in the serum, a failure of antibody production in response to antigenic stimulation and high susceptibility to recurrent bacterial infections [4]. XLA patients are usually healthy in the newborn period but come down with recurrent bacterial infections between 3 and 18 months of age after the decline of maternal antibodies. Mortality rate of patients suffering from XLA without therapy is 100 % due to the increased susceptibility to a variety of encapsulated bacteria and enterovirus infections finally leading to death in early childhood. For that reason, a lifelong substitution of XLA patients with human IgG administered intravenously or subcutaneously is necessary, but the life expectancy of XLA patients is still below average. Long-term studies revealed a cumulative risk of more than 80 % for XLA-patients after 17 years with IgG substitution for development of chronic lung disease that is characterized by the presence of recurrent or chronic symptoms associated with abnormal lung morphology like peribronchial thickening, segmental atelectasis and bronchiectasis [5-7]. Also in the year 1993, two independent studies could demonstrate that a point mutation in the *Btk* gene causes a similar although less

severe syndrome in the mouse, called X-linked immunodeficiency (*Xid*) [8,9]. Gene mapping localized the *BTK* gene at the midportion of the long arm of the human X chromosome at Xq22. Early sequence comparisons of BTK showed a similarity to the Src family of cytoplasmic protein-tyrosine kinases, since several Src homology (SH) domains could be identified. However, the N-terminal myristoylation signal, characteristic for Src kinases, is missing in Btk. A closer look on structure lead to the grouping of BTK into a new family of cytoplasmic protein-tyrosine kinases named Tec family [2].

1.1.1 Tec Family of Kinases

The Tec kinases are the second largest family of non-receptor tyrosine kinases and so far, besides Btk four more mammalian members of Tec family kinases have been described, namely Bmx tyrosine kinase (bone marrow kinase gene on the X chromosome [10]), IL-2 inducible T cell kinase (Itk) [11], Rlk/Txk tyrosine kinase (resting lymphocyte kinase/T and X cell expressed kinase [12,13]) and eponymous Tec kinase (Tyrosine kinase expressed in hepatocellular carcinoma [14]) [15]. The characteristic feature of Tec family kinases is the N-terminal part that is composed of a pleckstrin homology (PH) domain and a short Tec homology (TH) domain consisting of a Btk homology (BH) motif and one or two proline-rich regions (PRR). The PH domain alone is able to bind to certain types of phospholipids as well as proteins and the PH domain together with the BH motif constitute a binding site for GTP-bound "active" Gα subunits of heterotrimeric G proteins [16,17]. Moreover, the $\beta\gamma$ subunits of heterotrimeric G proteins can be bound, which activate Btk [18]. Further hallmarks of Tec kinases are the Src homology domains SH2 and SH3, known to bind to sequences containing phosphorylated tyrosine residues or to interact with proline-rich sequences, respectively [19]. The catalytic kinase domain found in the C-terminal portion of the protein, also shares some similarity to the Src kinase catalytic domain. However, Tec kinases do not have a C-terminal negative regulatory tyrosine residue and lack the N-terminal myristoylation signal of Src kinases that is essential for posttranslational myristoylation and subsequent localization to the membrane. Instead, the PH domain of Tec kinases can bind to phospholipids, which results in physical tethering of Tec kinases to the cell membrane comparable to the myristoylation signal of Src kinases (Figure 1.1) [15].

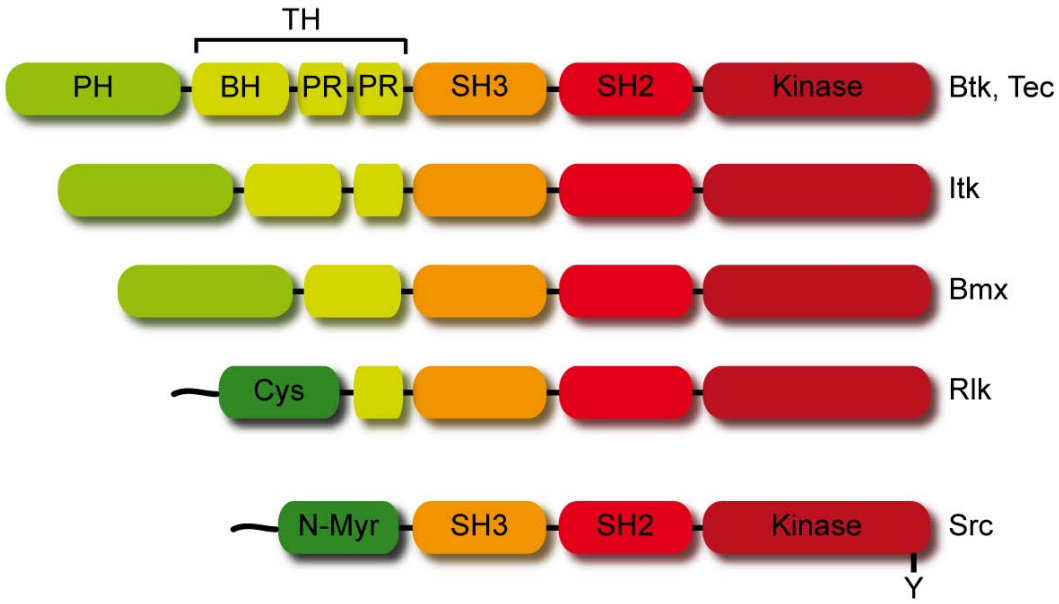


Figure 1.1 Structural comparison of Tec family and Src family kinases.
Similar to Src kinases, Tec kinases possess a C-terminal kinase domain, followed by an SH2 and SH3 domain. Tec kinases differ from Src kinases in their N-terminal regions by means of the Tec domain consisting of a BH motif and proline-rich regions followed by the PH domain. Tec family kinases do not contain a negative regulatory tyrosine at the C-terminus as well as a N-terminal myristoylation. PH, Pleckstrin homology domain; TH, Tec homology domain; BH, Btk homology motif; PR, proline-rich region; SH, src homology domain; N-Myr, N-terminal myristoylation signal. Figure adapted from Schmidt et al. [15].

1.1.2 Activation of Btk

Tec family kinases are activated following ligand binding to a variety of cell surface receptors, such as antigen receptors, receptor tyrosine kinases, cytokine receptors, G protein-coupled receptors and integrin adhesion receptors. Possibly, the best-studied activation cascade of Tec family kinases is the signaling downstream of antigen receptors, which includes Btk activation after cross-linking of the B cell antigen receptor (BCR) in B cells [20]. First, Btk has to be translocated to the cell membrane via interaction of the PH domain with phosphatidylinositol-3,4,5-triphosphate (PIP_3), a product of activated phosphoinositide-3-kinase (PI3K) [21,22]. Mutations within the PH domain of Btk prevent its activation due to impaired PIP_3-binding and can cause both XLA in humans and *Xid* in mice [23]. Recent studies on Btk and Itk have shown that interaction of the PH domain with PIP_3 leads to the targeting of the kinase to specific membrane microdomains, known as lipid rafts [24]. Additionally, other proteins have been shown to interact

with Btk via the PH domain, including heterotrimeric G proteins, F-actin and focal adhesion kinase (FAK) [25]. Once at the membrane, phosphorylation of the tyrosine residue 551 (Y551) within the activation loop of the kinase domain occurs, which is mediated by Src family kinases. This phosphorylation leads to an increase in Btk kinase activity, which is essential for full Btk function [26,27]. The two-step activation mechanism is common for most members of the Tec kinase family. Additionally, in the case of Btk, activation also causes the autophosphorylation at tyrosine residue 223 (Y223) within the SH3 domain [28]. Phosphorylation of this site alters the affinity of the SH3 domain for certain binding partners [29] and may hamper an intramolecular interaction between the SH3 domain and the PRR of the TH domain. Two independent studies have demonstrated that in the resting state Btk forms homodimers as a result of stable intermolecular interactions between the PRR of one Btk protein and the SH3 domain of the second Btk protein. Possibly, these intermolecular interactions are involved in the regulation of the kinase functions by interfering with enzymatic activity or with protein localization [30-32].

1.1.3 Downstream Effectors of Btk

The diversity of interaction domains found in Btk, as well as the recruitment into lipid rafts in a PI3K activity-dependent manner implies a role of Btk as an adaptor and amplifier within a multimolecular signalosome. For Btk, numerous interacting factors have been described and several cellular processes have been linked to Btk function (Figure 1.2). One of the best-studied roles of Btk is the requirement of the kinase for sustained Ca^{2+} influx from extracellular sources in B cells, also known as capacitative Ca^{2+} entry.

Engagement of the BCR causes the rapid activation of Src kinases followed by the recruitment and activation of Syk kinase, which in turn phosphorylates the adaptor protein BLNK/SLP-65 (B cell linker protein/SH2 domain–containing leukocyte protein of 65 kD). Simultaneous stimulation of PI3K results in an increase of PIP_3 levels within the cell membrane that prompts the recruitment of Btk to the membrane and subsequently enables the Src kinase-mediated phosphorylation of Btk at position Y551. Following the recruitment, a multimolecular signalosome complex forms within the lipid rafts, where BLNK/SLP-65 serves as core for a complex that additionally includes Btk, phospholipase Cγ2 (PLCγ2) and other proteins. Formation of the signalosome complex is a crucial step for Btk-

dependent phosphorylation and activation of PLCγ2 finally leading to calcium mobilization as well as to activation of the protein kinase C (PKC) via the second messengers inositol-3,4,5-triphosphate (IP_3) and diacylglycerol (DAG), respectively [33]. Further evidences emphasizing the function of Btk for BCR-dependent signaling originated from a study describing a novel protein, termed BCAP (B cell adaptor for PI3K), which connects antigen-induced BCR activation and PI3K activation. BCAP is phosphorylated by Btk and Syk upon BCR engagement and is involved in the subsequent recruitment of PI3K into lipid rafts via binding of the regulatory subunit p85 of PI3K [34]. Another study has demonstrated the necessity of Btk for the production of the PI3K- and PLCγ2-substrate phosphatidylinositol-4,5-bisphosphate (PIP_2) by recruitment of the synthesizing enzymes phosphatidylinositol-4-phosphate 5-kinases (PIP5K) into lipid rafts [35].

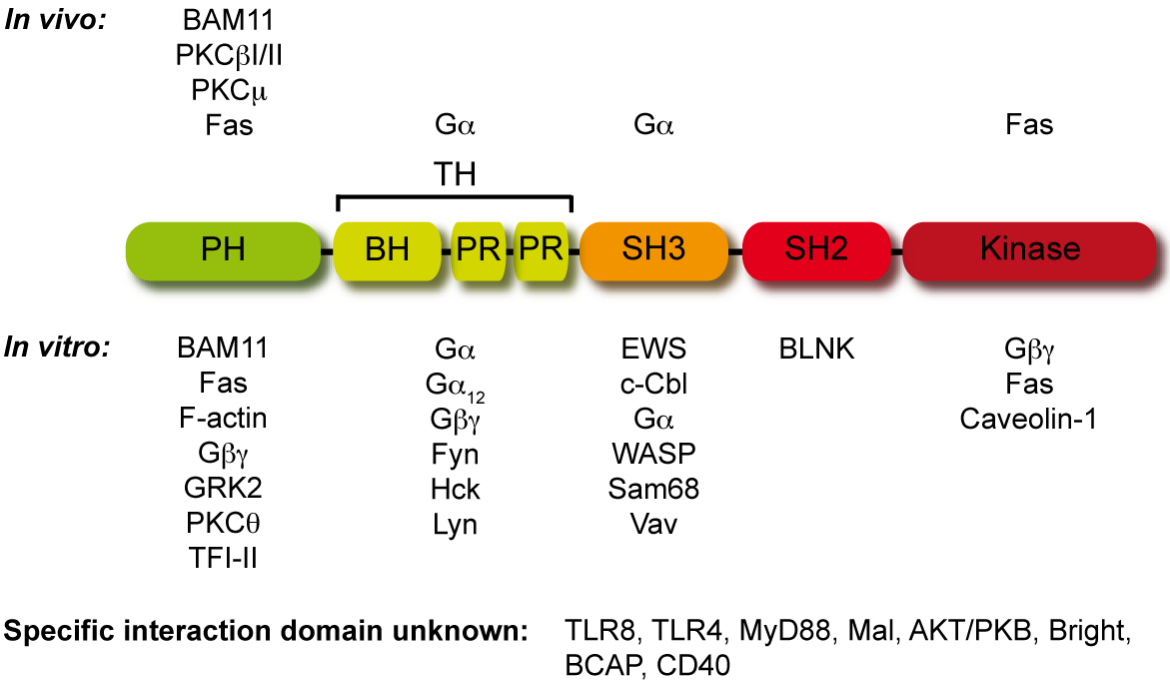


Figure 1.2 Interaction partners of Btk.

Proved *in vivo* and *in vitro* interaction partners of Btk and their corresponding interaction domains are depicted in the scheme. BAM11, Btk-associated molecule 11; GRK2, G protein-coupled receptor kinase 2; EWS, Ewing sarcoma breakpoint region 1; c-Cbl, cellular Casitas B-lineage lymphoma, TLR, Toll-like receptor; MyD88, myeloid differentiation primary response gene 88; AKT/PKB, protein kinase B. Figure adapted from Lindvall et al. [20].

Additionally, further studies also have revealed the importance of functional Btk in the regulation of several other signaling pathways such as the nuclear factor κB (NFκB) pathways [36,37] as well as the mitogen-activated protein kinase (MAPK) pathways, particularly for extracellular signal-regulated kinase 2 (ERK2) and c-jun NH_2-terminal kinase 1 (JNK1) [38,39]. Nevertheless, Btk not only indirectly regulates gene transcription via activation of numerous signaling pathways, it can also directly associate with the transcription factors BAP135/TFII-I (Btk-associated protein of 135 kDa/general transcription factor II-I), Bright and signal transducer and activator of transcription 5A (STAT5A) [40-43]. Supporting evidence for a direct contribution of Btk to transcriptional activation emerged from a study demonstrating nucleocytoplasmic shuttling of Btk [44].

Furthermore, Btk has been shown to be involved in cytoskeletal reorganization after antigen receptor ligation. Upon BCR engagement, Btk activation is required for the control of actin cytoskeleton dynamics [45] by activation of Wiskott-Aldrich syndrome protein (WASP), a known actin regulator, and phosphorylation of the Rho family GTPase guanidine nucleotide exchange factor (GEF) Vav, another regulator of cytoskeletal rearrangement [46]. Finally, evidences argue that Btk influences cell survival pathways. Although defects in these pathways in mutant cells may be related to changes in antigen-induced molecules such as Fas, Fas ligand (Fas-L) and p38, the observation that Btk directly interferes with Fas binding to FADD (Fas associated via death domain) suggests a more direct involvement in survival versus apoptotic processes [47].

1.1.4 Function of Btk in Hematopoietic Cells

The expression of Tec family kinases is predominantly restricted to cells of the hematopoietic system except for Bmx and Tec, which also have been observed in endothelial and liver cells. Also, Btk was found additionally expressed in several neuronal cell lines. But according to the current knowledge, the majority of Btk expression is restricted to the hematopoietic system. In detail, Btk expression has been described in hematopoietic stem cells (HSC), multipotent progenitor cells and in any myeloid cell type, like erythroid cells, platelets, monocytes, macrophages, granulocytes and dendritic cells (DC). In the lymphoid lineage, Btk has been observed in the B cell lineage, but not in T cells or natural killer cells (NK cells) [15].

The function of Btk in B cells and B cell development has been evidenced by XLA due to the fact that this primary immunodeficiency is elicited by defects in early B cell development. Profound hypoagammaglobulinemia and markedly reduced or absent B cells in the peripheral circulation are characteristic features of XLA, which are caused by a severe block in differentiation at pro-B cell to pre-B cell transition in the bone marrow, before the production of surface immunoglobulin-positive B cells starts. The mouse syndrome *Xid*, caused by the R28C point mutation within the PH domain that hampers membrane recruitment, is characterized by a failure of B cells to become phenotypically and functionally mature [8] and resembles partially the XLA phenotype. Several studies have demonstrated the activation of Btk through a variety of cell surface molecules, including BCR and pre-BCR, mentioned above, as well as interleukin 5 (IL-5) and interleukin 6 (IL-6) receptors on B cells. However, the block in B cell differentiation found in XLA patients provides a strong support for the importance of Btk in signaling through the pre-BCR and BCR. To date, there is no evidence that signaling through any of the other receptors contributes to the pathophysiology of XLA [48].

Besides the role of Btk in B cells, the expression of Btk in all cell types of myeloid origin indicates an important role of Btk also within the myeloid compartment. A plethora of studies has demonstrated a variety of signaling pathways leading to Btk activation in cells of the myeloid lineage, like Fcε receptor I (FcεRI) in mast cells, collagen receptor in platelets, erythropoietin receptor in erythroid cells, fMLP receptor in neutrophils, as well as Toll-like receptor 4 (TLR4) and macrophage-colony stimulating factor (M-CSF) receptor in macrophages to mention only a few [15]. But there are also studies claiming that there is no evidence for any functional importance of Btk in myeloid cells *in vivo*. These controversial results concerning Btk function in myeloid cells clearly show a lack of knowledge about the detailed function of Btk in the different signaling pathways in neutrophils, macrophages or dendritic cells and of course in the interplay of these different cell types with each other and also with the adaptive immune system. Additionally, almost nothing is known about the role of Btk in HSC and multipotent progenitors, although expression of Btk in the stem cell compartment implicates a potentially important function in hematopoietic precursor proliferation or differentiation.

1.2 Hematopoiesis

The hematopoietic system is traditionally categorized into two separate lineages, the lymphoid lineage responsible for adaptive immunity and the myeloid lineage including morphologically, phenotypically and functionally distinct cell types like innate immune cells as well as erythrocytes and platelets. Mature hematopoietic cells, except some rare lymphoid cell types, are relatively short-lived with life spans ranging from few hours for granulocytes to a couple of weeks for erythrocytes demanding a continued replenishment of functional cells. This process is named hematopoiesis and takes place primarily in the bone marrow, where few hematopoietic stem cells give rise to a differentiated progeny following a series of more or less well-defined steps of multipotent progenitors and lineage-restricted precursors leading to a hierarchical structure of the process. During the course of hematopoiesis cells lose their proliferative potential as well as multilineage differentiation capacity and progressively acquire characteristics of terminally differentiated mature cells.

1.2.1 Hematopoietic Stem Cells

In the murine hematopoietic differentiation hierarchy, the most primitive cells with highest multipotent activity are long-term repopulating HSC (LT-HSC). One of the first definitions of true HSC meaning LT-HSC came from bone marrow transplantation experiments in mice determining HSC by their capacity to reconstitute several times the hematopoietic system of lethally irradiated adult organisms. Such experiments have demonstrated, that HSC possess multi-potentiality as well as the ability to produce exact replicas upon cell division, named self-renewal capacity. In contrast to real HSC, short-term repopulating HSC (ST-HSC) defined by their ability to contribute transiently to the production of lymphoid and myeloid cells in lethally irradiated recipients, are often described misleadingly as self-renewing cells. Therefore, the contemporary model of hematopoietic hierarchy proposes the affiliation of ST-HSC to the group of multipotent progenitors (MPP), which are characterized by a more limited proliferative potential, but retain the ability to differentiate into various hematopoietic lineages [49,50] (Figure 1.3).

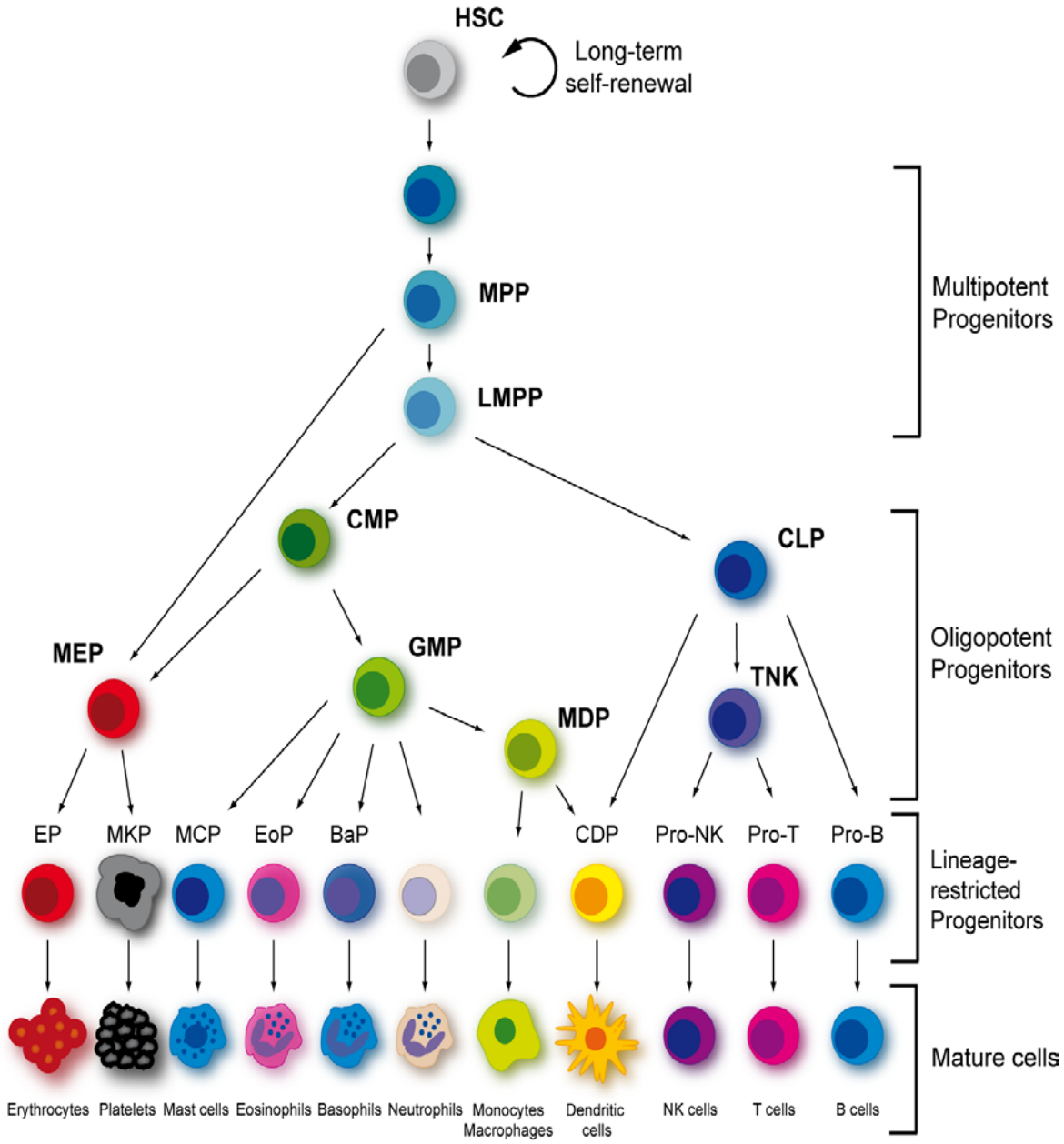


Figure 1.3 Model of the hematopoietic hierarchy in the mouse.

The developmental course shown in the scheme are proposed using results generated by prospective isolation and characterization of different progenitors. HSC, hematopoietic stem cell; MPP, multipotent progenitor; LMPP, lymphoid-primed multipotent progenitor; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; MEP, megakaryocyte-erythrocyte progenitor; GMP, granulocyte-macrophage progenitor; MDP, monocyte-dendritic cell progenitor; TNK, T cell NK cell progenitor; EP, erythroid progenitor; MKP, megakaryocyte progenitor; MCP, mast cell progenitor; EoP, eosinophil progenitor; BaP, basophil progenitor; CDP, common dendritic cell progenitor. Figure adapted from Weisman and Shizuru [50], Iwasaki and Akashi [51] as well as Geissmann [52].

Nevertheless, at the moment a defined model for the hierarchy of multipotent progenitors is not available, because several studies have demonstrated different types of multipotent progenitors with lymphoid as well as myeloid potential [51]. Additionally, a lot of research concerning prospective isolation and characterization of HSC and multipotent progenitors has provided insight into the surface marker expression on these types of cells leading to the definition of HSC and multipotent progenitors as cells being mainly negative for lineage markers but positive for the surface markers Sca1 and Kit. This fraction of bone marrow cells is also named LSK-fraction ($Lin^-Sca1^+Kit^+$) and comprises all stem cell capacity of the hematopoietic system, but of course there are more surface markers published, permitting a more precise definition of HSC and MPP as depicted in Table 1.1. For the present work only the Slam (signaling lymphocyte activation molecule) family receptors CD150 and CD48 will be of special interest, which allow to distinguish inside the LSK-fraction between HSC ($CD150^+CD48^-$) and multipotent progenitors ($CD150^-CD48^-$), whereas $CD48^+$ cells represent the most restricted progenitors [53].

Population	Surface Marker
HSC	$Lin^-Kit^+Sca1^+CD150^+CD48^-CD34^-Flt3^-$
ST-HSC	$Lin^-Kit^+Sca1^+CD150^+CD48^-CD34^+Flt3^-$
MPP	$Lin^-Kit^+Sca1^+CD150^-CD48^-CD34^+Flt3^+$
LMPP	$Lin^-Kit^+Sca1^+CD150^-CD34^+Flt3^{hi}$
CLP	$Lin^-Kit^{lo}Sca1^{lo}IL7R^+Flt3^{hi}$
CMP	$Lin^-Kit^+Sca1^-CD34^+Fc\gamma RII/III^{lo}$
MEP	$Lin^-Kit^+Sca1^-CD34^-Fc\gamma RII/III^-$
GMP	$Lin^-Kit^+Sca1^-CD34^+Fc\gamma RII/III^+$

Table 1.1 Surface markers of hematopoietic stem cells and progenitors.
Definition of surface marker composition for individual progenitor populations is adapted from Weissman and Shizuru[50], as well as Kiel et al. [53].

Under homeostatic conditions in adult mice, the number of HSC remains relatively constant and the majority of HSC stays in a quiescent state that contributes not

only to their long-term maintenance, but also allows a rapid cell cycle entry upon a variety of differentiation cues. The minority of HSC is in an active and dividing state and gives rise to all hematopoietic cells meaning that these few active HSC not only have to self-renew, but also have to produce all differentiated progeny. These different cell fates can only be achieved by an asymmetric division of the HSC, which allows the generation of two non-identical daughter cells, one maintaining stem cell identity and the other becoming a differentiated cell. Two different mechanisms are proposed by which asymmetry could be achieved: first by divisional asymmetry that is introduced by unequally redistributed cell-fate determinants in the cytoplasm [54]. An alternative possibility would be the environmental asymmetry, which is caused by different extrinsic signals provided by distinct local microenvironments and provokes different cell fate decisions of two identical daughter cells [55].

1.2.2 Lineage-Committed Progenitors

Downstream of the HSC and MPP populations with high proliferative and self-renewal capacity starts the differentiation process in murine hematopoiesis leading to oligopotent and later on to lineage-committed progenitors with a diminished proliferation but increased differentiation. The contemporary model of murine hematopoiesis assumes that the decision for differentiation into the lymphoid/myeloid or megakaryocyte/erythrocyte lineages probably occurs very early in hematopoiesis. Several studies have demonstrated that multipotent progenitors like the granulocyte-monocyte-lymphoid progenitor (GMLP) or the lymphoid-primed multipotent progenitor (LMPP) retain only minor megakaryocyte/erythrocyte lineage potential, whereas the vast majority of progenitors appears to be committed to the granulocyte/monocyte as well as the lymphoid lineage [56]. In the next step of ongoing differentiation oligopotent progenitors with differentiation capacity for several hematopoietic lineages develop from an ancestor, the common lymphoid progenitor [57] (CLP) and the common myeloid progenitor [58] (CMP). The CLP is the earliest population in the lineage-negative fraction that upregulates the receptor for interleukin 7 (IL-7), an essential cytokine for T and B cell development. Furthermore, the CLP carries differentiation potential for all types of lymphoid cells including B cells, T cells and NK cells. The surface marker profile of CLP was defined as $Lin^{-}Sca1^{lo}Kit^{lo}IL7R\alpha^{+}$ [57]. In contrast

to CLP, the CMP resides in the $Lin^-Sca1^-Kit^+$ population in bone marrow that can be further fractioned by expression of the Fcγ receptor II/III (FcγRII/III) and CD34 leading to three distinct progenitor populations. The CMP is defined as $Fc\gamma RII/III^{lo}CD34^+$ and can give rise to all types of myeloid colonies in clonogenic assays, while the $Fc\gamma RII/III^{hi}CD34^+$ granulocyte-monocyte progenitor (GMP) is restricted to granulocytes and macrophages. The $Fc\gamma RII/III^{lo}CD34^-$ megakaryocyte-erythrocyte progenitor (MEP) is delimitated to megakaryocytes and erythrocytes [58].

Still a matter of dispute is the dendritic cell (DC) development, because DC mainly are the progeny of GMP, but can also be generated from lymphoid progenitors such as CLP and pro-T cells under certain conditions [59]. However, the majority of plasmacytoid DC (pDC) and conventional or myeloid DC (mDC) develop successively by several commitment steps downstream of the GMP in the bone marrow. The first step is the development of the monocyte/macrophage and DC precursor [60] (MDP) out of the GMP that has lost differentiation potential for granulocytes and expresses the FcγRII/III and CD34 at a comparable level to the GMP, but is also $Kit^{lo}CX_3CR1^+$. Further differentiation of MDP, which is accompanied by the loss of monocyte potential, leads to the common DC precursor (CDP) defined as $Lin^-Kit^{int}Flt3^+M\text{-}CSFR^+$ population that can only give rise to pDC and mDC [52,61,62]. Besides the characterization of MDP and CDP by several studies, further progenitor populations for eosinophils, basophils and mast cells have been isolated downstream of the GMP and their position in the hematopoietic hierarchy is depicted in Figure 1.3. Moreover, the monopotent megakaryocyte lineage-committed progenitor (MKP) [63] and erythroid progenitor (EP) [64] have been described downstream of the MEP. Only for the monocyte/macrophage lineage and the neutrophil granulocytes, a putative committed precursor downstream of the GMP has not been identified to date [51].

1.2.3 Regulation of Hematopoiesis

The differentiation process of quiescent HSC towards different progeny of mature hematopoietic cells is associated with a variety of cell fate choices at every single step of hematopoiesis and comprises quiescence, self-renewal or differentiation at HSC level as well as proliferation, differentiation and lineage choice at the progenitor level. Of course, different cell fate choices require at each step in the

hematopoietic hierarchy a process of decision-making that is presumed to be dependent on and regulated by a combination of intrinsic and extrinsic factors.

1.2.3.1 Maintenance of HSC

For the maintenance of HSC with respect to quiescence, self-renewal and suppression of differentiation, the major intrinsic factors belong to the Bmi1-p53 axis of cell cycle regulators and the PI3K signaling pathway. Bmi1 is a member of the Polycomb group gene family that controls cell proliferation via repression of the *Ink4/Arf* locus. Therefore, Bmi1 supports self-renewal by suppressing transcription of the cell cycle inhibitors $p16^{Ink4a}$ and $p19^{ARF}$, which are encoded in *Ink4/Arf* locus. Whereas the tumor suppressor p53 contributes to the regulation of HSC quiescence via inhibition of cell cycle [65]. In contrast, the PI3K signaling pathway controls cell proliferation, growth and survival via integration of numerous upstream signals, including growth factors, nutrients and oxygen status.
Additionally, several extrinsic factors have been identified that are necessary for HSC fate decision. The extrinsic regulators embrace soluble membrane-bound extrinsic factors including cytokines (fms-related tyrosine kinase 3-ligand, stem cell factor), chemokines (CXCL12) and growth factors (Angiopoietin-1, granulocyte-CSF, granulocyte-macrophage-CSF), as well as Wnt (wingless type), Notch, Hedgehog and the TGFβ (transforming growth factor β) family of cytokines. These extrinsic factors are provided by a specialized microenvironment in the bone marrow, the so-called stem-cell niche that resides in the endosteal and vascular compartments of the bone. In these areas, the bone marrow cells of hematopoietic and non-hematopoietic origin like megakaryocytes, osteoblasts, endothelial cells and CXCL12-abundant reticular (CAR) cells create a supportive microenvironment via physical interaction with HSC and production of soluble factors [65].

1.2.3.2 Cell-Intrinsic Regulation of Lineage Commitment

The differentiation process downstream of HSC involves selective activation and silencing of genes that introduces lineage commitment. Probably, these lineage-determining programs are dependent on extrinsic as well as intrinsic signals, or on both at different developmental stages. Nevertheless, gene regulatory networks are predicted to have a leading role in directing cell fate choice and lineage restriction.

These gene regulatory networks are composed of several master transcription factors joining special features, as mutual regulation of activity by antagonism as well as lineage-determining functions via activation of lineage-specific genes and repression of lineage-foreign genes (Figure 1.4). The first example pointing out the importance of these transcription factors is the transition from self-renewing HSC towards more committed MPP that is dependent on the transcription factor CAAT-enhancer binding protein α (C/EBPα). The prototype of the C/EBP family displays all characteristic features of the transcription factor family, such as the N-terminal transactivation domain as well as the C-terminal DNA-binding domain consisting of a highly conserved basic region and a leucine zipper dimerization domain. Prerequisite for binding of C/EBPα to the cognate DNA-site is the homo- or heterodimerization with another transcription factor via the leucine zipper domain that in turn allows the basic region to bind to the CAAT motif [66,67]. Evidences for the function of C/EBPα in hematopoietic differentiation revealed from studies on conditional C/EBPα-deficient mice, which demonstrated a competitive advantage of C/EBPα-deficient HSC over wild type HSC in reconstitution experiments. Further analyses of the transcriptome of C/EBPα-deficient HSC have confirmed that the expression of the self-renewal factor Bmi1 is increased in these cells, suggesting C/EBPα as a pro-differentiation factor in HSC fate decision [68].

Probably, the next step in decision-making during differentiation is the choice for erythroid versus myeloid-lymphoid lineage restriction at the transition from MPP to LMPP/GMLP or MEP that is regulated by the E-twenty six (Ets) family transcription factor PU.1 and the transcription factor GATA-binding protein 1 (GATA-1). GATA-1 is expressed in erythroid, megakaryocyte, mast as well as eosinophil lineages and contains zinc fingers, which mediate DNA binding to the WGATAR DNA sequence as well as protein-protein interaction [69,70]. In contrast to GATA-1, PU.1 is restricted to monocyte as well as B lymphoid lineages and consists of a N-terminal transactivation domain, a PEST-domain (proline, glutamic acid, serine and threonine rich sequence) and the eponymous Ets-domain at the C-terminus, which mediate DNA binding to an 11 bp sequence with a central GCA motif [71,72]. Additionally, both transcription factors are detectable in MPP and gene disruption studies have demonstrated the indispensable functions of GATA-1 and PU.1 for megakaryocyte/erythrocyte and myeloid/lymphoid development, respectively.

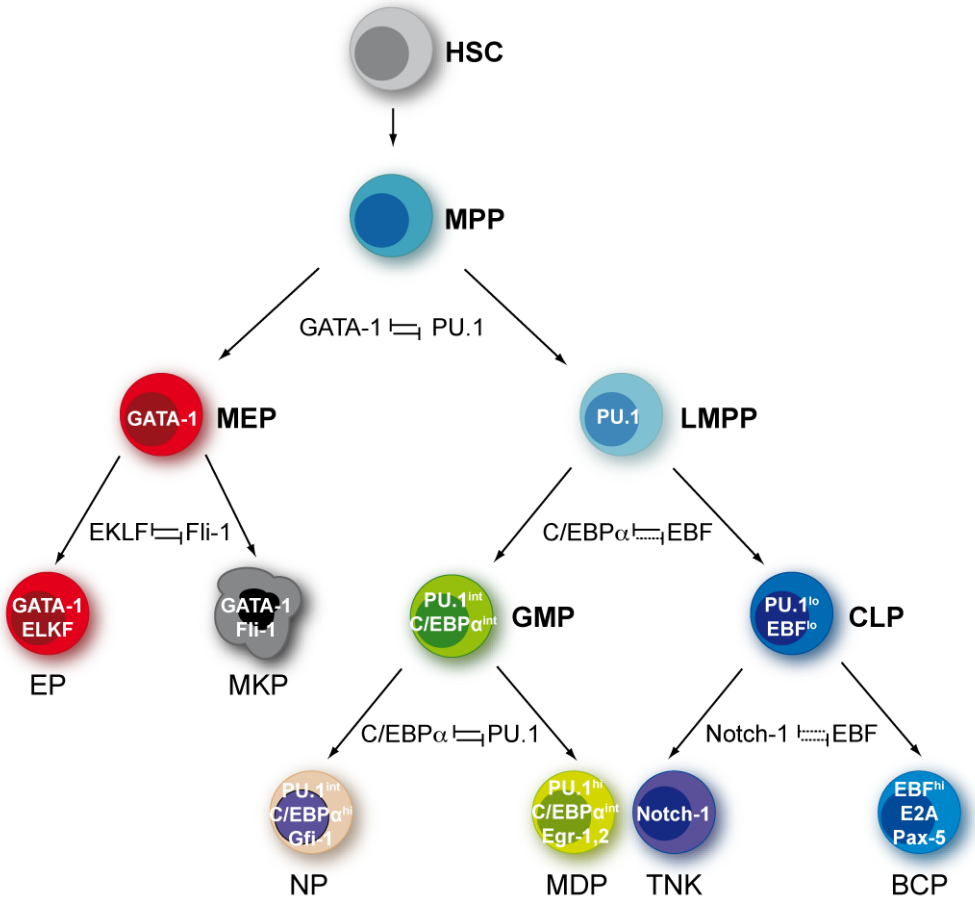


Figure 1.4 Intrinsic regulation of hematopoiesis.

The scheme displays a simplified overview of gene regulatory networks, which have a major influence on hematopoietic lineage choice during hematopoiesis. Supposed (dashed lines) and proved (continuous lines) cross-antagonisms between key transcription factors which function to regulate binary cell fate choices are noted in the scheme. Additionally, transcription factors that are important for the generation of particular intermediates are noted in white. HSC, hematopoietic stem cell; MPP, multi-potent progenitor; LMPP, lymphoid myeloid-primed progenitor; MEP, megakaryocyte-erythrocyte progenitor; EP, erythroid progenitor; MKP, megakaryocyte progenitor; GMP, granulocyte-macrophage progenitor; CLP, common lymphoid progenitor; NP, eosinophil progenitor; MDP, monocyte-dendritic cell progenitor; TNK, T cell NK cell progenitor; BCP, B cell progenitor. Figure adapted from Laslo et al. [73].

Analyses of systemic PU.1-deficient mice revealed a complete loss of CMP, GMP and CLP populations but normal numbers of MEP causing impaired lymphoid and myeloid cell development as well as retained megakaryocyte/erythrocyte development [74]. In contrast, GATA-1-deficient mice die between embryonic day 10.5 and 11.5 due to severe anemia resulting from a maturation arrest of erythroid

cells [75]. Further support for the lineage instructive role of GATA-1 originated from forced expression of GATA-1 in lineage-committed progenitors like GMP and CLP exclusively leading to megakaryocyte/erythrocyte development [76]. Several other studies dealing with certain aspects of the molecular interaction of PU.1 and GATA-1 as well as their gene regulatory capacity revealed the cross-antagonism between these proteins involving direct physical interaction of both factors that results in an inhibition of the transactivation potential of the counterpart [73]. Based on these findings, GATA-1 is prospected as the erythroid/megakaryocyte lineage determinant, whereas PU.1 is regarded as the myeloid/lymphoid lineage determinant.

Downstream of LMPP or GMLP, lineage choice embraces myeloid, as well as B or T lymphoid lineage and mainly depends on the transcription factors PU.1, early B cell factor (EBF) and Notch. For myeloid lineage restriction, a high expression level of PU.1 is necessary, whereas low levels of PU.1 plus EBF expression establish the B lymphoid lineage restriction and Notch instructs the T lymphoid lineage choice. The B cell fate determinant EBF has been demonstrated to initiate the early B cell lineage program of gene expression (*B29, VpreB* and *Pax5*) and to antagonize expression of myeloid lineage genes (*Cebpa, Sfpi1* and *Id2*) [77]. Additionally, the same regulatory properties have been shown for the T cell fate determinant Notch that inhibits B cell development via repression of EBF function as well as Pax5 expression, a secondary B cell lineage determinant [73].

Regarding granulocyte and monocyte development, besides PU.1, the transcription factor C/EBPα has to be enumerated. Studies have demonstrated that conditional deletion of C/EBPα in bone marrow cells of mice using the Mx1-Cre system lead to a total lack of mature granulocytes and a partial lack of monocytes due to a differentiation block at the transition from CMP to GMP [68]. Moreover, lineage choice between monocytes and granulocytes depends on the expression level of PU.1 and C/EBPα, which has been shown by studies using different mouse as well as *in vitro* models for diminished PU.1 expression in the hematopoietic system. In all experimental setups, reduced expression of PU.1 is followed by an augmented granulopoiesis to the disadvantage of monopoiesis. Additionally, gene expression analyses of PU.1-deficient progenitors have demonstrated a decreased or even absent expression of several monocyte-specific genes, like the macrophage scavenger receptor or the M-CSF receptor.

Furthermore, the need for C/EBPα during the transition from CMP to GMP is possibly due to the transcriptional upregulation of PU.1, since forced C/EBPα expression in hematopoietic progenitors favors monopoiesis and not granulopoiesis, whereas exogenous C/EBPα in myeloid cell lines directs granulopoiesis [78]. Nevertheless, C/EBPα is probably indispensable for granulocyte development due to the transcriptional upregulation of several granulocyte-specific factors. One of these factors is the transcription factor growth factor independent-1 (Gfi-1), which is necessary for the repression of proliferation as well as monocyte lineage-promoting factors such as M-CSF, miR21 or miR196b [79]. Another important target of C/EBPα is the transcription factor C/EBPε that is important for terminal granulocyte differentiation, because of the transcriptional control of granule-specific genes (lactoferrin and gelatinase) as well as cell cycle regulation [79]. Besides the upregulation of other transcription factors, C/EBPα force granulocyte development additionally by transactivation of various genes, such as G-CSF receptor [80,81] or myeloperoxidase (MPO) [82] as well as miR223 [83], and downregulation of proliferation by direct interaction with the cell cycle regulator E2F [84,85]. In line with these experimental results is the association of inactivating C/EBPα mutations with hematopoietic malignancies like acute myeloid leukemia and high-risk myelodysplastic syndrome proposing that C/EBPα possesses the ability to arrest cell proliferation and to drive terminal differentiation [86].

Taken together the plethora of studies implicates the following model for monocyte versus granulocyte lineage choice. First of all, C/EBPα is needed for the transition from CMP to GMP by induction of PU.1 expression. High protein levels of PU.1 induce monopoiesis via interaction with other transcription factors like interferon regulatory factor 8 (IRF8) or activating protein-1 family transcription factors (AP-1/Jun proteins) and the transcriptional activation of monocyte-specific genes [78]. However, AP-1 family transcription factors are also able to heterodimerize with C/EBPα [87] implicating an inhibition mechanism of PU.1 for granulocyte development by sequestering the binding partners of C/EBPα. In contrast to the high protein levels of PU.1 that favor monopoiesis, insufficient activation of PU.1 transcription allows C/EBPα to induce the granulopoiesis program accompanied by suppression of monopoiesis. In summary, these data propose an indispensable function of lineage-determining transcription factors for hematopoietic differentiation due to their function as genetic switches. But it is still

a matter of dispute to which extend extrinsic factors particularly cytokines influence the outcome of hematopoiesis by regulating the expression of lineage-determining transcription factors.

1.2.3.3 Extrinsic Regulation of Lineage Commitment

Cytokines are a large family of extracellular ligands that stimulate several responses after binding to structurally and functionally conserved cytokine receptors. Biological responses provoked by cytokines cover a broad spectrum of different biological activities, for example survival, proliferation, differentiation, or maturation. In the case of the hematopoietic system, the most important cytokines are interleukins and colony-stimulating factors with supportive functions for several lineages as well as erythropoietin (EPO) and thrombopoietin (TPO) that act on single lineages [88] (Figure 1.5).

Besides the requirement of cytokines for regulation of basal hematopoiesis, they are also essential for controlling emergency hematopoiesis in response to infections or blood loss. This is reflected by the different origins of cytokines, secreted for example by activated immune cells or by stroma cells as well as by organs, like liver and kidney. Further support for the plasticity of hematopoiesis with regard to emergency situations delivered a recent study, which has demonstrated that activation of Toll-like receptors (TLR) on hematopoietic progenitor cells induces their differentiation as well as proliferation and shapes the lineage commitment of hematopoietic cells. TLR are activated in response to pathogen-associated molecular patterns, for example lipopolysaccharide, double-stranded RNA or flagellin, and are crucial mediators of innate immunity [89].

In steady-state conditions, serum concentrations of cytokines are low, but they can be elevated up to 1000-fold by challenging the immune system and possess high picomolar affinities for their corresponding receptors [88]. On the binding of cytokine molecules follows the activation of the receptor via homodimerization (G-CSFR), oligomerization with a common signaling subunit (GM-CSFR, IL-6R) or conformational changes in preformed receptor dimers (EPOR), which finally leads to activation of Janus kinases (JAK). Upon activation of the tyrosine kinases of JAK family, the cytokine receptors as well as the kinases themselves are phosphorylated to generate docking sites for SH2 domain containing proteins. One example are proteins of the STAT family that promote transcriptional activation of target genes after phosphorylation by JAK [90,91]. Additionally, other signaling

molecules can be recruited to the cytokine receptors, such as Src kinases, protein phosphatases or PI3K, which mediate the activation of numerous signaling pathways like MAPK-ERK, Ras or PI3K [92].

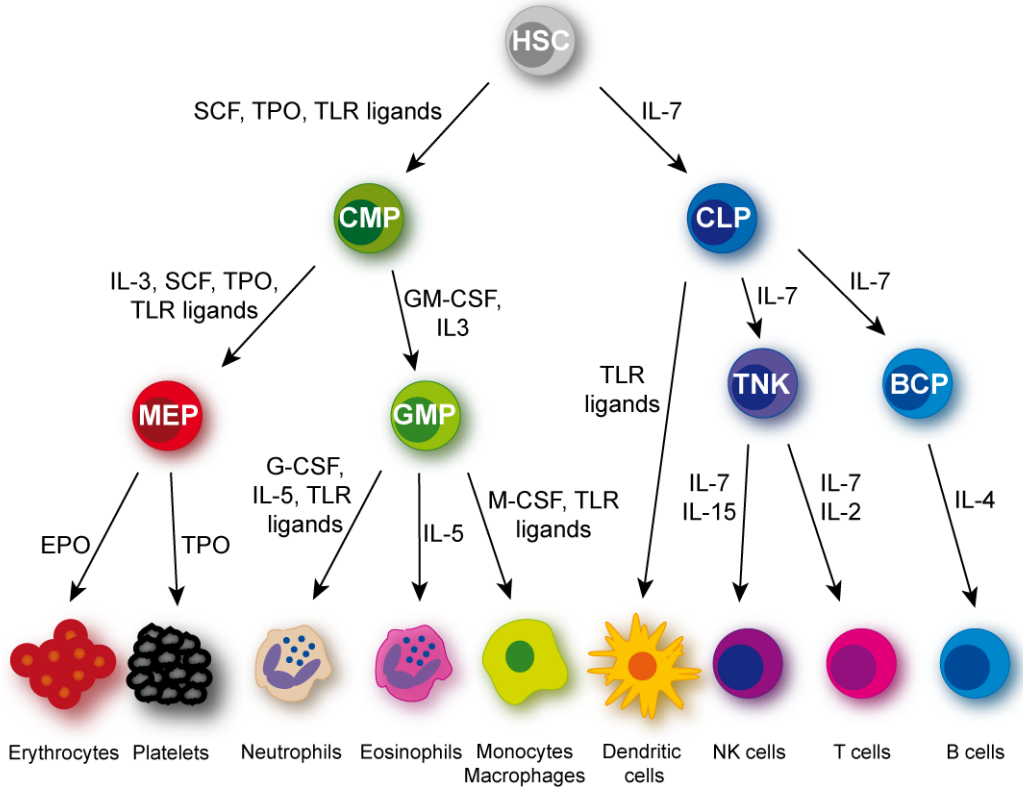


Figure 1.5 The role of cytokines in hematopoiesis.
Cytokines act on both multipotent progenitors as well as committed progenitors and provide survival and proliferation signals. The scheme supplies a simplified overview of proved cytokine activities to illustrate potential functions of different cytokines without claiming to be complete. HSC, hematopoietic stem cell; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte-erythrocyte progenitor; GMP, granulocyte-macrophage progenitor; TNK, T cell NK cell progenitor; BCP, B cell progenitor. Figure adapted from Robb [91], as well as McGettrick and O'Neill [93].

To date, basically two hypotheses exist concerning the role of cytokines in hematopoiesis. The instructive model, proposes that cytokines transmit specific signals to multipotent progenitors to direct their lineage commitment. In contrast, the permissive or stochastic model suggests that cytokines only provide permissive growth and survival signals to intrinsically determined and lineage-committed progenitors. Supportive data for the permissive as well as the

instructive model of cytokine function originated from different studies, where cytokine receptors were ectopically expressed in lineage-committed progenitors.

Studies in favor of the permissive model have demonstrated that viral transduction of fetal liver cells with the M-CSF receptor results in the generation of erythroid colonies upon M-CSF administration [94]. Similar results have been obtained by the restoration of definitive erythropoiesis in EPOR-deficient fetal liver cells via the expression of the human GM-CSFR plus GM-CSF treatment [95]. Furthermore, replacement of the intracellular domain of the G-CSFR with the intracellular domain of EPOR induces no alterations in lineage commitment [96].

Oppositional results emanated from the ectopic expression of IL-2Rβ in CLP, which results in rapid generation of granulocytes and macrophages in the presence of IL-2 [97]. Additionally, ectopic expression of the human GM-CSFR in IL-7-deficient CLP was not able to restore lymphopoiesis upon GM-CSF administration [98]. Experiments with single GMP cultured in the presence of M-CSF or G-CSF have further supported the hypothesis of lineage instruction by cytokines due to the almost solely development of either macrophages or granulocytes, respectively [99].

Nevertheless, gene deletion studies for several cytokine receptors have shown the indispensable function of the most cytokines for hematopoiesis. For example, the knockout of EPO and EPOR in mice leads to embryonic death at E13.5 due to severe anemia, even if erythroid progenitor cells were present [100,101]. Analysis of IL-7Rα-deficient mice revealed a lethal phenotype as a result of a severe hypoplasia of all lymphoid lineages, but retained development of the earliest unipotent T and B cell precursors [102]. In contrast, mice bearing deletions of colony-stimulating factor receptors demonstrated no lethal phenotypes. Disruption of the G-CSFR in mice results in ineffective granulopoiesis, with chronic neutropenia due to a decrease of mature myeloid cells in the bone marrow and a modest reduction of progenitor cells [103], whereas deletion of the common β-chain of IL-3, IL-5 and GM-CSF receptor in mice only lead to reduced numbers of eosinophils [104,105].

Taken together, the present available data do not resolve the question if cytokines only have permissive functions in the guidance of hematopoiesis, especially with regard to differentiation from HSC to restricted progenitors or not. But the plasticity of the transition from multipotent progenitor cells to restricted progenitor cells, especially regarding adaption of hematopoiesis in emergency situations suggests,

that cytokines have to some extend instructive functions. Furthermore, almost nothing is known about potential functions of cytokines and cytokine signaling regarding gene expression or posttranslational regulation of lineage-determining transcription factors, which would provide a possible link between intrinsic and extrinsic regulation of hematopoiesis.

1.3 Neutrophils

Neutrophils together with basophils and eosinophils form the class of polymorph-nucleated cells (PMN) based on the characteristic multilobulated shape of the nucleus, and are the most abundant type of immune cells. A further name for this class of cells is “granulocytes”, derived from the excessive enrichment of different types of storage vesicles in the cytoplasm, which are named granules and filled with proteolytic and bactericidal proteins. Neutrophils together with macrophages display the first line of defense against invading microorganisms, accomplished by patrolling with the blood stream until activation in response to infections. After the conquest of the physical barriers, provided by skin and mucus membranes and infiltration of tissue by pathogens, neutrophils become activated by signals that are provoked by resident macrophages at the site of infection. The activated macrophages release cytokines that in turn activate locally endothelial cells and enable the capture of bypassing neutrophils to guide them into the infected tissue. Following the arrival at the site of infection, neutrophils combat microorganism via phagocytosis and degranulation as well as neutrophil extracellular trap formation. Besides the function of granules as storage bags for anti-microbial proteins that are kept in store until liberation to the outside or into the phagolyosome, they are also important reservoirs for membrane proteins. Particular, surface receptors are stored that were incorporated into the surface membrane upon degranulation. Furthermore, neutrophils are able to regulate the immune response via recruitment and activation of additional neutrophils, macrophages and T cells. A tremendous amount of neutrophils is generated daily in the bone marrow to allow a continuous replacement of the short-lived population of neutrophils circulating in the blood.

1.3.1 Differentiation of Neutrophils

The development and differentiation of neutrophils from multipotent progenitor to mature neutrophils entirely occurs in the bone marrow, where approximately

two-third of the developmental capacity is devoted to granulopoiesis. The process of neutrophil development can be subdivided into early granulopoiesis embracing differentiation from a multipotent progenitor to a lineage-committed progenitor and terminal granulopoiesis describing the production of mature granulocytes downstream of the committed progenitor (Figure 1.6). During maturation, different subsets of granules are formed sequentially and segmentation of the nucleus increases, allowing the definition of different developmental stages based on morphological changes.

Terminal granulopoiesis starts with the myeloblast and promyelocyte state, where the switch from proliferation to differentiation takes place, displayed by the loss of ability for cell division after the promyelocyte state. Moreover, the formation of the first granules starts, which are named primary or azurophilic granules. Primary granules are defined by a high content of myeloperoxidase (MPO), bactericidal permeability-increasing protein, defensins, and a family of structurally related serine proteases, namely cathepsin G, neutrophil elastase (ELANE) and proteinase 3 [106]. The most important transcription factors at myeloblast/promyelocyte stage are C/EBPα and Gfi-1, which are necessary for the suppression of monocyte development and proliferation as well as for the transcriptional activation of granulocyte-specific genes like *MPO*, *ELANE* or *CEBPE* [79,85,86]. The importance of Gfi-1 and ELANE has been demonstrated by studies analyzing the genetic background of severe congenital neutropenia (SCN) and other forms of neutropenia. These studies revealed that one of the major causes for loss of neutrophil differentiation beyond promyelocyte state are mutations in the *ELANE* gene [107,108], but in rare cases of SCN also mutations of the *GFI-1* gene have been described [109]. Detailed analyses of Gfi-1 in mice further supported the function of Gfi-1 as molecular switch towards granulocyte development by suppression of monocyte-specific genes, like *Csf1* (M-CSF) and *Csf1r* (M-CSFR) [110].

Ongoing differentiation beyond promyelocytes leads to the development of myelocytes and metamyelocytes, which are defined by the beginning of nuclear segmentation and the appearance of secondary (also called specific) granules as well as the exit from cell cycle. Secondary granules are characterized by high amounts of lactoferrin, cathelicidin, collagenase (MMP8) as well as leukolysin and lysozyme in the matrix [106,111]. The regulation of secondary granule protein

expression and the exit from cell cycle mainly depends on the transcription factor C/EBPε, whose expression peaks in myelocytes and metamyelocytes [85,112].

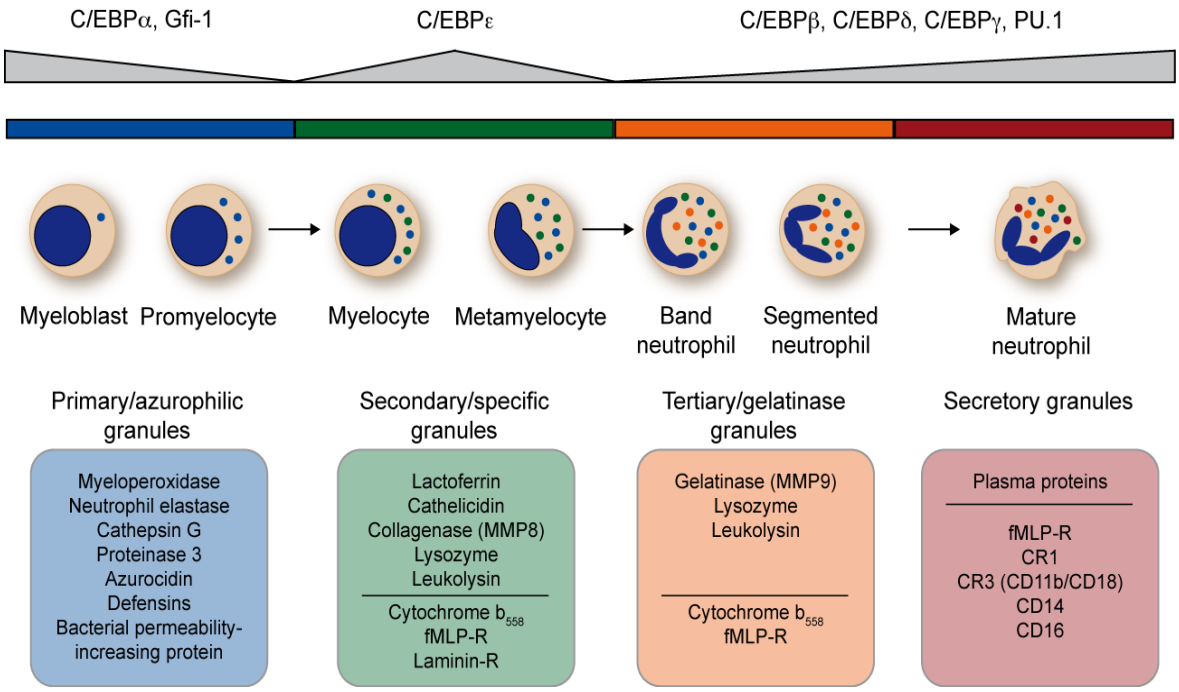


Figure 1.6 Terminal granulopoiesis in the bone marrow.

The terminal granulopoiesis that is characterized by sequential formation of different granule types and segmentation of the nucleus starts at the myeloblast/promyelocyte stage and ends with mature neutrophils. Granule types not only differ in the time point at which they are formed, but also in their specific content, which is described at the bottom of the figure. Above the line in the boxes matrix content is depicted and beneath the proteins that are located to the vesicle membrane. At different stages of terminal granulopoiesis several transcription factors, which are indicated on top of the figure, are important for the regulation of maturation and timed expression of granule proteins. Figure adapted from Borregaard [79], Pham [106], as well as Borregaard and Cowland [111].

Based on studies using C/EBPε-deficient mice, which displayed neutrophil-specific defects including bilobed nuclei, abnormal respiratory burst and compromised bactericidal activity as well as impaired chemotaxis [113,114], the genetic cause of a very rare congenital disorder named neutrophil specific granule deficiency (SGD) has been delineated to the *CEBPE* locus [115]. Additional studies have revealed the essential functions of C/EBPε for the expression of secondary and tertiary granule proteins [113,116] and demonstrated the direct interaction of C/EBPε with E2F1 and Rb protein, finally leading to cell cycle exit [117].

The last step of terminal granulopoiesis, the differentiation into band and segmented neutrophils leads to mature neutrophils with finally segmented nuclei and tertiary as well as secretory granules. Tertiary granules are mainly defined by a high content of gelatinase (MMP9) as well as leukolysin and lysozyme, whereas secretory granules only contain plasma proteins in their matrix. The most important feature of secretory vesicle is their membrane that is highly enriched for receptors such as Mac-1/CD11b (integrin αM), CD14, FcγRII (CD16) or formyl-peptide receptor (fMLP-R) [111]. In the course of neutrophil terminal differentiation, C/EBPα expression gradually diminishes during the myeloblast stage. C/EBPε peaks at the myelocyte/metamyelocyte stage, whereas the expression level of the transcription factors PU.1, C/EBPβ, C/EBPδ and C/EBPγ increases subsequent to the metamyelocyte stage [112]. However, gene deletion studies using C/EBPβ- or C/EBPδ-deficient mice revealed no hematopoietic abnormalities with regard to terminal granulopoiesis. Still, Hirai and colleagues have demonstrated the indispensable role of C/EBPβ during emergency granulopoiesis in response to cytokine treatment or fungal infection in contrast to C/EBPα and C/EBPε, which were not upregulated under these conditions [118]. In the case of the transcription factor PU.1, a conditional gene deletion model has evidenced a PU.1-dependent transcriptional activation of $gp91^{phox}$, a component of the NADPH oxidase, as well as of Mac-1/CD11b [119].

After finishing maturation, neutrophils are retained in the bone marrow to be ready for immediate release in response to infections. Retention is achieved by activation of the chemokine receptor CXCR4 [120] that is expressed on mature neutrophils and binds to the ligand CXCL12/SDF-1 present on the surface of bone marrow endothelial cells [121]. Attenuation of CXCR4 signaling leads to the entry of neutrophils into the circulation, which occurs partially due to the downregulation of CXCL12 expression on endothelial cells in response to G-CSF and antagonistic functions of the chemokine receptor CXCR2 [122] on neutrophils like heterologous desensitization [123] and receptor internalization of CXCR4 [124].

1.3.2 Immune Functions of Neutrophils

For appreciation of the important functions of neutrophils concerning protection of the host against invading pathogens and the unique characteristics of neutrophils

facilitating this immune defense it is necessary to shed light on the single steps enabling neutrophils to combat against microorganisms.

1.3.2.1 The Leukocyte Adhesion Cascade

The first step required for neutrophil activation is the guidance of neutrophils from blood circulation to the site of infection in the tissue, which is named the leukocyte adhesion cascade. This multi-step process describes the sequential and partially overlapping stages mediating neutrophil migration from vascular lumen to extravascular tissue [125]. Leukocyte adhesion cascade starts with the initial attachment of neutrophils to endothelial cells at postcapillary venules, a process also named rolling, which is elicited by the expression of P-selectin and E-selectin on the luminal surface of endothelial cells [125]. Surface expression of selectins on endothelial cells is mediated by activating stimuli such as TNFα, IL-1β and IL-17 that are generated during infection or inflammation for example by resident tissue macrophages. Both selectins bind to the P-selectin ligand-1 (PSGL-1), which is constitutively expressed on the surface of neutrophils, whereas only E-selectin binds to the E-selectin ligand-1 (ESL-1) and CD44 [126] also expressed on the surface of neutrophils [79]. Interaction of selectins with their cognate ligands enables neutrophils to adhere to the inflamed endothelium. Interestingly, the adherence process requires the shear stress occurring in the venules during blood flow to support adhesion by strengthening the bond between selectin and ligand. After capturing of neutrophils by endothelial cells, selectin signaling pathways are induced by activation of the Src family kinases Hck, Fgr and Lyn [126] that in turn phosphorylate and activate immunoreceptor tyrosine-based activation motifs (ITAM) in the two adaptor proteins DAP12 (NDAX activation protein of 12 kDa) and FcRγ (γ chain of Fc-receptors) [127]. Phosphorylation of ITAM leads to activation of Syk mediating activation of PLCγ, PI3K as well as p38, finally inducing integrin activation and cytoskeletal rearrangement in neutrophils [125,128].

The transition between primarily selectin-mediated rolling to integrin-mediated firm arrest is named slow rolling and involves the activation of the β_2-integrins LFA-1 ($\alpha_L\beta_2$, lymphocyte function-associated antigen-1) and Mac-1 ($\alpha_M\beta_2$, CD11b/CD18), constitutively expressed on neutrophils. Both integrins are engaged by the binding of their cognate ligands ICAM-1 (intercellular adhesion molecule-1) and ICAM-2 that are present on endothelial cells after stimulation by TNFα, IL-1β and IL-17.

Rolling on E-selectin or P-selectin and activation of PSGL-1 and CD44 signaling induces the extended, but not the high affinity form of LFA-1, which is able to mediate rolling, but not to firm arrest to ICAM-1. Prerequisite for full LFA-1 activation is the engagement of cytokine receptors on neutrophils by ligands such as chemokines or chemoattractants, secreted by various cell types including activated endothelial cells, leukocytes and platelets. Chemokines and chemoattractants bind with high affinity to specific G protein-coupled receptors (GPCR) and drive the activation of trimeric G proteins finally leading to the activation of several signaling events. The most important signaling molecule being activated after chemokine binding is PI3K that in turn mediates the activation of small GTPases, the MEK/ERK cascade as well as PLC. Particularly, PLC activation is accompanied with production of the second messengers IP_3 and DAG as well as calcium release from the endoplasmatic reticulum. The signals indispensable for full activation of integrin signaling, referred to as inside-out signaling, occur immediately after chemokine receptor engagement and are not fully understood [129,130]. Nevertheless, studies with $G\alpha_{i2}$-deficient neutrophils have demonstrated a contribution of trimeric G protein signaling to firm arrest of neutrophils in response to chemokine binding [131].

Simultaneously with rolling and arrest, neutrophils start to discharge their secretory vesicles. Probably, this process is stimulated by chemoattractants like formyl peptides (fMLP), leucotriene B4 (LTB4) or complement factor C5a [132] and causes the incorporation of different integrin and chemokine receptors into the plasma membrane and further augments signaling strength. The proclivity for exocytosis of the different granules is regulated by the density of vesicle-associated membrane protein 2 (VAMP-2). The concentration of VAMP-2 is highest in secretory vesicles and decreases from tertiary via secondary granules and is lowest in primary granules. Upon increase in intracellular calcium the v-SNARE (N-ethylmaleimide-sensitive factor attachment protein receptors) family member VAMP-2 is activated and provokes the exocytosis of granules [133,134].

After firm arrest, adhesion strengthening and spreading takes place, which are elicited by the full activation of β_2-integrin signaling named outside-in signaling, again leading to the activation of the Src family kinases Hck and Fgr [135]. Further important factors for adhesion are probably the GEFs Vav1 and Vav3 [136,137], WASP [138] as well as the PI3Kγ [139], because studies with neutrophils deficient for

these factors have demonstrated an accelerated detachment of adherent neutrophils under flow conditions. Additionally, concomitant with the induction of adhesion neutrophils undergo polarization into a leading edge lamellipodium, where receptors for chemokines and phagocytosis are concentrated. Polarization mainly depends on the actin-reorganization, which is regulated by signals from the PI3Kγ via PIP_3 and by the Rho GTPases Rac, RhoA and Cdc42 [79,140,141].

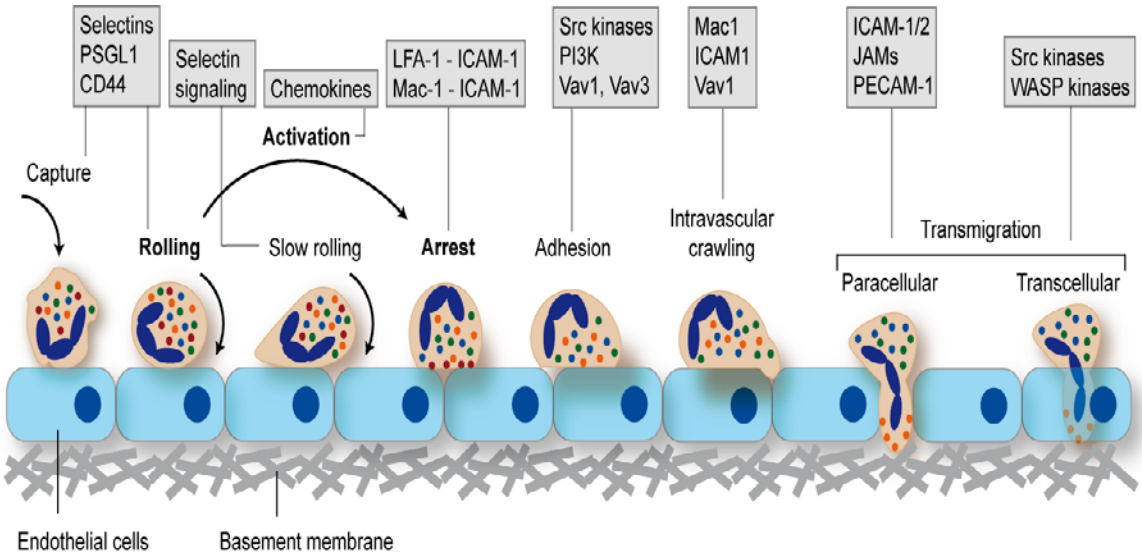


Figure 1.7 The leukocyte adhesion cascade.

The classical steps of the leukocyte adhesion cascade are highlighted in bold and include rolling, activation and arrest. Additional research led to the definition of more transition stages such as slow rolling, adhesion, intravascular crawling and transmigration. Key molecules involved in each step are indicated in the boxes. ESAM, endothelial cell-selective adhesion molecule; ICAM11, intercellular adhesion molecule 1; JAM, junctional adhesion molecule; LFA-1, lymphocyte function-associated antigen 1; Mac-1, macrophage antigen 1; PSGL1, P-selectin glycoprotein ligand 1; PECAM1, platelet/endothelial-cell adhesion molecule 1; PI3K, phosphoinositide-3-kinase; WASP, Wiskott-Aldrich syndrome protein. Figure adapted from Ley et al.[125], Borregaard[79], Woodfin et al.[128].

The final step of leukocyte adhesion cascade is the transendothelial migration occurring along the paracellular road, whereby neutrophils squeeze between two endothelial cells, or along the transcellular road, whereby neutrophils penetrate one individual endothelial cell. Before neutrophils can migrate via the paracellular road, they have to seek for preferred sites of transmigration by intravascular

crawling, a process primarily determined by the interaction of Mac-1 with ICAM-1 and Vav1 activation [137,142]. Inhibition of intravascular crawling is accompanied by a delayed transmigration preferentially the transcellular road at the expanse of paracellular transmigration [142]. The interaction of neutrophil integrins (LFA-1, Mac-1) with their ligand ICAM-1 activates signaling from the cytoplasmic tail of ICAM-1 in endothelial cells, which elicits the activation of the GTPases RhoG and RhoA and the formation of small membrane protrusions called docking structures or transmigratory cups [143-145]. These structures are endothelial-cell projections rich in ICAM-1 and VCAM-1, as well as cytoplasmic molecules such as ERM (ezrin, radixin and moesin) proteins and cytoskeletal components (vinculin, α-actin, and talin-1)[125] and are necessary for both roads of transmigration. Currently, only the molecular mechanisms mediating paracellular transmigration of neutrophils are known in great detail and the following sequence is postulated. The integrin ligands ICAM-1 and ICAM-2 accumulate at the cell-cell junctions and guide neutrophils to these structures via their β_2-integrin partners LFA-1 and Mac-1 [146]. Endothelial cell-intrinsic signaling downstream of ICAM-1 activates Src and Pyk-2 tyrosine kinase followed by the phosphorylation of VE-cadherin and destabilization of the VE-cadherin bonds, which finally yield in the ease of endothelial cell-cell junctions [145,147,148]. A further key player in loosening of endothelial cell junctions may be ESAM (endothelial cell-selective adhesion molecule), because the lack of ESAM results in decreased Rho signaling activity and reduced neutrophil transmigration. Ongoing penetration between the cells is elicited by ICAM-2, JAM-A (junctional adhesion molecule A) and PECAM-1 (platelet endothelial cell adhesion molecule-1), which was demonstrated by the use of neutrophils deficient for these adhesion molecules. These neutrophils have evidenced the arrest of paracellular transmigration between endothelial cells as well as between endothelial cells and the basement membrane, respectively [149,150]. Simultaneously with transmigration occurs the liberation of tertiary and to some extend secondary granules that allows the break through the basement membrane as not only matrix metalloproteinases are released, but also the surface expression of the laminin receptor is increased [151]. Furthermore, analysis of unstimulated mouse cremasteric venules identified regions of low expression of matrix proteins within the endothelial basement membrane, which appear to be permissive to emigrating neutrophils [125,152].

1.3.2.2 Tissue Neutrophils

After arrival of neutrophils in the interstitial tissue, cells have to move to the site of inflammation by chemotactic migration along a gradient of chemoattractants. Neutrophils are capable of detecting a plethora of chemoattractants, which are produced by bacteria, dying cells, various stromal or epithelial cells as well as immune cells and are immobilized via attachment to extracellular matrix components because of their negative charge. Proper navigation and migration through such a complex chemoattractant field that is generated by the different stimuli released at the site of inflammation assumes polarization of the neutrophil. Moreover, a multistep signaling process is necessary to respond to one agonist source after the other. This is regulated via the crosstalk between the chemoattractant receptors and their signaling pathways. Indeed, stimuli derived from targets or their close proximity such as formyl peptides released by bacteria, mitochondria of dying cells or complement factor C5a dominate the signals derived from regulatory cells like bioactive peptides (LTB4) as well as chemokines (IL-8) [153-155]. The main receptors mediating migration are β_2-integrins in concert with β_1-integrins (laminin receptor $\alpha_6\beta_1$, fibronectin receptor $\alpha_5\beta_1$) as well as β_3-integrins (vitronectin receptor $\alpha_V\beta_3$). Upon chemokine stimulation and transendothelial migration, integrins are liberated from the granules of neutrophils, where they are mostly stored [156-158]. Similar to the earlier described signaling cascade, integrin activation results in tyrosine kinase as well as small GTPase activation and it finally induces actin reorganization, which is essential for the crawling of neutrophils along the matrix fibers.

Once the neutrophil has arrived at the site of inflammation, the clearance program starts. It is facilitated by the simultaneous activation of microbial phagocytosis, respiratory burst and degranulation. Appropriate destruction and clearance of microorganisms by neutrophils can only be achieved through concerted activation of all three mechanisms that leads to phagocytosis of pathogens into the phagolysosome, where they can be destroyed by reactive oxygen species and antimicrobial peptides. The crucial activation is achieved by the integration of several signals like chemokines activating their cognate receptors, which are essential for respiratory burst and degranulation [155], as well as phagocytosis related receptors such as complement receptors (CR1 and CR3 for complement factors) or Fcγ-receptors (FcγRIIA and FcγRIIIB for immune complexes) [159].

Phagocytosis is induced by Syk, Vav, Rac, Cdc42 and WASP activation as well as PIP_2 and PIP_3 formation, which result in actin reorganization. Finally, this cascade leads to the enclosure of a microorganism in the phagolysosome, a place endowed with a sophisticated armamentarium to eliminate and degrade pathogens. One major part in the defense plays the NADPH oxidase, a protein complex consisting of a transmembrane heterodimer ($gp91^{phox}$ and $p22^{phox}$, cytochrome b_{558}) stored in the granule membranes, which assembles with three cytosolic subunits ($p40^{phox}$, $p47^{phox}$, $p67^{phox}$) at the phagolysosomal membrane after activation [160]. The active oxidase produces superoxide anions by transferring electrons from cytosolic NADPH to molecular oxygen and liberates them into the phagolysosomal lumen. Within the phagolysosome, superoxide anions are the precursors for several reactive oxygen species (ROS) such as hydroxyl radicals, singlet oxygen or hydrogen peroxide that can also be converted by myeloperoxidase into hypochlorous acid and chloramines. The importance of ROS in pathogen elimination is highlighted by chronic granulomatous disease (CGD), which is caused by inactivating mutations in the subunits of the NADPH oxidase complex and is characterized by severe recurrent infections that can result in death [159]. But not only ROS production is essential for the clearance of pathogens. Also, the antimicrobial proteins and peptides that are stored in the granules and released into the phagolysosome are necessary inventories of the pathogen destruction cascade. These proteins can be grossly subdivided into two categories: the first one hampers microbial growth and the second compromises the integrity of the microorganism. Growth prevention is accomplished by limiting the availability of essential nutrients for example by secretion of scavengers like lactoferrin, which sequesters iron. Breakdown of microbial integrity in turn involves defensins, cathelicidins, lysozyme and proteases that are also stored to a great extend in the neutrophil granules [159]. Defensins bind to negatively charged molecules on the microbial surface and subsequently induce membrane permeabilization of Gram-positive and Gram-negative bacteria by forming multimeric ion-permeable channels [161]. The precursors of cathelicidins are processed by neutrophil elastase and act through permeabilizing the cell wall and the membranes of bacteria [162]. Furthermore, phagolysosomes are also equipped with an assortment of endopeptidases, exopeptidases and hydrolases that degrade various microbial components [159]. Recently, neutrophil extracellular traps

(NETs) have been described as additional weapon for pathogen clearance, which are released by neutrophils. These NETs consist of decondensed chromatin covered by antimicrobial granule proteins and are able to kill a variety of microbes including bacteria, fungi and parasites [163].

Besides the destruction of intruders, neutrophils are also a key component of the inflammatory response. They recruit and activate macrophages and dendritic cells as well as B and T cells. For example, the granule proteins like cathepsin G and azurocidin are released from neutrophils and induce chemotaxis of monocytes and DCs. Furthermore, neutrophils are able to produce cytokines such as TNFα that drive DC and macrophage maturation and activation. They also secrete B-lymphocyte stimulator (BLyS) or interferon-γ, which help to drive proliferation and maturation of B cells and differentiation of T cells, respectively [164]. After the clearance mission has been completed, neutrophils have to undergo apoptosis and to be resolved by attracted macrophages, since the extended neutrophil activation within tissue is related to unwanted damage to host and inflammatory diseases such as acute respiratory distress syndrome, sepsis or acute coronary artery disease. One main activator of neutrophil apoptosis is ROS generation, which causes the loss of mitochondrial transmembrane potential, the release of cytochrome c as well as apoptosis-inducing factor and subsequently the activation of caspases [165].

Nevertheless, the outstanding functions of neutrophils as a first line of defense against invading pathogens and their important immunomodulatory functions are illustrated by several genetic disorders such as SCN, SGD or CGD that affect neutrophil function and lead to life-threatening recurrent infections. Neutrophils are not only indispensable for the clearance of tissue from invading pathogens, they also have pro-inflammatory functions leading to the activation of the innate as well as the adaptive immune system, but also an anti-inflammatory role after mission is accomplished. This plethora of functions makes neutrophils to an essential component of the immune system of a healthy organism.

1.4 Aims of the Study

Today, the need for Bruton tyrosine kinase in B cell development and function is well described. However, the function of Btk in hematopoietic stem cells as well as in myeloid cells, where Btk is also expressed, is discussed controversial.
In order to evaluate the role of Btk for hematopoietic differentiation and myeloid cell function, particularly for neutrophil granulocyte development and function, the present work was designed to answer the following questions:

1.) Is there a function of Btk for myeloid cell development *in vitro* and *in vivo*?
2.) Particularly, is there a need of Btk expression for the development of neutrophil granulocytes out of myeloid precursor cells as well as for neutrophil function?
3.) What are the molecular mechanisms by which Btk might regulate myeloid differentiation and function?

2 Material and Methods

2.1 Mice

2.1.1 Mice Strains

For the described experiments two different kinds of Btk-deficient mouse strains were used, the CBA/CaHN-*Btk*^{xid}/J mice and the Btk-deficient mice (B6;129S-*Btk*^{tm1Wk}/J). The CBA/CaHN-*Btk*^{xid}/J is an inbreed CBA strain carrying the spontaneous point mutation R28C within the PH domain of *Btk* gene, which hampers PIP_3-dependent membrane recruitment and prohibit activation of Btk [8]. Whereas the Btk-deficient mouse is a genetically modified C57/Bl6 mouse, where Btk expression is abolished after gene targeting of the exons 13 and 14 of the *Btk* gene with a neomycin cassette. The deletion led to a disruption of the open reading frame as well as to the introduction of an additional stop codon and results in complete abrogation of Btk protein expression [166,167]. Both mice strains were purchased originally from The Jackson Laboratory. As controls for Btk-deficient mice wild type littermates were taken. For CBA/CaHN-*Btk*^{xid}/J mice sex- and age-matched CBA/J mice, also purchased from The Jackson Laboratory, were used. All animals were maintained in the animal facility under SFP conditions and used for experiments at the age of 8 to 18 weeks of age.

2.1.2 Isolation of Genomic DNA from Mouse Tail Tips

For isolation of genomic DNA from the tail tips of mice, first a proteinase K digestion was performed over night at 56 °C. Next, genomic DNA was enriched by the addition of 6 M NaCl. After centrifugation at 13 000 rpm for 5 min at room temperature the upper phase was transferred to a fresh tube. Precipitation was performed with isopropanol according to conventional protocols for DNA precipitation.

2.1.3 Genotyping

Btk-deficient mice were genotyped by PCR using primers specific for the wild type or knockout allele (Table 2.1). The primer pair Btk-oIMR0013 and Btk-oIMR0014 was used to amplify a 280 bp fragment of the *Btk*-knockout allele, whereas the

primer pair Btk-oIMR0367 and Btk-oIMR0368 amplified an 820 bp fragment of the wild type allele.

The point mutation of CBA/CaHN-*Btk*^{xid}/J mice were detected by PCR-RFLP, a method published by Takagi et al. [168]. Briefly, a second artificial *HypCH4 V* restriction site surrounding the *Btk*^{xid} mutation site was introduced by PCR amplification with primers presented in Table 2.1.

Primer	Sequence
Btk-oIMR0013	ctt ggg tgg aga ggc tat tc
Btk-oIMR0014	agg tga gat gac agg aga tc
Btk-oIMR0367	cag aga agg ttc cat gtc gg
Btk-oIMR0368	gcc att agc cat gta ctc gg
Btk-xid-genotyping forward	agt aca caa aca agt tcc aga gag
Btk-xid-genotyping reverse	tgt gta cag tca aga gaa aca tgc

Table 2.1 Primers for Genotyping of Mice

Afterwards, the different *Btk* alleles could be distinguished by restriction fragment length polymorphism due to the second restriction site for *HypCH4 V* in the *Btk*^{xid} allele. Digestion of the PCR product from *Btk*^{xid} allele with *HypCH4 V* produced one additional fragment (76 bp, 39 bp and 23 bp) in comparison to the digestion of the wild type amplicon (98 bp and 39 bp). For a sufficient separation of low molecular fragments TAE agarose gels containing 4 % MoSieve-Agarose were used.

2.2 Isolation of Hematopoietic Cells from Mice

2.2.1 Sorting of Hematopoietic Progenitor and Stem Cells

Bone marrow cells were obtained under sterile conditions from femur and tibia of mice by flushing the bones with 10 mL MACS buffer (1 x PBS, 2 mM EDTA, 0.5 % BSA) using a 1 mL SubQ syringe (BD Biosciences). After washing of cells by centrifugation at 1200 rpm for 5 min total cell numbers were determined. Lineage-negative cells were enriched using a mixture of rat-IgG antibodies against CD11b, CD19, Ter119 and Goat-anti-Rat IgG Dynabeads (Invitrogen). Enrichment was

performed according to manufacturer's instructions for Dynabeads. For fluorescence activated cell sorting of hematopoietic stem and progenitor cell populations, lineage-depleted bone marrow cells were blocked with 1 µg mouse IgG (Jackson ImmunoResearch) per 10^6 cells and stained with following PE-conjugated lineage markers: anti-CD3ε, anti-CD4, anti-CD8α, anti-CD45R/B220, anti-Gr-1, anti-CD19, anti-CD11b and anti-Ter119. The different stem and progenitor cell populations were subdivided using the following surface markers: PE-conjugated anti-IL-7Rα, PE-Cy7-conjugated anti-c-Kit, PE-Cy5.5-conjugated anti-Sca-1, APC-conjugated anti-FcγRII/III and FITC-conjugated anti-CD34. Cells were sorted on a FACSAria (BD Biosciences) using 1.4 mL U-tubes (Micronic) for low volume.

2.2.2 Purification of CD11b$^+$ Myeloid Cells

In some experiments, CD11b$^+$ myeloid cells were purified from the bone marrow of femur and tibia of mice using rat-IgG anti-CD11b antibody (BD Pharmingen) and anti-rat IgG MicroBeads (Miltenyi Biotec). Enrichment was performed according to manufacturer's instructions with LS columns (Miltenyi Biotec). Myeloid cell viability was > 95 % as assessed by trypan blue exclusion test and enrichment was > 60 % as analyzed by flow cytometry according to the expression of the following surface markers: CD11b for myeloid cells, Ter119 for erythroid cells and CD45R/B220 for lymphoid cells.

2.2.3 Preparation of Erythroid Cell-Depleted Bone Marrow Cells

The outcome of GM-CSF treatment on differentiating murine myeloid cells was analyzed in erythroid cells depleted bone marrow cells. For depletion of erythroid cells, bone marrow cells isolated from femora and tibiae using HBSS were loaded onto Histopaque 1119 (Sigma-Aldrich) and centrifuged at 1600 x g for 30 min without brake at room temperature. Bone marrow cells without erythroid cells were harvested from the interface and washed twice with HBSS by centrifugation at 1200 rpm for 5 min. Myeloid cell viability was > 95 % as assessed by trypan blue exclusion test and enrichment was > 60 % as analyzed by flow cytometry according to the expression of the following surface markers: CD11b for myeloid cells, Ter119 for erythroid cells and CD45R/B220 for lymphoid cells.

2.2.4 Isolation of Murine Bone Marrow PMN

Basically, polymorph nucleated cells (PMN) were purified from bone marrow according to the protocol published by Siemens et al. [169]. In detail, bone marrow cells isolated from femur and tibia of mice were resuspended in a volume of 3 mL mouse neutrophil buffer (1 x HBSS, 0.1 % BSA, 1 % glucose) and loaded on top of a freshly prepared discontinuous Percoll™ gradient (2 mL 50 %/2 mL 55 %/2 mL 62 %/3 mL 81 %) and centrifuged at 1600 x g for 30 min without break. The discontinuous Percoll™ gradient was layered from top to bottom using 5 mL syringes (Terumo) and 20 G x 2 ¾``needles (Terumo) into a 15 mL conical tube. Afterwards, PMN were harvested from the 62 %/81 % interface and washed with buffer by centrifugation at 1200 rpm for 5 min. Subsequently, PMN were resuspended again in a volume of 3 mL neutrophil buffer and loaded onto 3 mL Histopaque 1119 and centrifuged at 1600 x g for 30 min without break. Finally, PMN were harvested from the interface and washed again twice with neutrophil buffer by centrifugation at 1200 rpm for 5 min. Isolation and centrifugation were done at room temperature and freshly prepared mouse neutrophil buffer served as buffer for the whole isolation procedure. In addition, the 100 % Percoll™ stock solution was freshly prepared by mixing Percoll™ (GE Healthcare) and 10 x HBSS (without Mg^{2+}/Ca^{2+}) at a ratio of 9:1 (v/v) and the different Percoll™ solutions were obtained by mixing a appropriate volume of 1 x HBSS with 100 % Percoll™ stock solution. PMN viability was > 95 % as assessed by trypan blue exclusion test and enrichment was > 70 % as analyzed by flow cytometry according to the expression of following surface markers: CD11b and Gr-1 for PMN, Ter119 for erythroid cells and CD45R/B220 for lymphoid cells.

2.3 Cell Culture

2.3.1 General Cell Culture Procedures

All procedures were done under a laminar flow and all lab material was either purchased sterile (plastic consumables, solutions), sterile filtrated using Stericups™ with 0.2 µm pore size (solutions), autoclaved (solutions) or heat sterilized (glass ware).

2.3.2 Cultivation of Myeloid Cells

To culture bone marrow cells or enriched myeloid cells isolated from mice for a short time, cells were cultivated in RPMI (complete medium without FCS: RPMI, 1 % L-Glutamine, 1 % Penicillin/Streptomycin, 2 % FCS) at 37 °C in a humidified atmosphere with 5 % CO_2 and where indicated supplemented with 50 ng/mL rmGM-CSF for induction of differentiation.

2.3.3 Cultivation of Bone Marrow-Derived PMN

For the cultivation of bone marrow-derived PMN isolated from mice for a short time (for up to two days), cells were cultured in RPMI (RPMI, 1 % L-Glutamine, 1 % Penicillin and Streptomycin, 2 % FCS, 10 mM HEPES) or HBSS with Mg^{2+}/Ca^{2+} (1 x HBSS, 1 mM $MgCl_2$, 2 mM $CaCl_2$) at 37 °C in a humidified atmosphere with 5 % CO_2.

2.3.4 Colony Forming Assay

Methylcellulose-based MethoCult® M3231 medium purchased from Stem Cell Technologies was used to quantify the proliferation and differentiation potential of myeloid progenitors *in vitro*. Briefly, 500 GMP sorted from the bone marrow of mice where cultured in 1 mL MethoCult® M3231 supplemented either with 50 ng/mL rmSCF, 10 ng/mL rm-IL-3 and 25 ng/mL rmGM-CSF, or 50 ng/mL rmSCF, 10 ng/mL rmIL-3, 100 ng/mL rmFlit-3 ligand and 10 µg/mL LPS or 1 µg/mL Pam_3CSK_4 for 6 to 8 days at 37 °C in a humidified atmosphere with 5 % CO_2 and additionally some sterile H_2O in an extra dish to avoid the dehydration of the semi-solid media. The preparation of methylcellulose medium with supplements as well as the performance of the assay was done according to manufacturer's instructions. In detail, MethoCult® M3231 medium was thawed overnight under refrigeration at 4 to 8 °C. Supplements plus IMDM medium was added and the solution was mixed by shaking vigorously for 60 s. Afterwards the bottle was kept untouched stand for at least 5 min to allow bubbles to rise and finally MethoCult® medium was dispensed up to a volume of 3.9 mL into 4 mL cryo-tubes (VWR) using 5 mL syringes (Terumo) with 16 gauge blunt-end needles (Stem Cell Technologies) and was stored at -20 °C. For seeding of GMP, the appropriate amount of tubes were thawed overnight under refrigeration and 1500 GMP plus additional supplements (rmGM-CSF, Flit3 ligand, LPS, Pam_3CSK_4)

were added in a volume of 400 µL IMDM. Then, the tubes were mixed and bubbles were allowed to dissipate. Finally, 1 mL medium was dispensed into pre-tested 35 mm culture dishes (Stem Cell Technologies) using a 1 mL syringe (Terumo) with a 16 gauge blunt-end needle.

For the quantification of colony forming units (CFU) per 500 GMP, growing colonies were counted using a Leica Microscope and in some cases photographed using a Leica Microscope DM IRB equipped with a digital camera ResPag C14 (Jenoptik) at 10-fold magnification and Openlab software. In a second approach, the cells from single dishes were harvested by diluting methylcellulose with 10 mL PBS and counted in order to obtain the average cell number per CFU. A certain part of in that way harvested cells was proceeded to flow cytometry to analyze surface marker expression.

2.4 Neutrophil Function Assays

2.4.1 Migration Assay

To gain insight into ability of neutrophils to migrate towards a chemokine gradient bone marrow derived PMN were isolated as described earlier in 2.2.4. Migration was assessed in RPMI (RPMI, 1 % L-Glutamine, 1 % Penicillin and Streptomycin, 2 % FCS, 10 mM HEPES) using equilibrated Costar permeable supports (6.5 mm Transwell® with 5 µm pore polycarbonate membrane insert) and non-TC-treated 24-well plates. PMN were used at a density of 10^5 per 100 µL and assayed for transmigration towards 10 µM fMLP solved in 600 µL RPMI present in the lower chamber. Migration assay was done over a time of 4 h at 37 °C in a humidified atmosphere with 5 % CO_2. After incubation, cells migrated to the lower chamber were harvested and counted by the use of a BD FACSCantoII over 1 min by high speed.

2.4.2 Transendothelial Migration Assay

For evaluation of the transendothelial migration ability of bone marrow derived PMN *in vitro*, mouse b.End5 brain endothelioma cells were used for establishment of an endothelial monolayer. Mouse b.End5 cells were cultured in DMEM (DMEM, 1 % NEAA, 1 % Penicillin and Streptomycin, 10 % FCS) and used at passage 9 – 12. Endothelial cells were seeded at a density of 2.5×10^5 per well in a volume of

100 µL on Costar permeable supports (6.5 mm Transwell® with 5 µm pore polycarbonate membrane insert) – these were coated with 0.1 % gelatin solved in PBS for 30 min at 37 °C. The permeable supports were placed in non-TC-treated 24-well plates filled with 600 µL DMEM medium. The integrity of the b.End5 monolayer was monitored daily by a trypan blue permeability test [170]. In detail, medium was removed and 100 µL of undiluted trypan blue was added onto the endothelial cell monolayer, followed by an incubation for 30 min at 37 °C. Afterwards, the amount of trypan blue diffusing through the endothelial monolayer was assessed by calorimetric determination with an ELISA reader at 600 nm wavelength (SpectraMax190, Molecular Devices) in comparison to a control without endothelial cells. Confluence of the monolayer was achieved, when trypan blue permeability was less than 10 % in comparison to control. The b.End5 monolayer was activated overnight using 5 ng/mL h-TNFα and at the next day washed twice with HBSS with Mg^{2+}/Ca^{2+} (1 x HBSS, 1 mM $MgCl_2$, 2 mM $CaCl_2$). Freshly isolated bone marrow derived PMN were loaded at a density of 2×10^6 cells per 100 µL HBSS with Mg^{2+}/Ca^{2+} to the upper chamber and their migration was initiated by 10 µM fMLP added to lower chamber containing 600 µL HBSS with Mg^{2+}/Ca^{2+}. After incubation for 4 h at 37 °C PMN, that had transmigrated through the endothelial monolayer into bottom chamber, were harvested and counted using a FACSCantoII for 1 min at high speed. Transendothelial migration was assessed relative to the total input of PMN.

2.4.3 Immune Complex-Induced Degranulation

The release of elastase and gelatinase after binding of neutrophils on immune complexes (IC) by neutrophils was measured in the following way. First, large precipitating immune complexes were simulated by immobilizing IC on 96-well-plates (Fluoronunc). For this purpose, wells were initially treated with 0.1 mg/mL poly-D-lysine solved in H_2O for 1 h and then fixed with 2.5 % glutaraldehyde (in H_2O) for 20 min. After washing three times with PBS, BSA control wells and IC wells were coated with 1 mg/mL BSA in PBS for 30 min, followed by 2 h incubation with 0.1 M glycine in PBS (all from Sigma Aldrich) to reduce unspecific binding. Finally, 20 µg anti-BSA (Sigma) was coated for 1 h on IC and IC control wells to obtain fixed IC [171]. For the degranulation assay 5×10^5 isolated PMN were incubated in the coated plate together with the fluorescence-quenched substrate

DQ-elastin or DQ-gelatin (EnzCheck Kit, Invitrogen) at a final concentration of 0.15 mM in HBSS with Mg^{2+}/Ca^{2+} (1 x HBSS, 1 mM $MgCl_2$, 1 mM $CaCl_2$) at 37 °C. Elastase/Gelatinase release as a parameter of degranulation was evaluated by measuring at certain time points fluorescence intensity of digested fluorescent substrate at 530 nm using a fluorescence reader (SpectraMax GeminiEM, Molecular Devices).

2.4.4 Annexin-V Apoptosis Assay

After isolation, PMN undergo very quickly apoptotic cell death due to their short lifetime and high turnover *in situ*. For analysis of normal age-dependent apoptosis and stimulus-induced rescue from apoptosis, freshly isolated bone marrow derived PMN were cultured in RPMI (RPMI, 1 % L-Glutamine, 1 % Penicillin and Streptomycin, 2 % FCS, 10 mM HEPES). PMN were left unstimulated or treated with different kind of stimuli (Table 2.2) for several days. The degree of apoptosis was assessed by staining PMN with the Annexin-V FITC detection Kit I (BD Pharmingen) according to manufacturer's instructions. Samples were analyzed by the use of a FACSCantoII.

Stimulus	Concentration
rmGM-CSF	25 ng/mL
fMLP	10 µM
LPS	100 ng/mL

Table 2.2 Inducer used in the Apoptosis Assay

2.4.5 Reverse Passive Arthus Reaction in Skin

The reverse passive Arthus reaction (Art-r) was done according to the protocol published by Sunderkötter et al. [172]. In our experiments the Art-r was elicited by injecting 2.5 mg BSA in 200 µL PBS intraperitoneally as well as immediately afterwards 10 mM/20 µL rabbit anti-BSA-IgG (Sigma-Aldrich) subcutaneously in left ear. The development of the Arthus reaction was evaluated over a time period of 8 h using different macroscopic criteria for severity of inflammation and vessel damage. Ear swelling as a parameter for edema formation and neutrophil granulocyte infiltration was assessed by measuring ear thickness with a spring-

loaded Oditest caliper (Kreoplin) after 3 h, 6 h and 8 h. Contralateral ears served as controls. Petechia or hemorrhage formation as a parameter for the extent of vascular destruction was scored semi-quantitatively after 8 h by evaluating number and size of hemorrhages per ear (Table 2.3). Pictures of whole ears were taken using a stereomicroscope equipped with an AxioCam MRc (Carl Zeiss).

1 = 1 – 10 min petechiae (size of the tip of a pin) or 5 small petechiae (head of a pin)
2 = 5 – 15 small petechiae, or hemorrhagic macula (up to 0.5 mm diameter)
3 = hemorrhagic maculae between 0.5 – 1 mm, or 2 hemorrhagic maculae of 0.5 mm
4 = hemorrhagic area covering 50 % of ear surface

Table 2.3 Scoring System for Evaluation of the Degree of Hemorrhage Development.

2.5 Generation of Bone Marrow Chimeras

To gain insight into the function of hematopoietic stem and progenitor cells, as well as from precursor derived myeloid cells, bone marrow transplantation experiments were done by transferring the bone marrow of wild type or Btk-deficient C57BL/6 mice carrying the *Ly5.2* allele (CD45.2) into lethally irradiated C57BL/6-Ly5.1 mice (CD45.1) purchased from Charles River Laboratories. In detail, donor wild type and Btk-deficient male mice were sacrificed, bone marrow cells were resuspended in PBS and nucleated cell count was done using phase contrast light microscopy. Simultaneously, male recipient mice were lethally irradiated with two dosages of 550 rad in a time window of 1.5 h. Afterwards 5 million nucleated bone marrow cells of wild type and Btk-deficient donor mice were transferred in different ratios. Four weeks after injection, engraftment of donor bone marrow cells was tested by analyzing the blood for CD45.1 antigen expression. Blood was taken from tail vein and analyzed by flow cytometry for lineage markers and CD45.1/CD45.2 expression.

2.6 Flow Cytometry

2.6.1 Analysis of Murine Spleen

For evaluation of spleen leukocyte composition, regarding the occurrence and frequency of different hematopoietic cell populations, flow cytometric analysis of cells for different surface markers was performed. Spleens were prepared from mice and single cell suspension was obtained by collagenase D digestion. In detail, 500 µL collagenase D solution (10 mM HEPES/NaOH pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 2 mg/mL collagenase D (Roche)) was injected into spleen. Subsequently, spleens were cut in small pieces and transferred to 1.5 mL Eppendorf tubes. Next, samples were incubated at 37 °C for up to 30 min and mixed in between several times. After passing cell suspension through 40 µm meshes (BD Falcon), it was centrifuged at 1500 rpm for 5 min in a 15-mL conical tube. Erythrocyte depletion was done by treating the cells with 5 mL 0.16 M NH_4Cl for 5 min. After washing cells with PBS, total cell numbers were determined. For flow cytometry, one million cells were block with 1 µg mouse IgG (Jackson ImmunoResearch) and stained for different hematopoietic surface markers. Samples were analyzed on a FACSCantoII.

2.6.2 Analysis of Murine Bone Marrow

To gain insight into the cellular composition of the bone marrow, as well as into the hematopoietic stem and progenitor cell compartment, bone marrow cells were isolated from femurs of mice. Afterwards, total cell numbers were obtained and 1 - 3 x 10^6 cells were blocked with 1 µg mouse IgG (Jackson ImmunoResearch) and stained for characteristic hematopoietic cell surface markers. Samples were analyzed on a FACSCantoII.

2.6.3 Analysis of Murine Blood

Analysis of whole blood for white blood count, differential white blood count and red blood cell parameters was performed using the ABC animal blood counter (Scil animal care company GmbH) or by flow cytometry using antibodies directed against specific leukocyte surface markers. Whole blood was collected from tail vein into sample tubes containing EDTA (Kabe Labortechnik) to prevent coagulation and analyzed directly by the ABC. For flow cytometry 25 µL EDTA-

treated whole blood from tail vein were mixed with 75 µL PBS containing 2 % EDTA and with fluorochrome-conjugated antibodies for 15 min in the dark. Subsequently, BD FACS™ Lysing Solution (BD Biosciences) was applied according to manufacturer's instructions to lyse erythrocytes. Cells were washed twice with PBS containing 2 % EDTA and transferred into 5 mL polystyrene round-bottom tubes with cell strainer cap (BD Falcon) prior to analysis by the use of a FACSCantoII.

2.6.4 BrdU Incorporation

Cell proliferation *in vivo* was assessed by BrdU incorporation into the DNA. After a single intraperitoneal injection (1 mg/6 g body weight), BrdU was administered with the drinking water (1 mg/mL) on 4 consecutive days. Afterwards BrdU incorporation in bone marrow cells was analyzed by flow cytometry with the APC-BrdU Flow Kit (BD Pharmingen) according to manufacturer's instructions.

2.6.5 Antibodies for Flow Cytometry

Usually, 10^6 cells were stained in a volume of 100 µL PBS or FACS buffer (PBS, 0.1 % BSA, 0.1 % sodium azide) with the appropriate amount of antibodies listed in point 2.11.4 in this chapter.

2.7 Microscopy

2.7.1 Cytospin Preparation and Pappenheim Staining

The composition of single colony-forming units grown in methylcellulose-based medium was analyzed by cytospin preparation and Pappenheim stain. For cytospin preparation a single CFU was picked out of the semisolid media and transferred to a 1.5 mL tube containing 100 µL PBS with help of a 20 µL micropipette and a light microscope. After the assembly of the Cytoclip with one glass slide, filter card and Cytofunnel sample chamber, cell suspension was transferred into the sample chamber. Cells were centrifuged onto the glass slide at 500 rpm for 5 min with medium acceleration in a Cytopin3 Cytocentrifuge (Thermo Shandon). Subsequently, cytospins were dried over night at room temperature and stored at -20 °C.

The Pappenheim staining, also named May-Grünwald-Giemsa was performed as following: first, slides were stained with May-Grünwald solution (Sigma Aldrich) for 5 min at room temperature and washed in PBS for 1.5 min. Then, slides were stained in Giemsa solution (diluted in PBS at a ratio of 1:20, Sigma Aldrich) for 17 min and washed with Aqua dest. Afterwards slides were air dried and mounted with Entellan.

For evaluation of single CFU cell composition, cytospins were analyzed with a Leica Microscope DM IRB or Olympus Scan^R screening station. In detail, for every CFU-cytospin 100 cells were analyzed and according to their morphology grouped into neutrophil or monocyte. Finally, single CFU was determined as CFU-M, if more than 90 % of the cells were monocytes, or as CFU-G, if more than 90 % of the cells were granulocytes. When both cell types were present the CFU was determined as CFU-GM. In some cases cytospins were photographed with a Leica Microscope DM IRB equipped with a digital camera ResPag C14 (Jenoptik) at 20-fold magnification using Openlab software 4.0.4.

2.7.2 Immunofluorescence

To analyze neutrophil invasion after induction of the reverse passive Arthus reaction, treated and control ears of mice were excised for immunofluorescence staining 8 h after eliciting the r-Art. Ears were embedded on a small piece of nitrocellulose membrane in Tissue-Tek O.C.T compound (Sakura), frozen in a dry-ice/isopentane bath and stored at -80 °C. Several sections of 6 µm thickness were prepared using a Cryostat CM1900 (Leica) and air dried over night at room temperature. For immunofluorescence staining sections were fixed first in -20 °C acetone for 10 min and dried for 15 min at room temperature. Blocking was done with $F_c\gamma$R-block (1:100, BD Pharmingen) diluted in PBS/0.1 % BSA for 1 h at room temperature. In next step, the primary antibodies – biotinylated anti-CD31 (1:100, BD Pharmingen) and FITC-conjugated anti-Gr1 (1:75, BD Pharmingen) diluted in PBS/0.1 % BSA – were applied for 1 h at room temperature. The sections were washed three times with PBS and subsequently stained using the PE-conjugated Streptavidin (1:250, eBioscience) for 25 min at room temperature. After washing, sections were mounted with 50 µL ProLong Gold Antifade (Invitrogen) supplemented with 0.1 µL DAPI (0.1 µg/mL, Roche). In the end, sections were

analyzed with an Axiovert 200M fluorescence microscope (Carl Zeiss) equipped with an AxioCam MR3 digital camera (Carl Zeiss) and AxioVision software 2.2.5.

2.7.3 Toluidin Staining of Mast Cells

For analysis of mast cell numbers in the ear tissue of mice toluidin staining of paraffin-embedded untreated ears was done. First, ears were fixed with Carnoy solution (60 % ethanol, 30 % chloroform, 10 % acetic acid) overnight at 4 °C and subsequent incubated in 100 % ethanol overnight at 4 °C again. Embedding in paraffin was performed automatically with a Leica TP1020 tissue processor and cutting in 5 µm sections was done with a Microm 355S (Thermo Scientific). Before staining with toluidin blue solution (3 % in aqua bidest.) for 10 min, sections were dewaxed and rehydrated in a descending alcohol row. After staining, sections were dehydrated in an ascending alcohol row, cleared in xylol and mounted with Entellan. In the end, sections were analyzed with an Axiovert 200M microscope (Carl Zeiss) equipped with an AxioCam MR3 digital camera (Carl Zeiss) and AxioVision software 2.2.5.

2.7.4 Transmission Electron Microscopy

To visualize the ultrastructure of granules in neutrophils transmission electron microscopy (TEM) was performed in the following way: PMN were isolated from bone marrow as described in 2.2.4 and up to 3×10^6 cells were resuspended in 100 µL HBSS. This high-density cell suspension was fixed on sapphire plates by high-pressure freezing and freeze substitution as described elsewhere [173,174]. The ultrathin sections were analyzed in a Philips 400 TEM at 80 kV.

2.8 Immunoblot (Western Blot)

For immunoblotting of cell extracts, whole-cell protein lysates were prepared using RIPA buffer. In detail, 10^7 cells were mechanically lysed in 100 µL RIPA buffer, kept on ice for 10 min and centrifuged at 13 000 rpm for 15 min on 4 °C. Afterwards, the protein content of lysates was determined according to Bradford. Briefly, 900 µL Bradford solution were mixed with 100 µL 150 mM NaCl plus 1 µL of protein extract and incubated for up to 15 min at room temperature. Absorption of Bradford mixture was measured at 595 nm in a spectrophotometer (Ultrospec 3000, Pharmacia) and total protein was calculated in comparison to a

BSA calibrator. Equal amount of protein was separated on 10 % or 12.5 % SDS-polyacrylamide gels and electroblotted on nitrocellulose membrane (0.45 µm pore size, Whatman). For protein detection several antibodies were used in combination with the respective peroxidase conjugated secondary antibodies. Immunoreactivity was analyzed by enhanced chemiluminescence using SuperSignal West Dura Extended Duration Substrate (Thermo Scientific).

2.8.1 Antibodies for Immunoblotting

Generally, membranes were incubated with primary antibodies overnight at 4 °C with exception of ERK2, which was applied for 1 h at room temperature. All secondary antibodies were applied on membranes for 1 h at room temperature. Detailed information for all antibodies used can be found below.

Antibody	Supplier	Dilution	Buffer
Akt	Cell Signaling	1:1000	5 % BSA TBS-T
Phospho-Akt (T308)	Cell Signaling	1:1000	5 % BSA TBS-T
Phospho-Akt (S473)	Cell Signaling	1:1000	5 % BSA TBS-T
Bcl-x_L (H-62)	Santa Cruz Biotech.	1:1000	5 % NDM TBS-T
Btk	BD Transduction	1:250	5 % NDM TBS-T
Btk (pY551)	BD Pharmingen	1:500	5 % BSA TBS-T
c-Rel (N)	Santa Cruz Biotech.	1:1000	5 % NDM TBS-T
C/EBPα	Cell Signaling	1:1000	5 % NDM TBS-T
C/EBPβ (LAP)	Cell Signaling	1:1000	5 % BSA TBS-T
cyclin D1 (M-20)	Santa Cruz Biotech.	1:1000	5 % NDM TBS-T
ERK 2 (C-14)	Santa Cruz Biotech.	1:1000	5 % NDM TBS-T
p-ERK (E-4)	Santa Cruz Biotech.	1:1000	5 % BSA TBS-T
GSK-3β (27C10) Rabbit mAb	Cell Signaling	1:1000	5 % BSA TBS-T
Phospho-GSK3β (S9)	Cell Signaling	1:1000	5 % BSA TBS-T
JAK1 (HR-785)	Santa Cruz Biotech.	1:1000	5 % NDM TBS-T
JAK2 (M-126)	Santa Cruz Biotech.	1:1000	5 % NDM TBS-T
NFκB p50 (NLS)	Santa Cruz Biotech.	1:1000	5 % NDM TBS-T

Phospho-PI3K p85 (Tyr458)	Cell Signaling	1:1000	5 % BSA TBS-T
PU.1 (Spi-1) (T-21)	Santa Cruz Biotech.	1:1000	5 % NDM TBS-T
Rel-B (C-19)	Santa Cruz Biotech.	1:1000	5 % NDM TBS-T
Stat3	Cell Signaling	1:1000	5 % BSA TBS-T
Phospho-Stat3 (Y705)	Cell Signaling	1:1000	5 % BSA TBS-T
ImmunoPure Rabbit Anti-Mouse IgG, Peroxidase Conjugated	Thermo Scientific	1:10 000	5 % NDM TBS-T
ImmunoPure Goat Anti- Rabbit IgG, Peroxidase Conjugated	Thermo Scientific	1:20 000	5 % NDM TBS-T

2.8.2 Buffers and Solutions for Immunoblotting

Buffer	**Formulation**
RIPA Buffer	50 mM Tris/HCl pH 7.5 150 mM NaCl 1 mM EDTA/EGTA 1 % Triton X-100 MiniComplete and PhosStop (Roche)
Bradford Solution (1 L) (filtration necessary)	100 mg Comassie G-250 50 mL ethanol 100 mL 85 % phosphoric acid add 1 L H_2O
SDS-PAGE Separating Gel (10 % PAA) (45 mL)	11.25 mL 40 % acrylamide/bisacrylamide (37.5:1) 11.25 mL 1.5 M Tris/HCl pH 8.8 21.90 mL H_2O 225 µL 20 % SDS 450 µL 10 % APS 30 µL TEMED
SDS-PAGE Separating Gel (12.5 % PAA) (45 mL)	14.10 mL 40 % acrylamide/bisacrylamide (37.5:1) 11.25 mL 1.5 M Tris/HCl pH 8.8 19.05 mL H_2O 225 µL 20 % SDS 450 µL 10 % APS 30 µL TEMED

SDS-PAGE Loading Gel (4 % PAA)	1.5 mL 40 % acrylamide/bisacrylamide (37.5:1) 3.9 mL 0.5 M Tris/HCl pH 6.8 9.5 mL H_2O 75 µL 20 % SDS 150 µL 10 % APS 20 µL TEMED
SDS-PAGE Running Buffer (4 L)	12.1 g Tris 57.6 g glycine 20 mL 20 % SDS add 4 L H_2O
Semi-Dry Transfer Buffer (1 L)	5.8 g Tris 2.9 g glycine 1.85 mL 20 % SDS 100 mL methanol add 1 L H_2O
Wet Blot Transfer Buffer (1 L)	3.0 g Tris 14.4 g glycine 200 mL methanol add 1 L H_2O
Stripping Buffer (500 mL)	31.25 mL 1 M Tris/HCl pH 6.7 50 mL 20 % SDS 3.45 mL β-mercaptoethanol add 500 mL H_2O
TBS-T	25 mM Tris/HCl pH 7.5 150 mM NaCl 0.05 % Tween-20 in H_2O

2.9 Quantitative RT-PCR

Total RNA was isolated from 5×10^6 cells using the High Pure RNA Isolation Kit (Roche) according to the manufacturer's instructions. For isolation of total RNA from hematopoietic progenitor populations, sorted cells were directly lysed in 1 mL QIAzol (Qiagen) and supplemented with 10 µg tRNA from baker`s yeast (Sigma Aldrich) to increase isolation efficiency. The procedure to isolate total RNA with QIAzol was performed according to the standard protocol.

For cDNA synthesis between 250 ng and 1 µg total RNA was taken depending on the amount of available RNA. To avoid genomic DNA contamination, DNA digestion was performed with the RQ1 RNase-Free DNase (Promega) according to manufacturer's instructions, prior to cDNA synthesis. In the second step, reverse transcription was done at 42 °C using random hexamer primer (Roche), AMV reverse transcriptase (Roche) and RNasin Plus RNase Inhibitor (Promega). The semi-quantitative analyses were carried out using intron-spanning gene specific primers (see table below: 2.9.1) and Quantitec SYBR Green Mix (Qiagen) on a Lightcycler480 (Roche). Results were normalized with respect to housekeeping gene expression and calculation of gene expression ratios was done using the mathematical model published by Pfaffl et al. [175].

2.9.1 Primer for Quantitative RT-PCR

Gene	Forward Primer	Reverse Primer
Actb	caa cga gcg gtt ccg atg	gcc aca gga ttc cat acc ca
Btk	ggc cat caa gat gat cag aga	gct tct cat ggg aaa gat tca
Cebpa	aaa caa cgc aac gtg gag a	gcg gtc att gtc act ggt c
Cebpb	tga tgc aat ccg gat caa	cac gtg tgt tgc gtc agt c
Csf1r	gtc atg tct ctg ctg gtg ct	tgc ctt cgt atc tct cga tg
Csf2ra	aga gcc agg aag cac acc	cag tgc ttc atc ctc gtg tc
Csf3r	tat gct agg gtc cag cga gt	ggg agg ctc caa ttt cac a
Elane	tgg agg tca ttt ctg tgg tg	ctg cac tga ccg gaa att tag
Gapdh	gac ttc aac agc aac tcc cac	tcc acc acc ctg ttg ctg ta
Gata1	gaa tcc tct gca tca aca agc	ggg caa ggg ttc tga ggt
Hprt	cct aag atg agc gca agt tga a	cca cag gac tag aac acc tgc taa
Ltf	ggg caa gtg cgg ttt agt t	cca ttg ctt ttg gag gat tt
Mmp9	acg aca tag acg gca tcc a	gct gtg gtt cag ttg tgg tg
Mpo	gga agg aga cct aga ggt tgg	tag cac agg aag gcc aat g
Ngp	gcc taa aga ctg cga ctt cc	tga aga att tcc ctg tgc aa
Sfpi1	tct tct gca cgg gga gac ag	gga cga gaa ctg gaa ggt acc

2.10 Reagents and Chemicals

2.10.1 Chemicals

Item	Supplier
Acetic acid	Fluka, Switzerland
Acetic acid (glacial)	Merck, Germany
Acrylamide solution 40%	AppliChem, Germany
Agarose Ultrapure, Electrophoresis Grade	Gibco BRL, USA
APS	Sigma-Aldrich, Germany
β-Mercaptoethanol	Sigma-Aldrich, Germany
Boric acid	AppliChem, Germany
Butanol	AppliChem, Germany
Bromphenolblue	Carl Bittmann, Switzerland
BSA (Albumin Fraction V)	AppliChem, Germany
Di-sodium-hydrogen phosphate	AppliChem, Germany
DTT	AppliChem, Germany
EDTA – dihydrate	AppliChem, Germany
EGTA – dihydrate	AppliChem, Germany
Ethanol (absolute)	Sigma-Aldrich, Germany
Glycin	AppliChem, Germany
HEPES	Carl Roth GmbH, Germany
Hydrochloric acid 37%	Sigma-Aldrich, Germany
Hydrogen peroxide	AppliChem, Germany
Isopropanol	Merck, Germany
Methanol	Merck, Germany
Magnesium chloride	Sigma-Aldrich, Germany
Non-fat dried milk	AppliChem, Germany
Potassium chloride	Carl Roth GmbH, Germany
Potassium dihydrogen phosphate	Merck, Germany

SDS	AppliChem, Germany
Sodium azide	AppliChem, Germany
Sodium chloride	AppliChem, Germany
Sodium dihydrogen phosphate	Merck, Germany
Sodium hydroxide, pellets	AppliChem, Germany
Sodium orthovanadate	Sigma-Aldrich, Germany
TEMED	AppliChem, Germany
Tris (ultrapure)	AppliChem, Germany
Tri-sodium-citrate dihydrate	AppliChem, Germany
Tween 20	AppliChem, Germany

2.10.2 Kits

Item	Supplier
APC-BrdU Flow Kit	BD Pharmingen, USA
Annexin-V FITC detection Kit I	BD Pharmingen, USA
Enzcheck Elastase Kit	Invitrogen, USA
Enzcheck Gelatinase Kit	Invitrogen, USA
High Pure RNA Isolation Kit	Roche Diagnostics, Germany

2.10.3 Cell Culture

Item	Supplier
DMEM	Pan Biotech, Germany
FCS	Biochrom AG, Germany
10 x HBSS	Gibco, USA
Histopaque 1119	Sigma Aldrich, Germany
IMDM	Pan Biotech, Germany
L-Glutamin 200mM	Gibco, USA

Penicillin/Streptomycin	Gibco, USA
Percoll	GE Healthcare, USA
RPMI	Gibco, USA
0.4 % Trypanblue	Sigma Aldrich, Germany
rm-GM-CSF	Promokine, Germany
rm-IL-3	Stem Cell Technologies, USA
rm-SCF	Stem Cell Technologies, USA
M3231	Stem Cell Technologies, USA
Flit3-Ligand	Invivogen, USA
Pam_3Cys_4	Invivogen, USA
LPS	Invivogen, USA

2.10.4 Antibodies for Flow Cytometry

Antigen	Clone	Conjugation	Supplier	Dilution
B220/CD45R	RA3-6B2	PE	Miltenyi, Germany	1:11
B220/CD45R	RA3-6B2	PerCP	BD Pharmingen, USA	0.2 µg
CD3ε	145-2C11	PE	Miltenyi, Germany	1:11
CD4	H129.19	FITC	BD Pharmingen, USA	0.1 µg
CD4	H129.19	PE	BD Pharmingen, USA	0.1 µg
CD8α	53-6.7	PE	BD Pharmingen, USA	0.1 µg
CD11b	M1/70	PE	eBioscience, USA	0.05 µg
CD11b	M1/70	APC	eBioscience, USA	0.05 µg
CD11b	M1/70	Pacific Blue	eBioscience, USA	0.05 µg
CD11c	HL3	APC	BD Pharmingen, USA	0.1 µg
CD16/32	93	APC	eBioscience, USA	0.5 µg
CD19	6D5	PE	Miltenyi, Germany	1:11
CD34	RAM34	FITC	eBioscience, USA	3.75 µg
CD40	HM40-3	FITC	BD Pharmingen, USA	0.1 µg
CD45	30-F11	APC-Cy7	BioLegend, USA	0.5 µg

CD45.1	A20	Pacific Blue	eBioscience, USA	0.1 µg
CD45.2	104-2	PE-Cy7	BioLegend, USA	0.2 µg
CD48	HM48-1	PE-Cy7	BioLegend, USA	0.2 µg
CD80	16-10A1	FITC	BD Pharmingen, USA	0.1 µg
CD86	GL1	FITC	BD Pharmingen, USA	0.1 µg
CD117(Kit)	ACK2	PE-Cy7	eBioscience, USA	1 µg
CD150	TCF15-12F12.2	Pacific Blue	BioLegend, USA	0.2 µg
CXCR4(CD184)	2B11/CXCR4	PE	eBioscience, USA	1.25 µg
DX5(CD49b)	DX5	PE	BD Pharmingen, USA	0.1 µg
F4/80	BM8	PE-Cy7	eBioscience, USA	1.25 µg
Gr-1(Ly6G)	RB6-8C5	PE	Miltenyi, Germany	1:11
Gr-1(Ly6G)	RB6-8C5	Pacific Blue	eBioscience, USA	0.05 µg
ICAM-1(CD54)	3E2	PE	BD Pharmingen, USA	0.1 µg
IL-7Rα(CD127)	A7R34	FITC	eBioscience, USA	0.5 µg
Ly6C	1G7.G10	PE	Miltenyi, Germany	1:11
MHC II (I-A/I-E)	M5/114.15.2	FITC	eBioscience, USA	0.25 µg
NK.1-1	PK136	FITC	BD Pharmingen, USA	0.1 µg
Sca-1(Ly6A/E)	D7	PE-Cy5.5	eBioscience, USA	2.5 µg
Ter119	TER-119	PE	Miltenyi, Germany	1:11
VCAM-1(CD106)	429	Alexa-Flour 647	eBioscience, USA	1 µg

2.10.5 Other Reagents

Item	Company
10bp DNA ladder	New England Biolabs, USA
1kb plus DNA ladder	Invitrogen, USA
Medical X-Ray Film Super RX	Fujifilm, Japan
PageRuler protein ladder	Fermentas Life Sciences, Canada
Phosphatase Inhibitor, PhosStop	Roche Diagnostics, Germany
Protease Inhibitor, Complete-Mini	Roche Diagnostics, Germany

Nitrocellulose membrane 0.45 µm	Milipore, USA
Random hexamer primer	Roche Diagnostics, Germany
RNaseA	Amersham GE Healthcare, USA
RNase inhibitor, RNasin plus	Promega,USA
SYBR Green PCR master mix	Qiagen, USA
Taq DNA polymerase, GoTaq	Promega, USA
Trizol, QIAzol	Qiagen, Germany
Whatman paper	Biometra, USA

2.10.6 Plastic Ware

Item	Company
6 well Non-Tissue culture plate	BD Falcon, USA
24 well Non-Tissue culture plate	Greiner Bio-one, USA
Conical tube polypropylene 15 ml	BD Falcon, USA
Conical tube polypropylene 50 ml	BD Falcon, USA
Disposable tubes	Eppendorf, Germany
Nunc disposable tubes	Thermo Scientific, USA
Petri dishes	BD Falcon, USA
Polystyrene tube with cell-strainer cap	BD Falcon, USA
Polystyrene tube	BD Falcon, USA
35 mm culture dishes	Stem Cell Technologies, USA
1.4 mL U-tubes non coded	Micronic, The Netherlands
Cuvettes	Brand, Germany
96 well Fluronunc-plate	Nunc, USA
96 well plate	Roche, Germany
Lobind	Eppendorf, Germany
Transwell	Roche, Germany

2.11 Laboratory Equipment

Item	Company
Biofuge Pico	Heraeus Instruments, Germany
Block thermostat TCR 100	Roth AG, Germany
Centrifuge J2-21	Beckman, USA
Deep freezer -80°C, Class N / HFU 586	Heraeus Instruments, Germany
Gel Documentation GenoSmart	VWR, Germany
Ice-machine AF 10	Scotsman, Italy
Incubator	Heraeus Instruments, Germany
Laminar flow sterile bank	Heraeus Instruments, Germany
Magnetic stirrer RCT	Kika Labortechnik, Germany
Megafuge 1.0R	Heraeus Instruments, Germany
Microscope Leica DMIL	Leica, Germany
Mini Rocker MR-1	A. Hartenstein, Germany
Multichannel Pipette	Costar, USA
Multipipette	Eppendorf, Germany
PCR machine Primus 96 Plus	MWG Biotech, Germany
pH meter Calimatic 761	Knick, Germany
Pipetman; 10, 20, 100, 200, 1000 µl	Gilson, France
Pipette boy AccuJet	Brand, Germany
Pipette boy Pipetus	Hirschmann Laborgeräte, Germany
Power Supply PowerPac Universal	Bio-Rad Labratories, USA
Refrigerator	Liebherr, Germany
Scale / Sartorius BP-210-S	Sartorius AG, Germany
Scale / Scaltec	Scaltec, Germany
Shaker Polymax 1040	Heidolph Instruments, Germany
Shaker Reax	Heidolph Instruments, Germany
Spectrophotometer Ultrospec 3000	Pharmacia Biotech, Great Britain
Steam sterilizer	H+P Labortechnik GmbH, Germany

Trans-Blot SD	Bio-Rad Laboratories, USA
ViCellXR	Becton Coulter, USA
Vortex Genie-2	Scientific Industries, USA
Water bath	GFL, Germany
Water bath Julabo C	Julabo Labortechnik, Germany

2.12 Software

Company, Software, Version
Microsoft Office 2007
Thomson Endnote X
Adobe Creative Suite 2
Graphpad Prism 5
Roche Lightcycler480 Software 1.5.0
BD Bioscience FACSDiva 6.2.1
Molecular Devices SoftMax Pro 4.3
AxioVision 4.8

3 Results

3.1 Mouse Models for Btk Deficiency

Mutations in the murine *Btk* gene cause X-linked immunodeficiency (*Xid*) in mice that partially resembles the human disease X-linked agammaglobulinemia (XLA), and is characterized by a failure of B cells to become phenotypically and functionally mature [9]. In the present work two different mouse models were used to study the influence of Btk deficiency on murine hematopoietic stem cells as well as myeloid cells in detail. The first mouse model, named *Xid*-mouse, is an inbreed CBA strain carrying the spontaneous point mutation R28C within the PH-domain of the *Btk* gene, which hampers PIP_3-dependent membrane recruitment and prohibit activation of Btk [8]. The second mouse model, named Btk-deficient mouse, is a genetically modified C57BL/6 mouse, where Btk expression is abolished after gene targeting of the exons 13 and 14 of the *Btk* gene.

3.2 Btk Function in Hematopoiesis

3.2.1 Analysis of Myeloid Cell Populations in Btk-Mutant Mice

First of all, to gain insight into the possible functions of Btk in myeloid cells, it was of interest to analyze whether there are differences in the composition of the myeloid lineage in lymphoid tissues or blood. For that reason, spleen, blood and bone marrow of wild type and Btk-deficient mice were analyzed for cellularity as well as frequency of all hematopoietic cell types and particularly for the different myeloid cell types. The hematopoietic cells were analyzed by determining absolute cell counts as well as by flow cytometry for the characterization of lineage markers to classify the cell populations.

These experiments revealed a reduced cellularity in spleens of Btk-deficient mice accompanied by a strong decrease in peripheral B cell numbers, which has been described previously [166,167]. Additionally, in spleens of animals lacking Btk a significant reduction of myeloid cells was detected caused mainly by a significant decrease of neutrophil granulocyte and macrophage populations (Figure 3.1).

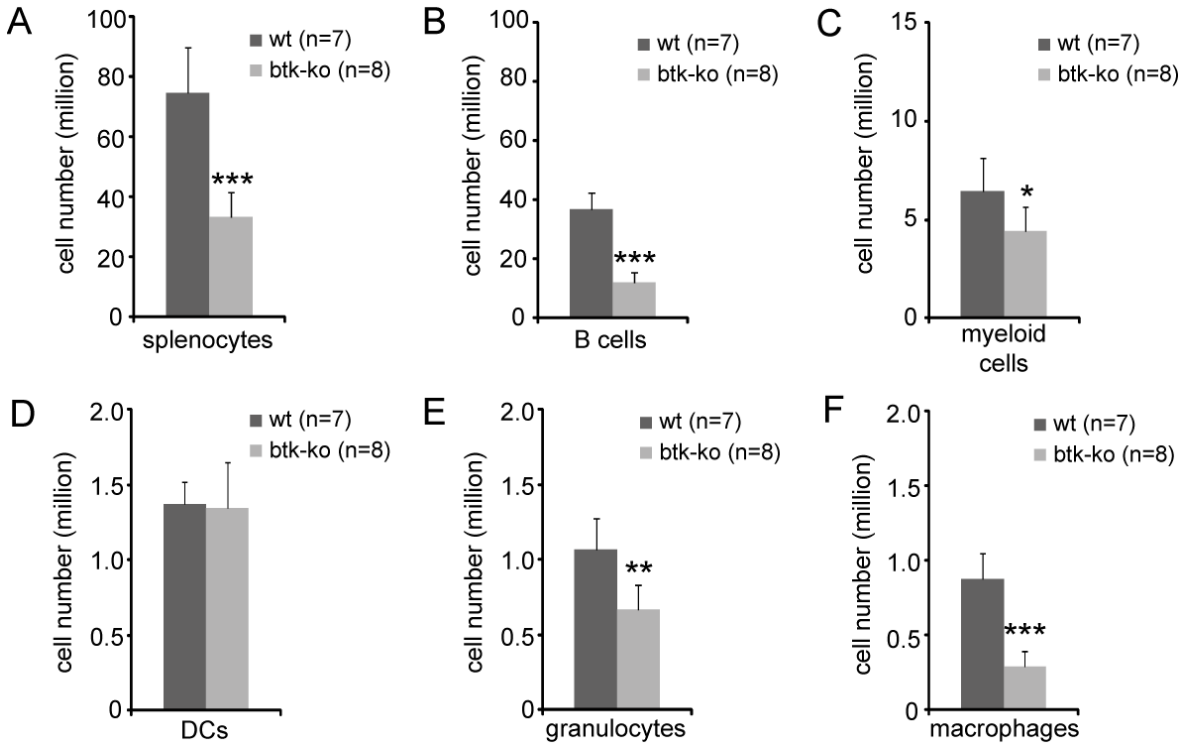


Figure 3.1 Frequencies of myeloid cell populations in spleens of Btk-deficient mice. (A-F) After erythrocyte lysis cell suspensions of spleens from wild type and Btk-deficient (*Btk*-ko) mice were (A) counted and stained with (B) B220 for B cells, (C) CD11b for myeloid cells, (D) CD11b/CD11c for dendritic cells (DCs), (E) CD11b/Gr-1 for granulocytes and (F) CD11b/F4/80 for macrophages. Data presented are mean values (± SD). **P* ≤ 0.05; ***P* ≤ 0.005; ****P* ≤ 0.0005. n represents the number of biological replicates.

For analyses of white blood cell frequencies and numbers blood samples were taken from the tail vein of mice and analyzed using an Animal Blood Counter (ABC). In contrast to the results for Btk-deficient spleens, no changes were observed in the blood of Btk-deficient mice concerning frequency or number of myeloid cells, neither for neutrophil granulocytes nor for monocytes (Figure 3.2A,B). Similar results were obtained for the blood of *Xid*-mice, where also no significant changes in the numbers of granulocytes or monocytes per blood volume were detected (Figure 3.2C,D).

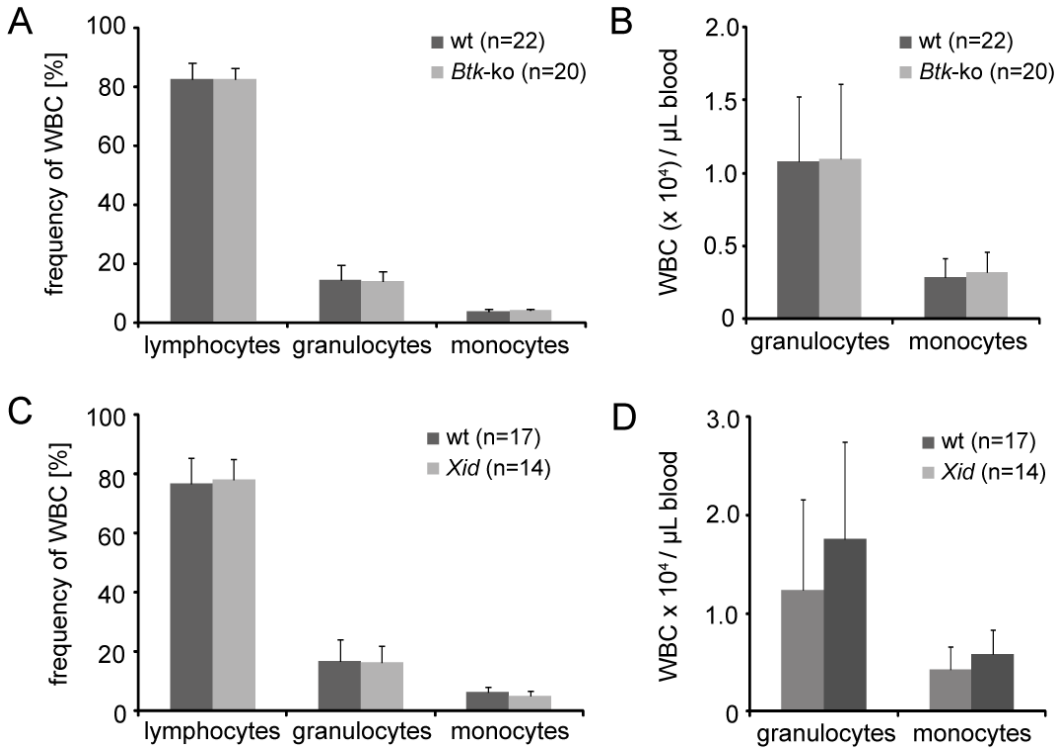


Figure 3.2 Frequencies of myeloid cells in the blood of Btk-deficient and *Xid*-mice. Blood taken from the tail vein of wild type (wt) and Btk-deficient (*Btk*-ko) (A-B) as well as *Xid*-mice (C-D) was analyzed using an Animal Blood Counter for determination of differential white blood cell (WBC) counts. Frequencies (A,C) and cell numbers per blood volume (B,D) of lymphocytes, granulocytes and monocytes are shown. Data presented are the mean values (± SD). n represents the number of biological replicates.

To examine the cellularity of bone marrow in Btk-deficient mice in comparison to wild type mice, the femurs were dissected and the bone marrow cells were flushed out, finally absolute cell counts and flow cytometry were performed. In contrast to the previous results, there was a marked increase of myeloid cell frequency in the bone marrow of mice lacking Btk expression. Detailed analysis of the cell phenotype revealed a significant increase in neutrophil granulocyte numbers, which are defined as positive for the surface markers CD11b and Gr-1 (Figure 3.3A-D). The significant increase in granulocyte population per femur in the bone marrow of Btk-deficient mice could be confirmed in *Xid*-mice by analyses of cell numbers for the different cell lineages in bone marrow (Figure 3.3E-H). Furthermore, elevated numbers of erythrocytes per femur were found in both mouse models for Btk deficiency, but the increase in erythrocytes was only significant in *Xid*-mice and not in Btk-deficient mice.

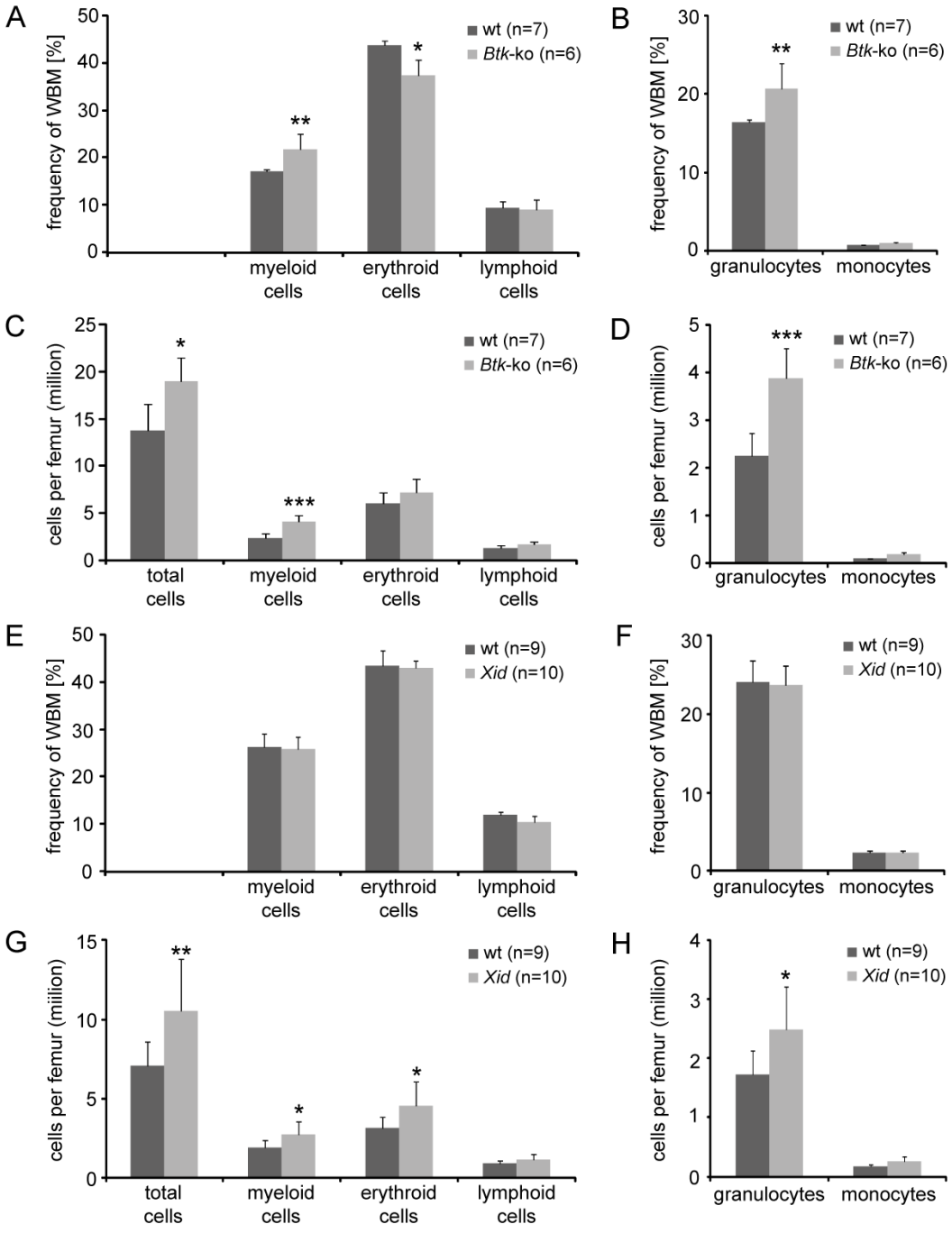


Figure 3.3 Cell numbers and frequencies of hematopoietic cell populations in the bone marrow of Btk-deficient and *Xid*-mice.

(A-D) Whole bone marrow (WBM) cells per femur of wild type (wt) and Btk-deficient (*Btk*-ko) as well as *Xid*-mice were counted and stained for the expression analysis of lineage-specific surface markers. (E-H) The myeloid compartment was further analyzed by the expression of $CD11b^{+}Gr\text{-}1^{+}$ (granulocytes) and $CD11b^{+}Gr\text{-}1^{-}$ (monocytes). The frequencies of cell populations were recalculated for defined cell numbers per femur (C,D,G,H). Data presented are mean values (± SD). **P* ≤ 0.05; ***P* ≤ 0.005; ****P* ≤ 0.0005. n represents the number of biological replicates.

The enlarged granulocyte as well as the extended erythrocyte population caused a significant increase in absolute whole bone marrow cell numbers in both types of Btk-mutant mice (Figure 3.3C,D,G,H). In summary, we have observed a significant augmentation in myeloid cells, particularly in granulocytes in the bone marrow of Btk-mutant mice, which does not result in higher numbers of granulocytes in blood or lymphoid organs. Moreover, in the spleen of Btk-deficient mice the numbers of granulocytes was even lower in comparison to wild type animals.

3.2.2 Analysis of Hematopoietic Stem Cell and Progenitor Populations in the Context of Btk Deficiency

Myeloid cells such as granulocytes, monocytes and dendritic cells are produced in the bone marrow by hematopoiesis starting from hematopoietic stem cells (HSC) that develop to myeloid progenitors that in turn give rise to mature progeny. Therefore, the increase in myeloid cells, especially in granulocytes detected in the bone marrow of Btk-deficient mice could be caused by an increase in myeloid progenitor numbers or hematopoietic stem cell as well as multipotent progenitor populations. HSC as well as multipotent progenitors and myeloid progenitors can be distinguished according to their surface marker profile. This allows a detailed analysis of these populations in Btk-deficient mice to evaluate the cause for the increase of granulocytes in the bone marrow.

First of all, we have examined the frequencies of long-term HSC and multipotent progenitor populations in the bone marrow, which both reside in the lineage$^-$Sca1$^+$Kit$^+$ fraction (LSK) and can be distinguished by their expression of CD150 and CD48 (Figure 3.4A). The detailed analysis of cell frequencies using flow cytometry revealed no significant differences in the frequencies, neither in the long-term hematopoietic stem cell compartment (LT-HSC), nor in the compartment of multipotent progenitors in Btk-deficient mice. But with respect to absolute cell numbers, which are significantly enhanced in mice lacking Btk, the multipotent progenitor population was slightly but not significantly increased (Figure 3.4B,C).

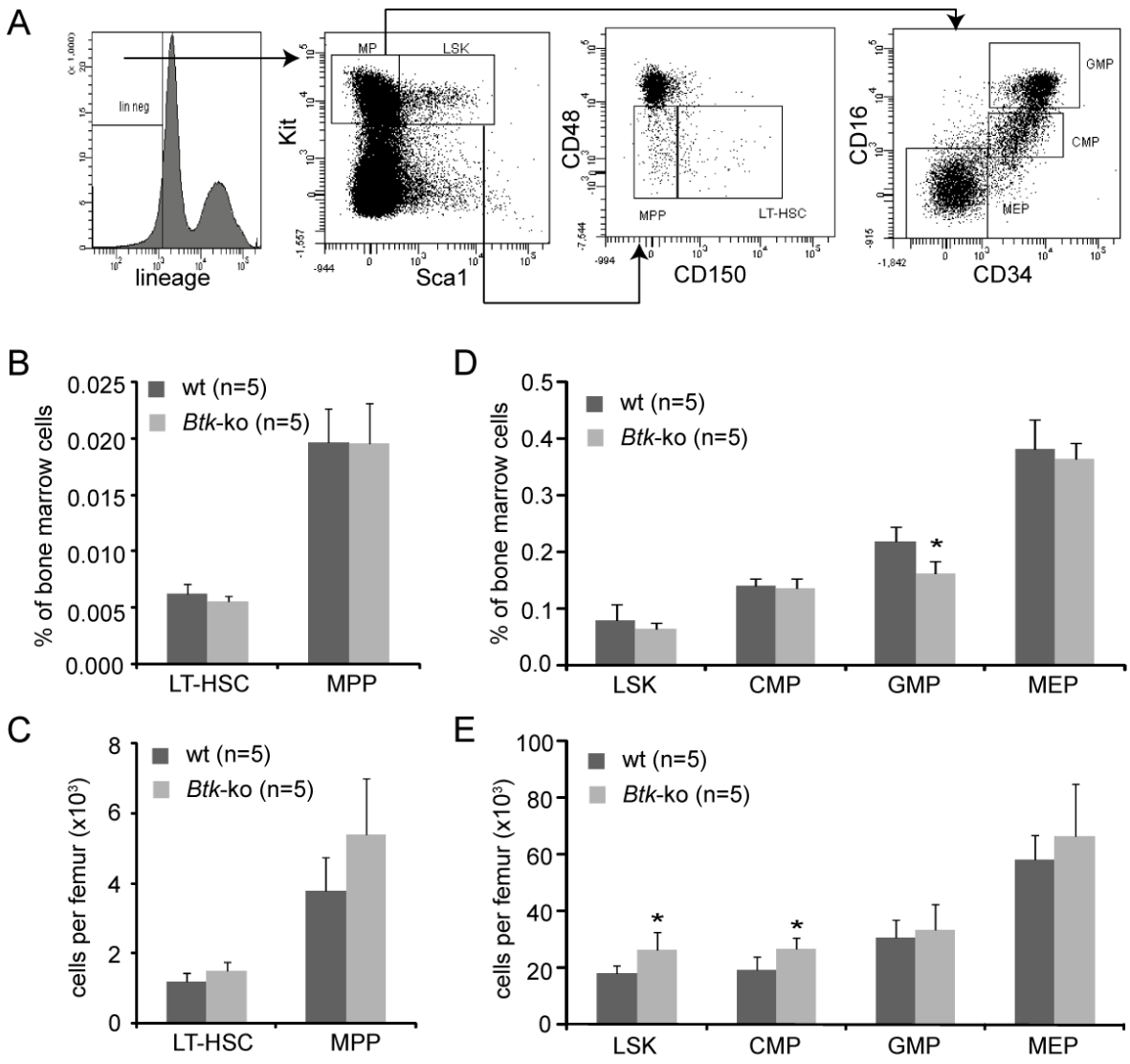


Figure 3.4 Frequencies and cell numbers of HSC and progenitor cell populations in the bone marrow of Btk-deficient mice.

(A) Bone marrow cells obtained from femurs of wild type (wt) and Btk-deficient (*Btk*-ko) mice were stained for lineage markers, Kit, Sca1, CD16, CD34, CD48 as well as CD150 and analyzed by flow cytometry. The lineage$^-$Sca1$^-$Kit$^+$ population (MP = myeloid progenitor) in the bone marrow was further examined for the myeloid progenitor subpopulations CMP, GMP and MEP by the expression of CD34 and CD16, and the lineage$^-$Sca1$^+$Kit$^+$ population (LSK) for the HSC and multipotent progenitor (MPP) compartment by the expression of CD48 and CD150. (B-E) Hematopoietic stem cell and progenitor cell subpopulations are shown as percentages of whole bone marrow cells or as absolute numbers per femur. Data presented are mean values (± SD). *$P \leq 0.05$. n represents the number of biological replicates.

The further restricted myeloid progenitors reside in the lineage$^-$Sca1$^-$Kit$^+$ fraction of the bone marrow and can be subdivided into common myeloid progenitor (CMP), megakaryocyte-erythrocyte progenitor (MEP) and granulocyte-monocyte progenitor (GMP) according to the CD16/CD32 (FcγRII/III) and CD34 expression level (Figure 3.4A). Analyses of the myeloid progenitor subpopulations within the bone marrow compartment of Btk-deficient mice revealed a slight but significant reduction of GMP frequency within the bone marrow compared to wild type mice. In contrast to the GMP decrease, normal frequencies of CMP and MEP were detected in bone marrow lacking Btk expression. However, calculation of absolute cell numbers per femur revealed comparable cell numbers for GMP and MEP for wild type and Btk-deficient mice. The absolute cell numbers for CMP showed even an increase in comparison to wild type animals (Figure 3.4D,E).

Taken together, the results concerning the hematopoietic stem cell compartment and myeloid progenitor populations could not explain the enlargement in the neutrophil granulocyte population that was observed in the bone marrow of Btk-deficient animals, because no increase in the direct precursor GMP could be detected. Moreover, the relatively lower frequency of GMP, in comparison to the other progenitor populations, and the augmented absolute cell numbers of multipotent progenitors and of the LSK fraction even indicated a decreased differentiation towards GMP under conditions of Btk deficiency.

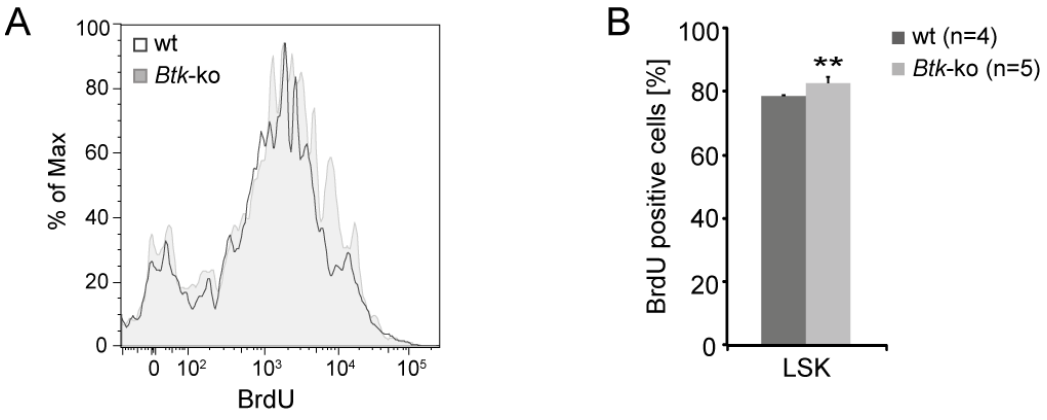


Figure 3.5 In vivo BrdU incorporation in Btk-deficient mice.

(A) Wild type (wt) and Btk-deficient (*Btk*-ko) mice were treated with BrdU for 4 days and analyzed by flow cytometry for BrdU incorporation within the LSK fraction of the bone marrow. (B) Quantification of BrdU incorporation is shown. Data presented are the mean values (± SD). ***P* ≤ 0.005. n represents the number of biological replicates.

Additionally, the increase in the LSK fraction, particularly in the MPP could also be caused by an enhanced proliferation of Btk-deficient HSC and MPP compared to wild type HSC and MPP. To analyze proliferation of HSC *in vivo*, BrdU incorporation assays were performed by BrdU injection intraperitoneally plus 4 days of BrdU administration with the drinking water. Afterwards BrdU incorporation in bone marrow cells was analyzed by flow cytometry. The assays revealed indeed a slight, but significant increase in BrdU-incorporation within the LSK fraction of bone marrow cells indicating an enhanced proliferation of HSC when Btk is missing (Figure 3.5).

3.2.3 Analysis of Myeloid Development in Btk-Deficient Mice

Another reason for the augmented granulocyte numbers in the bone marrow of Btk-deficient mice could be an enhanced proliferation or changed differentiation ratio downstream of the myeloid progenitor. To examine the developmental capacity of Btk-deficient myeloid progenitors in comparison to wild type progenitors, we have analyzed the myeloid differentiation of sorted progenitors *in vitro* using methylcellulose based medium. Because of the differences mainly found in the granulocyte and monocyte populations, we preferred granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin-3 (IL-3) as growth and differentiation promoting cytokines. IL-3 is known to support the growth of early myeloid progenitors of all lineages, especially in combination with other cytokines such as GM-CSF that is mainly necessary for the growth of neutrophil granulocytes and monocytes. Additionally, we used stem cell factor (SCF) as supplement to promote progenitor survival and expansion.

The GMP population was isolated from bone marrow cells, that were depleted for lineage-positive cells, by fluorescence-activated cell sorting (FACS) using the surface markers Kit, Sca1, CD16 and CD34. Next, equal numbers of purified GMP were plated in methylcellulose medium supplemented with SCF, IL-3 and GM-CSF. After 6 days, the number of colony-forming units (CFU) per plate was analyzed and surprisingly a reduction in the CFU number by approximately 50 % was observed in Btk-deficient GMP compared with wild type GMP. In contrast, the mean cell number per single colony was more than two-fold higher when Btk was not expressed indicating an extended proliferative potential of Btk-deficient GMP (Figure 3.6A-C).

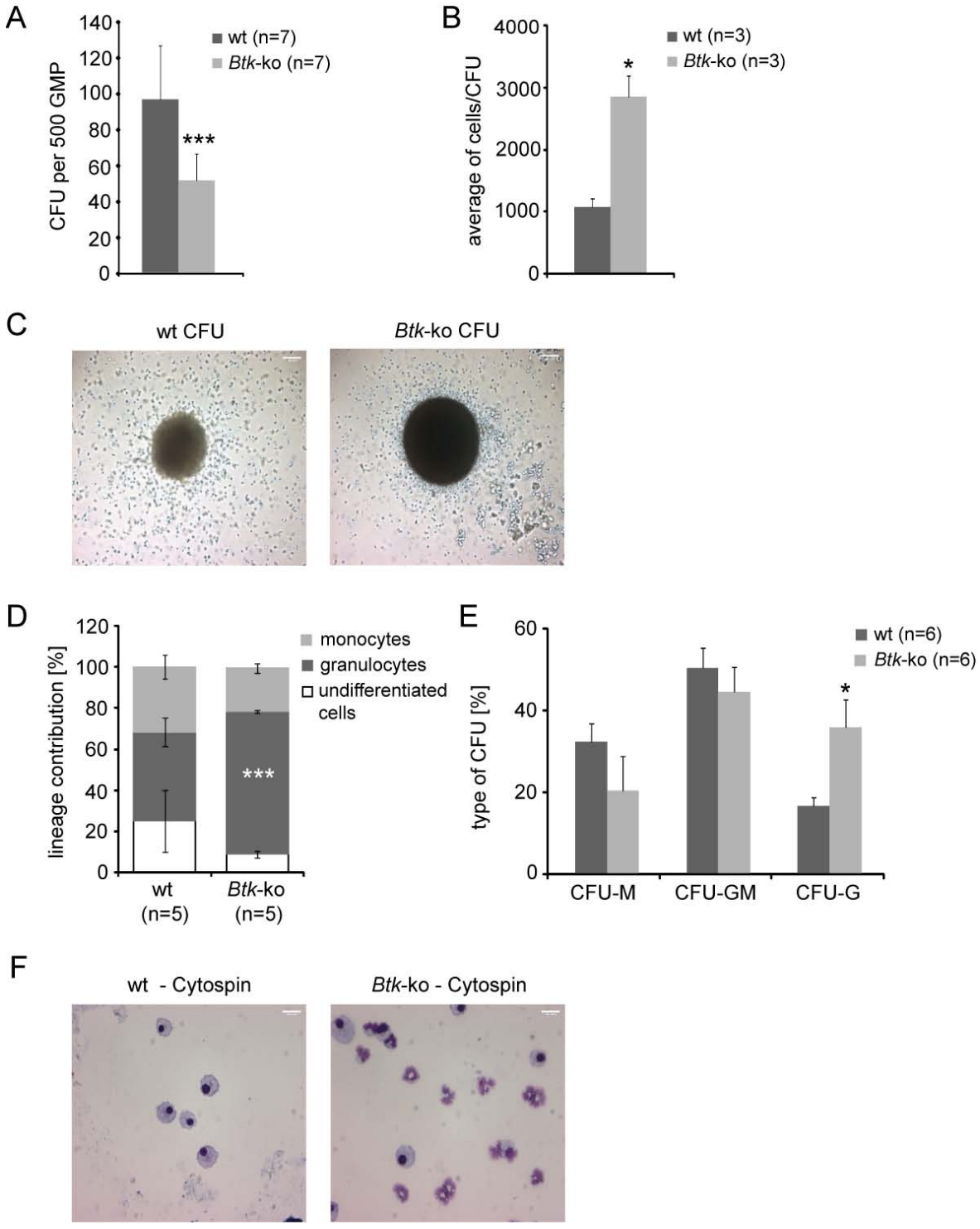


Figure 3.6 Myeloid development of purified Btk-deficient GMP *in vitro*.

500 GMP sorted out of individual wild type (wt) and Btk-deficient (*Btk*-ko) mice were seeded in methylcellulose medium supplemented with SCF, IL-3 and GM-CSF. The experiment was performed in quadruplicates. (A) After 6 days in culture colony-forming units (CFU) were counted. (B) The cell number per CFU was calculated. (C) One CFU with a representative size generated from wild type or Btk-deficient GMP was photographed and is presented. Scale bars represent 200 μm. (D) Differentiated cells

were analyzed by flow cytometry at day 8 of culture. Percentage of cells positive for the lineage markers is shown. Undifferentiated cells, $CD11b^+/Gr\text{-}1^-/F4/80^-$; granulocytes, $CD11b^+/Gr\text{-}1^+$; monocytes, $CD11b^+/F4/80^+$. Data presented are the mean (± SD). (E) Twenty to thirty individual CFU per biological replicate were processed for cytospins and Pappenheim staining and analyzed for the cell content by morphology. CFU-M, more than 90 % of the cells were macrophages; CFU-G, more than 90 % of cells were neutrophils; CFU-GM, the content of macrophages and granulocytes was in between 20 % to 80 %. (F) A representative cytospin per analyzed genotype is presented. Scale bar represents 50 μm. In (E) the data are presented as mean values (± SEM). Data were analyzed with Student's t-test. $*P \leq 0.05$, $***P \leq 0.0005$. n represents the number of biological replicates.

To gain further inside into the phenotype of generated cells, we analyzed a part of the obtained colony-forming units after 8 to 10 days using flow cytometry for several myeloid lineage markers such as CD11b (myeloid cells), Gr-1 (granulocytes) and F4/80 (macrophages). These analyses revealed a highly significant increase in granulocytes at the expense of monocytes and undifferentiated cells in cultures derived from Btk-deficient GMP (Figure 3.6D). Additional evidences for enhanced granulopoiesis were obtained by examination of the morphologic phenotype of single colony-forming units. For that purpose, several CFU were picked randomly from methylcellulose medium after 8 to 10 days in culture and fixed on glass slides using cytospin centrifugation. Finally, cytospins were stained using Pappenheim stain and analyzed by light microscopy in a blinded way. In the case of Btk-deficiency, the evaluation of cell morphology revealed again a significant increase in CFU consisting mainly of neutrophil granulocytes (purity > 90 %) at the expense of CFU formed mainly by monocytes/macrophages (Figure 3.6E,F).

Not only GM-CSF can drive myeloid development, especially towards granulocyte and monocyte populations, also Toll-like receptor (TLR) ligands can induce myeloid development in hematopoietic stem cells. Recently, a study has demonstrated TLR expression on hematopoietic progenitor cells and that activation of TLR signaling bypassed normal differentiation cues and led to monocyte/macrophage differentiation of myeloid progenitors [89]. In particular, the myeloid development in response to the ligands for TLR4 and TLR1/2 was analyzed.

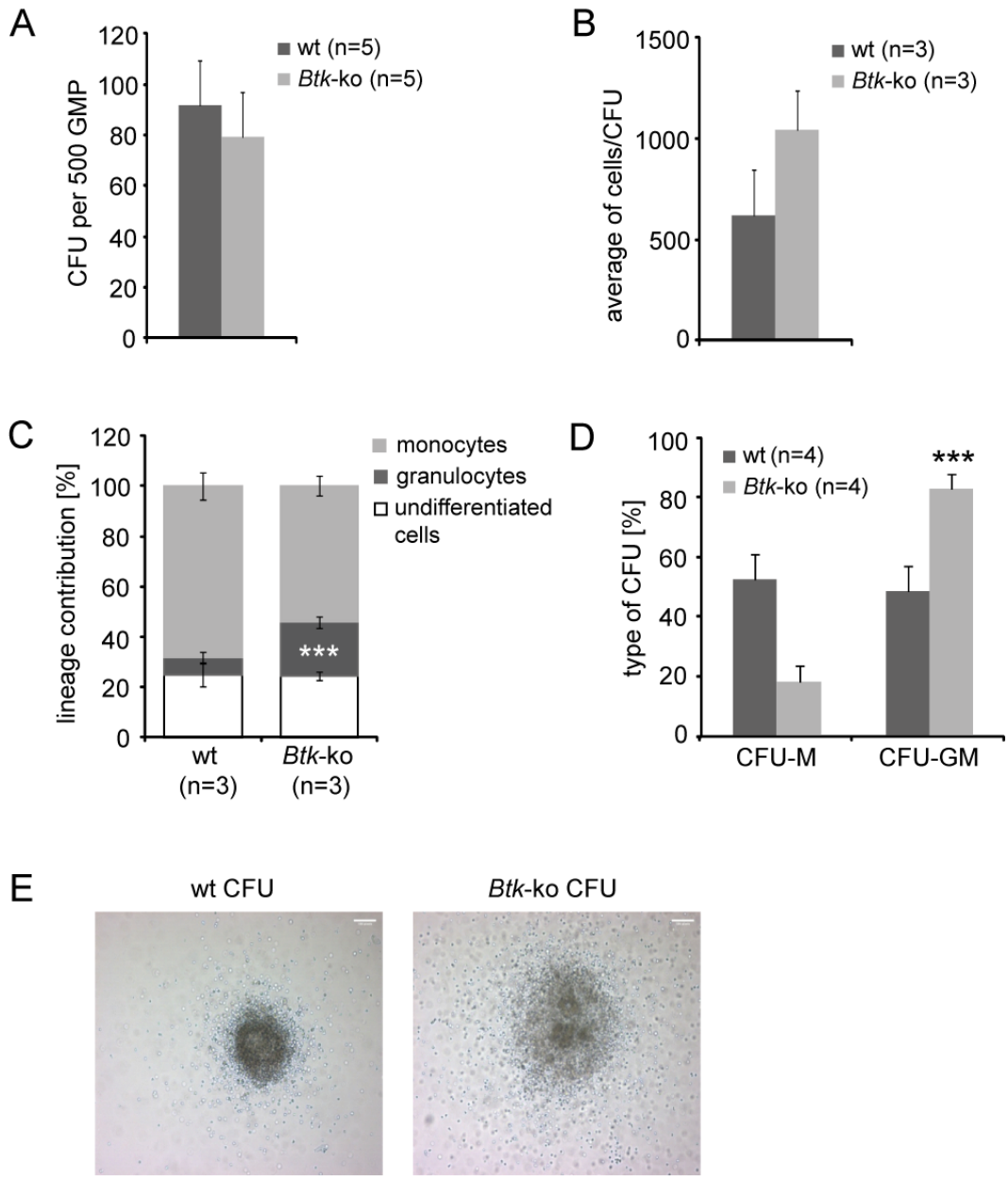


Figure 3.7 Myeloid development of Btk-deficient GMP upon TLR4 stimulation.

500 GMP sorted out of individual wild type (wt) and Btk-deficient (*Btk*-ko) mice were seeded in methylcellulose medium supplemented with SCF, IL3, Flit-3 ligand and LPS. The experiments were performed in triplicates. (A) After 6 days in culture CFU were counted and (B) the cell number per CFU was calculated by determining the number of cells developing from 500 seeded GMP per CFU. (C) Differentiated cells were analyzed by flow cytometry at day 8 of culture. Undifferentiated cells, $CD11b^+/Gr\text{-}1^-/F4/80^-$; granulocytes, $CD11b^+/Gr\text{-}1^+$; monocytes, $CD11b^+/F4/80^+$. (D) Twenty individual CFU per biological replicate were processed for cytospins and Pappenheim staining and analyzed for the cell content by morphology. CFU-M, over 90% of the cells were macrophages; CFU-GM, content of macrophages and granulocytes in between 20 % to 80 %. (E) One CFU with a representative size was photographed and is presented. Scale bars represent

200 μm. Data presented are the mean values (± SD). ****P* ≤ 0.0005. n represents the number of biological replicates.

For that reason, we examined the myeloid development of wild type and Btk-deficient GMP in response to the TLR4 ligand lipopolysaccharide (LPS) and the TLR1/2 ligand Pam_3CSK_4. The experiments were performed basically in the same way as described previously, but instead of GM-CSF Flit-3 ligand and one of the TLR ligands was used in combination with SCF and IL-3.

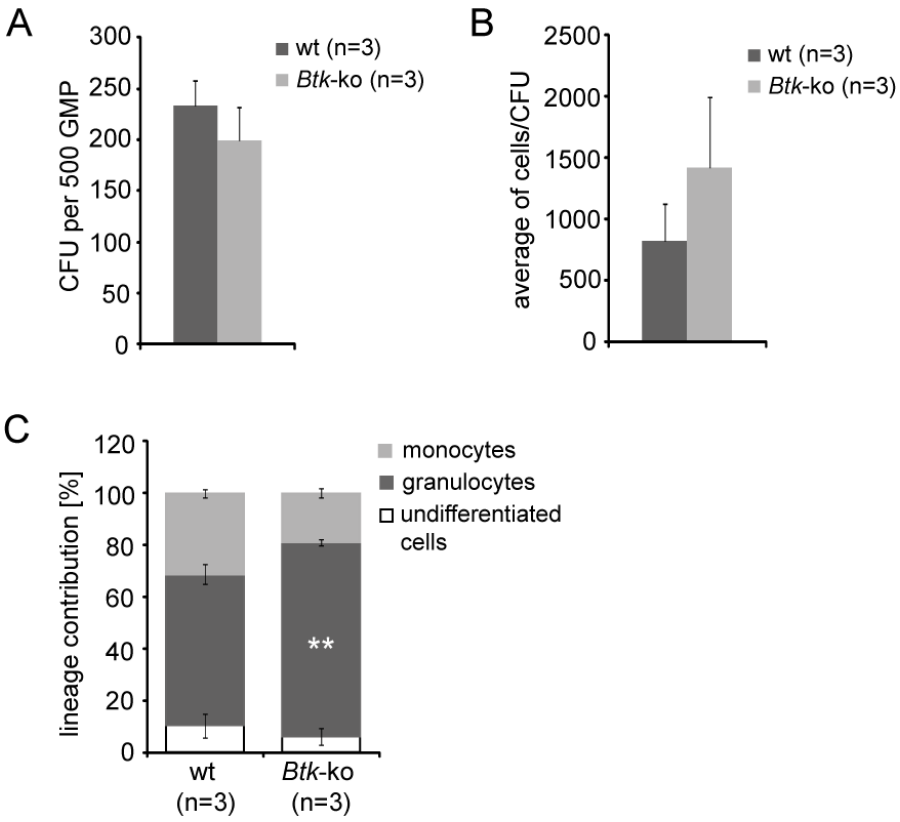


Figure 3.8 Myeloid development of Btk-deficient GMP upon TLR1/2 stimulation.
500 GMP sorted out the bone marrow of individual wild type (wt) and Btk-deficient (*Btk*-ko) mice were seeded in methylcellulose media supplemented with SCF, IL-3, Flit-3 ligand and Pam_3CSK_4. The experiments were performed in triplicates. (A) After 6 days in culture CFU were counted and (B) the cell number per CFU was calculated by determining the number of cells developing from 500 seeded GMP per CFU. (C) Differentiated cells were analyzed by flow cytometry at day 8 of culture. Differentiated cells were analyzed by flow cytometry at day 8 of culture. Legend: undifferentiated cells, $CD11b^+/Gr\text{-}1^-/F4/80^-$; granulocytes, $CD11b^+/Gr\text{-}1^+$; monocytes, $CD11b^+/F4/80^+$. Data presented are the mean values (± SD). ***P* ≤ 0.005. n represents the number of biological replicates.

Again, after 8 to 10 days of culture an increase in colony size was observed in the case when Btk-deficient GMP were seeded caused by an enhanced proliferation of Btk-deficient myeloid cells. Moreover, the shift towards granulopoiesis at the expanse of primarily monopoiesis was confirmed by flow cytometry of CFU differentiated in response to both TLR ligands (Figure 3.7, Figure 3.8). Interestingly, there was a clear difference in the differentiation outcome regarding the individual TLR ligands. In the case of LPS, mainly monocytes/macrophages were detected in the generated CFU, whereas Pam_3CSK_4 predominantly induced granulocyte cell development. Despite the differentiation cue, both TLR signals result in an increased granulocyte development downstream of Btk-deficient GMP. The analysis of myeloid developmental capacity downstream of isolated GMP revealed a significant shift in the differentiation towards granulocytes at the expanse of monocytes in myeloid progenitors lacking Btk irrespective of the used differentiation inducer. Moreover, Btk-deficient GMP displayed a higher proliferation rate indicated by the more than two-fold increase in cell number per colony upon GM-CSF stimulation, but also by the clearly enhanced cell numbers using TLR ligands as differentiation stimuli. In line with this augmented differentiation and proliferation of the granulocyte lineage found in Btk-deficient myeloid progenitors are the results concerning the size of the granulocyte population in the bone marrow. Additionally, the high proliferative potential of Btk-deficient neutrophil granulocytes could compensate for the marginal reduced numbers of GMP found in the bone marrow of these mice.

3.2.4 Btk Function in Terminal Differentiation of Neutrophils

To provide further evidence for an enhanced proliferation of developing granulocytes lacking Btk, we examined the proliferation of granulocyte precursors such as promyelocytes and myelocytes. These give rise to immature and later on to mature neutrophil granulocytes or shortly neutrophils and can be distinguished by the expression level of the surface marker CD11b and Gr-1 [176] (Figure 3.9A). To examine the proliferative potential of these subpopulations, *in vivo* BrdU incorporation assays were performed in the way described before. Indeed, the results revealed a slight but significant increase in proliferation of Btk-deficient promyelocytes and myelocytes (Figure 3.9B,C).

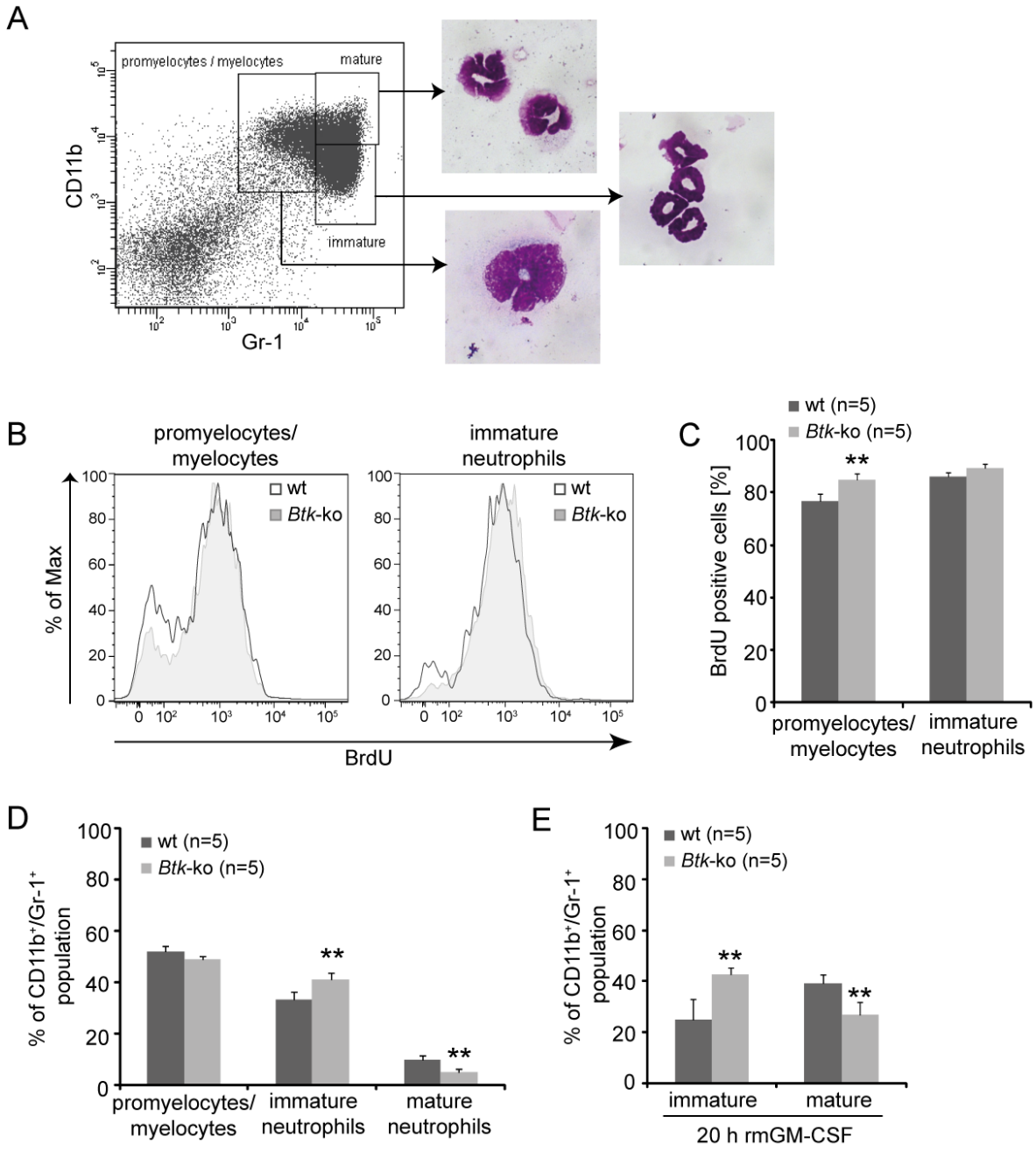


Figure 3.9 Proliferation and maturation of Btk-deficient neutrophils.

(A) The maturation status of neutrophils in the bone marrow can be distinguished by the expression level of the surface marker CD11b and Gr-1. To confirm settings, gated cells were sorted and Pappenheim-staining on cytospins of sorted cells was performed. (B,C) Wild type (wt) or Btk-deficient (*Btk*-ko) mice were treated with BrdU for 4 days and the $CD11b^+/Gr\text{-}1^+$ subpopulations, according to panel A, were analyzed and quantified for BrdU incorporation by flow cytometry. (D) The maturation phenotype of neutrophils was analyzed according to panel A on freshly isolated and erythrocytes-depleted bone marrow cells or (E) on for 20 h in the presence of GM-CSF-matured bone marrow cells. Data

presented are the mean values (± SD). $^{**}P \leq 0.005$. n represents the number of biological replicates.

Regarding the enhanced granulopoiesis observed in Btk-deficient mice as well as the contradictory decrease of neutrophils in the spleen, we wondered if Btk-deficient neutrophils are comparable to wild type neutrophils concerning their maturation status and function. Examination of neutrophil maturation, using the surface markers Gr-1 and CD11b to distinguish between immature and mature neutrophils (Figure 3.9A), revealed severe defects in the basal maturation of Btk-deficient neutrophils in the bone marrow accompanied with an increase in immature neutrophils (Figure 3.9D). Moreover, induction of neutrophil maturation by GM-CSF treatment of erythrocytes-depleted bone marrow cells for 20 hours could not rescue the diminished maturation of Btk-deficient neutrophils (Figure 3.9E). In summary, the analyses of maturation status of neutrophils in the bone marrow using flow cytometry clearly demonstrated an accumulation of immature neutrophils to the disadvantage of mature neutrophils in mice lacking Btk. One of the most important hallmarks of neutrophil terminal differentiation is the consecutive expression of granule proteins that are stored in four different types of granules forming at different time points of the differentiation process. From there, terminal differentiation is also associated with the appearance of granules in the cytoplasm of neutrophil granulocytes. At the promyelocyte state formation of the first granules starts, which are named primary granules and are defined by high content of myeloperoxidase (Mpo) and neutrophil elastase (Elane). Secondary granules appear at the myelocyte stage of differentiation and are characterized by high amounts of lactoferrin (Ltf) and collagenase (Mmp8). The last step in terminal granulopoiesis is differentiation into band and segmented neutrophils leading to mature neutrophils accompanied with the appearance of tertiary granules filled with gelatinase (Mmp9) and lysozyme as well as secretory granules, which are mainly loaded with receptors and plasma proteins [111].

To shed light on the maturation defect in Btk-deficient neutrophils, we first analyzed granule appearance and numbers as additional marker for neutrophil maturation. For that purpose, we used transmission electron microscopy of neutrophils isolated from bone marrow of wild type and Btk-deficient mice. The obtained photographs clearly demonstrated a significant decrease in granule numbers in neutrophils derived from mice lacking Btk expression (Figure 3.10).

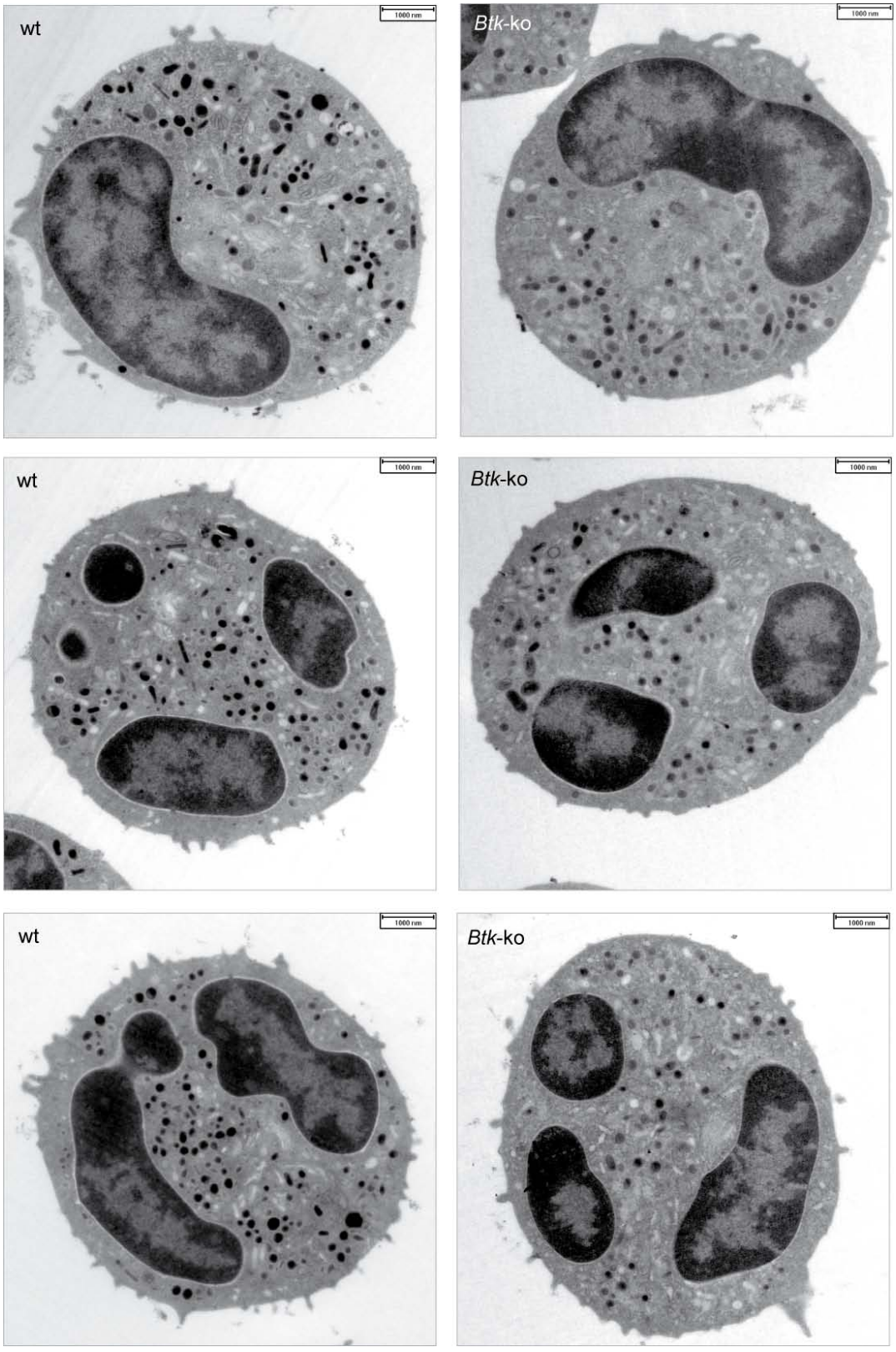


Figure 3.10 Analysis of Btk-deficient neutrophil morphology by transmission electron microscopy.

Transmission electron microscopy was performed using wild type (wt) and Btk-deficient (*Btk*-ko) neutrophils isolated from bone marrow. Representative images of each genotype are shown.

These reduction could be confirmed by the quantification of granule numbers per cell and granule numbers relative to cytosolic area using ImageJ software (Figure 3.11A,B). Moreover, the granules of Btk-deficient neutrophils displayed a lowered electron density, which indicated a diminished content of granule proteins. To confirm the results of transmission electron microscopy, mRNA expression of several granule-specific genes was analyzed using quantitative PCR of total RNA obtained from isolated neutrophils. These experiments revealed an obvious reduction in gene expression for the granule-specific genes neutrophil elastase as well as the precursor of lactoferrin and myeloperoxidase in Btk-deficient neutrophils (Figure 3.11C).

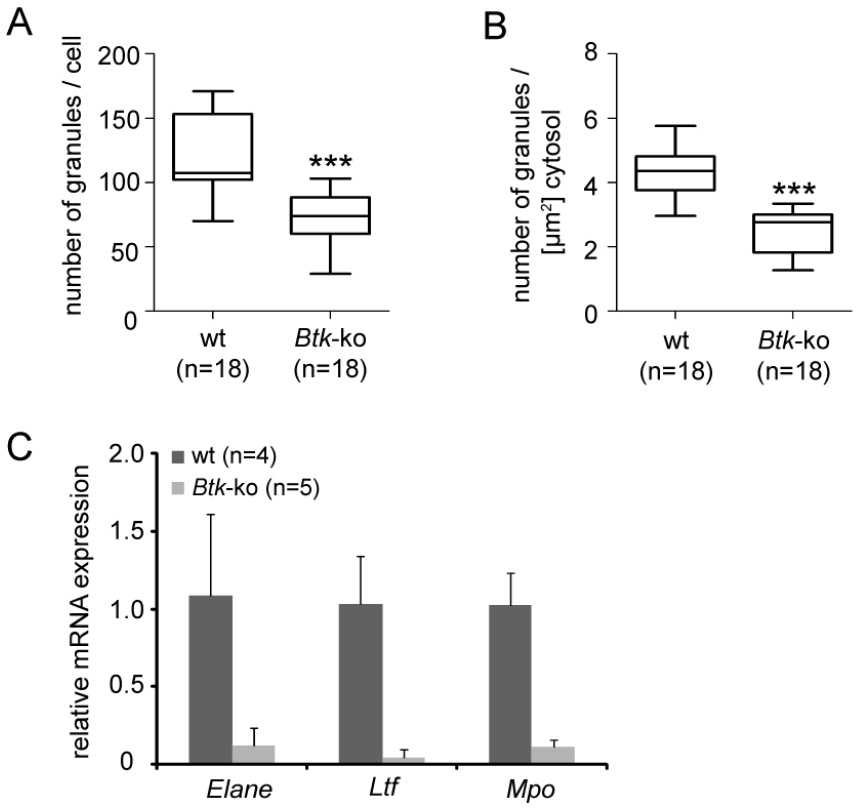


Figure 3.11 Granule numbers and granule-specific gene expression in Btk-deficient neutrophils.

(A,B) Wild type (wt) and Btk-deficient (*Btk*-ko) bone marrow derived neutrophils were analyzed by transmission electron microscopy and granule numbers per cell and per µm^2 cytosol were determined using ImageJ software. (C) The expression of several granule proteins such as neutrophil elastase (*Elane*), lactoferrin precursor (*Ltf*) and myeloperoxidase (*Mpo*) was analyzed by quantitative PCR relative to expression of β-actin. Data presented are the mean values (± SD). ***$P \leq 0.0005$. n represents the number of biological replicates.

Summarizing the collected data, Btk-deficient neutrophils evidently demonstrated a defect in terminal differentiation and maturation characterized by a significant decrease in granule numbers, as well as granule content shown by a clear reduction of granule protein expression.

3.3 Immune Functions of Btk-Deficient Neutrophils

3.3.1 Function of Neutrophils Lacking Btk in the Acute Inflammatory Response

Due to the observed maturation defects of Btk-deficient neutrophils associated with a reduction in granule number and granule protein expression, we wondered whether these defects are of any functional relevance. To test this issue in an *in vivo* approach, we decided to perform the reverse passive Arthus reaction in the ear. The Arthus reaction is the prototype of a type III hypersensitivity reaction, which emerges in response to soluble immune complexes (IC). Especially in the microcirculation of skin, glomeruli or alveoles, the deposition of immune complexes results in vascular destruction caused by massive neutrophil infiltration. In contrast to the classical Arthus reaction, the reverse passive Arthus reaction is elicited by administration of the specific antibody local at the preferred site of inflammation, for example the skin of an ear, and application of the appropriate antigen intraperitoneally or intravenously. After the formation of antigen-antibody complexes due to the antigen distribution by peripheral circulation, antibodies within the immune complexes interact with specific Fc receptors expressed on mast cells thereby inducing mast cell activation and degranulation. Additional to mast cell activation, the activation of classical pathways of the complement system occurs that are necessary for full elicitation of the inflammatory response. Both stimuli together are mandatory for recruitment of neutrophils to the site of IC deposition that leads to vascular destruction, displayed by the characteristic inflammatory trias of edema and hemorrhage formation as well as neutrophil infiltration [177]. This neutrophil infiltration is not just a consequence of a type III hypersensitivity reaction, but it is also its prerequisite. Sine, former studies using CD18-deficient mice have demonstrated that vascular destruction critically depends on the recruitment of neutrophils to the site of IC deposition [171].

Based on these findings, the function of Btk-deficient neutrophils was analyzed by the reverse passive Arthus reaction performed as following: the antigen BSA was applied intraperitoneally and the anti-BSA antibody was injected subcutaneously in the left ear of wild type or Btk-deficient mice. Over a time period of eight hours, the formation of edema was determined by measuring ear thickness and in the end ears were semiquantitative scored concerning petechiae and hemorrhage development.

Upon initiation of the reverse passive Arthus reaction, wild type mice showed a normal response with edema formation and hemorrhages that was visible on the treated ear. The signs of the inflammatory response, particularly edema and hemorrhage increased over time in wild type mice. In contrast, Btk-deficient mice showed significantly fewer signs of IC-mediated vascular destruction. Both characteristic features of the Arthus reaction, edema and hemorrhages are significantly decreased in mice lacking Btk (Figure 3.12).

To gain more insight in the behavior of neutrophils in Btk-deficient mice, treated ears were harvested 8 h after elicitation of the reverse passive Arthus reaction and analyzed by immunofluorescence staining with regard to infiltration of neutrophils into the site of inflammation. The obtained immunofluorescence pictures revealed that Btk-deficient neutrophils do not migrate into the inflamed tissue, although they were attracted to the site of inflammation and adhered to the luminal site of the blood vessel endothelium, and even some also transmigrated through the endothelium (Figure 3.13 arrowheads).

In summary, the analyzes of neutrophil function in reverse passive Arthus reaction, as a model for the acute inflammatory response demonstrated a significant impairment of Btk-deficient neutrophils in tissue migration at the site of immune complex deposition. This impairment led to a clear reduction in vascular destruction, edema formation and tissue damage.

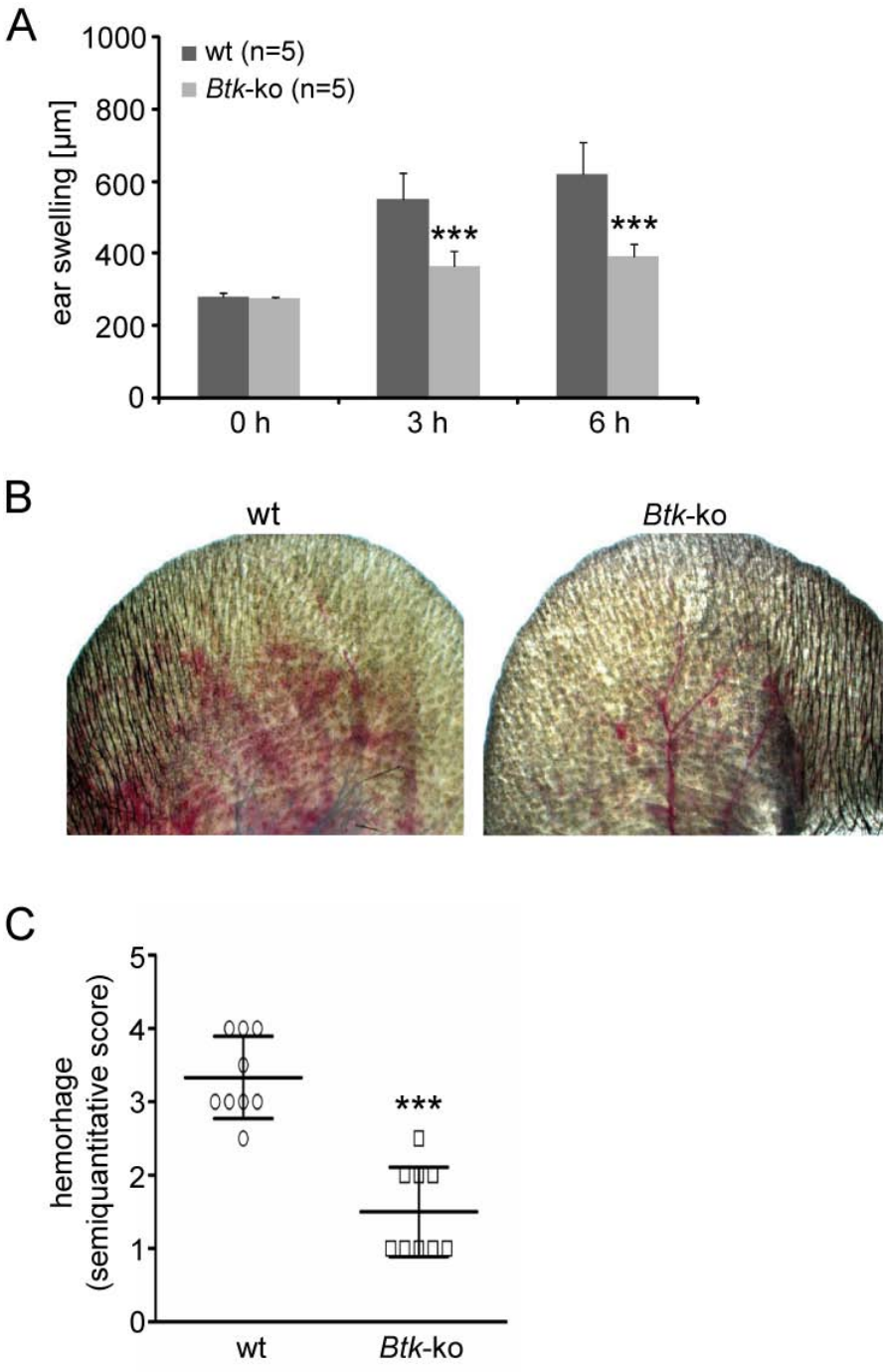


Figure 3.12 Vascular destruction after deposition of IC in the left ear of Btk-deficient mice.

The Arthus reaction was elicited in the left ear of wild type (wt) or Btk-deficient (*Btk*-ko) mice. (A) By measuring ear thickness before and during Arthus reaction, ear swelling was assessed as parameter of edema and neutrophil infiltration. Data presented are mean values of ear thickness (± SD). ***$P \leq 0.0005$. n represents the number of biological replicates. (B) After 8 hours, pictures of the treated ears were taken for (C) the semiquantitative scoring of petechiae and hemorrhage as marker of progressive, extensive vascular damage.

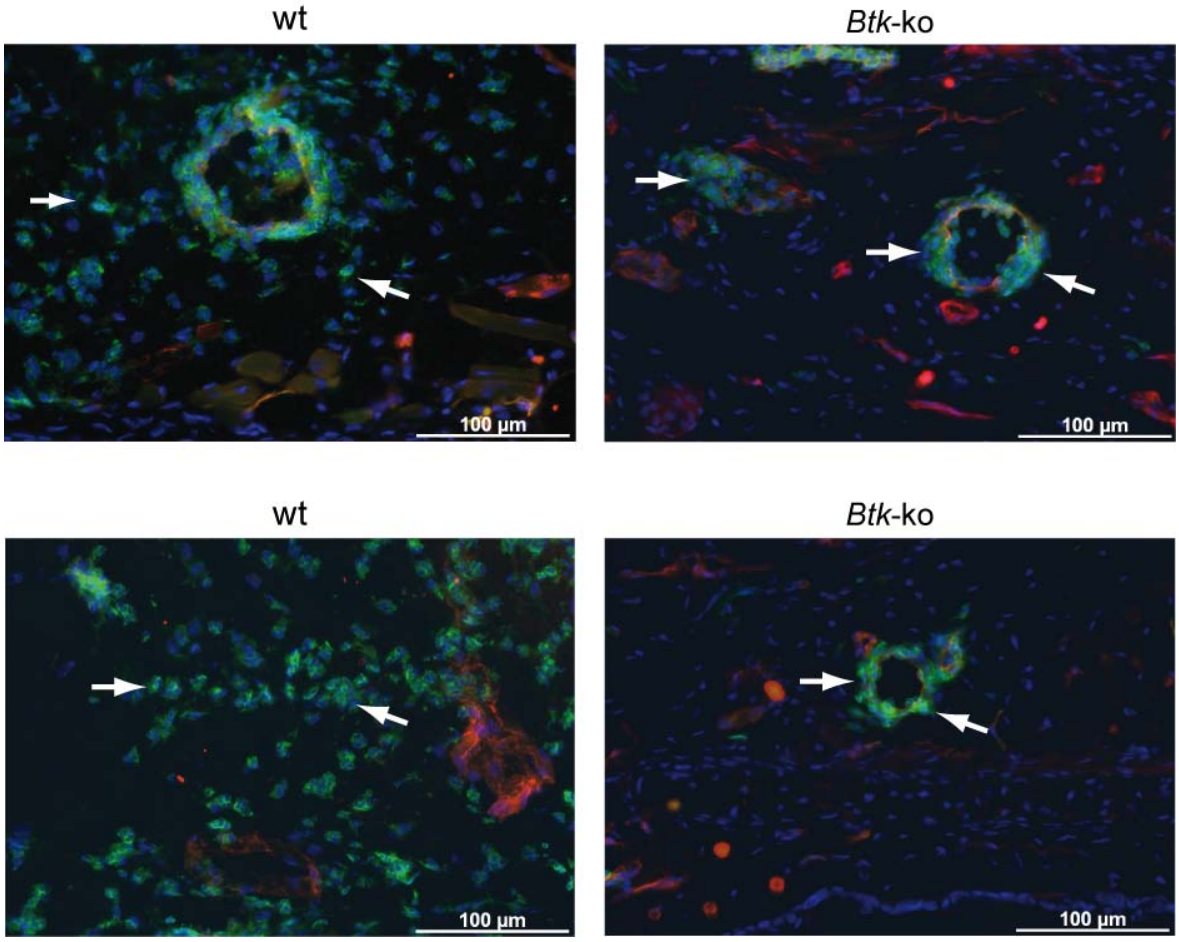


Figure 3.13 Infiltration of neutrophils in response to IC deposition in Btk-deficient mice.

Eight hours after the Arthus reaction was elicited in wild type (wt) and Btk-deficient (*Btk*-ko) mice, treated ears were harvested, embedded, and processed for immunofluorescence analyses. Slides were stained with anti-Gr-1 for neutrophils (green), anti-CD31 for endothelial cells of the blood vessels (red) and DAPI (blue). Arrowheads indicate the position of neutrophils.

3.3.2 Analysis of Immune Function of Btk-deficient Neutrophils *in vitro*

Still, it remains questionable, if the reduced expression of granule proteins leads to functional consequences regarding granule protein liberation and function after neutrophil activation. Therefore, the reduction in tissue migration of Btk-deficient neutrophils observed in the Arthus reaction could be caused by a decreased ability to destruct the basement membrane due to the diminished expression of granule proteins. In addition, the impaired terminal differentiation of neutrophils in Btk-deficient mice could also interfere with other processes needed for neutrophil function. Also, the survival of Btk-deficient neutrophils could be shortened caused

by the impaired terminal differentiation or missing Btk function and hamper the immune function of neutrophils.

To gain insight in these different issues of neutrophil biology several *in vitro* analyses were performed. First, we examined the age-induced apoptosis as well as the rescue from apoptosis by the cytokine GM-CSF, since GM-CSF is released to prolong the short basal lifespan of neutrophils during an acute inflammatory response.

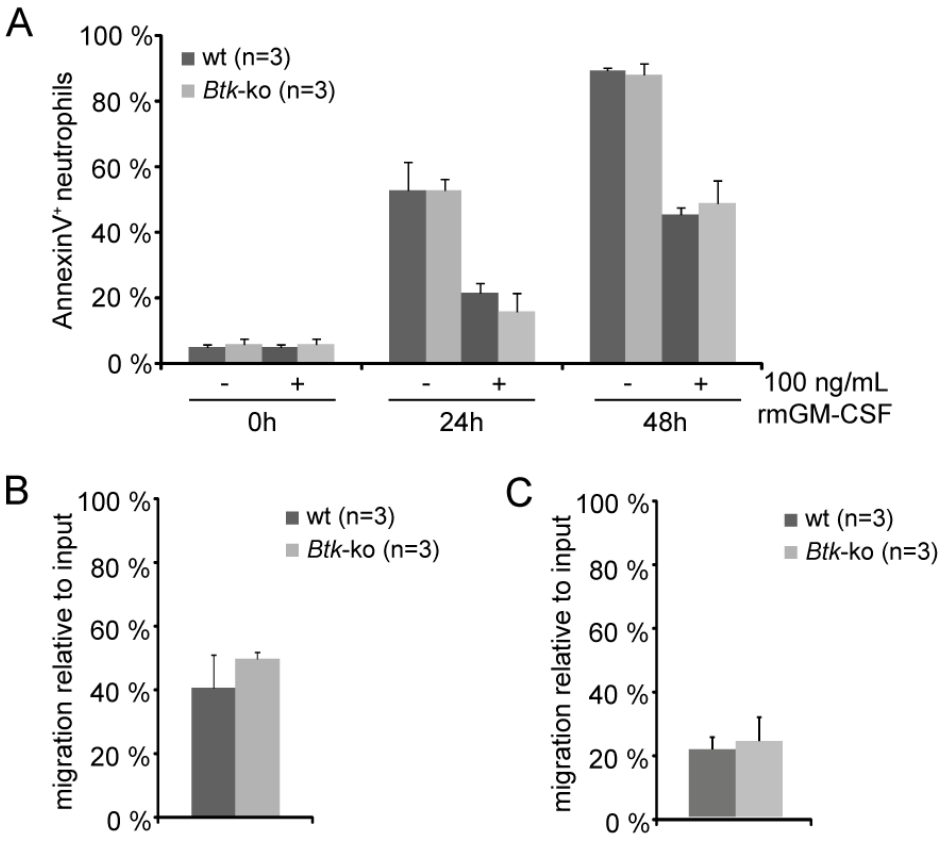


Figure 3.14 Apoptosis, chemotaxis and transendothelial migration of Btk-deficient neutrophils.

Neutrophils isolated from the bone marrow of wild type (wt) and Btk-deficient (*Btk*-ko) mice were (A) either left untreated or treated with GM-CSF as indicated to analyze protection from apoptosis under conditions mimicking an acute inflammatory response. (B) Isolated neutrophils were analyzed for their capacity to migrate towards an fMLP (10 µM) gradient in a migration assay. (C) Freshly isolated neutrophils were loaded onto a TNFα-activated endothelial monolayer and analyzed for their ability to transmigrate through the endothelium towards 10 µM fMLP. After incubation for 4 h the transmigrated cells were harvested and counted by flow cytometry relative to total input. Data presented are the mean values (± SD). n represents the number of biological replicates.

Therefore, neutrophils isolated from the bone marrow of wild type and Btk-deficient mice were cultured without any stimulus or in the presence of 100 ng/mL rmGM-CSF over a time course of 48 h. The amount of apoptotic cells was analyzed by flow cytometry for annexin-V surface expression (Figure 3.14A). The obtained data revealed no differences in apoptosis between wild type and Btk-deficient neutrophils neither for age-induced apoptosis nor for protection from apoptosis due to cytokine treatment.

Next, we evaluated the transendothelial migration and chemotaxis of neutrophil granulocytes *in vitro*, because both processes are essential for the recruitment of neutrophils from blood stream into tissue to the site of inflammation. To assess the transendothelial migration ability of neutrophils, an endothelial monolayer was established on permeable supports using mouse bEnd.5 endothelioma cells. After conformation of monolayer integrity and activation of the endothelial cells with TNFα over night, neutrophils isolated from bone marrow were loaded on top of the endothelium and transendothelial migration was induced using a chemoattractant gradient set by the bacterial product fMLP. After incubation for 4 h, amount of neutrophils migrated into the lower chamber was analyzed using flow cytometry. For analysis of chemotaxis, neutrophils isolated from bone marrow of wild type and Btk-deficient mice were loaded on permeable supports and migration towards the chemokine fMLP present in the lower chamber was measured after 4 h of incubation. In both experiments, we observed no obvious differences between wild type and Btk-deficient neutrophils (Figure 3.14B,C).

After exclusion of changes in apoptosis rate as well as alterations in transendothelial migration and in chemotaxis of Btk-deficient neutrophils as a cause for the impaired recruitment of neutrophils in the acute inflammatory response, the decreased expression of granule proteins still remains as a possible explanation. Upon activation of neutrophils the granule content is liberated into the phagolysosome to allow the destruction of phagocytosed particles via ROS production and proteases as well as bactericidal peptides. Additionally, granule content is also released into the extracellular space to destroy the extracellular matrix locally, which facilitates the motion of neutrophils in tissue. The process of granule liberation, also referred as degranulation, is induced by stimuli like opsonized particles that are recognized by complement receptors as well as Fcγ receptors, or by bacteria derived molecules like formyl peptides. To evaluate

the capacity to release granule proteins, immune complex-induced degranulation assays were performed *in vitro*. Neutrophils isolated from bone marrow of wild type and Btk-deficient mice were plated on 96-well plates coated with immune complexes that consisted of BSA and anti-BSA antibody and incubated with fluorescence-quenched elastin or gelatin. After degranulation, neutrophil elastase or gelatinase digested their cognate substrates, which resulted in the removal of the quencher and release of the fluorescence.

In line with our previous results obtained by RT-PCR or TEM, we found a clearly reduced release of neutrophil elastase and gelatinase upon IC-induced degranulation in neutrophils lacking Btk compared with wild type neutrophils (Figure 3.15). Together, these data demonstrated substantial alterations regarding neutrophil function that are due to the lowered granule numbers in Btk-deficient granulocytes as well as to the reduced expression and consequently the impaired release of granule proteins after neutrophil activation.

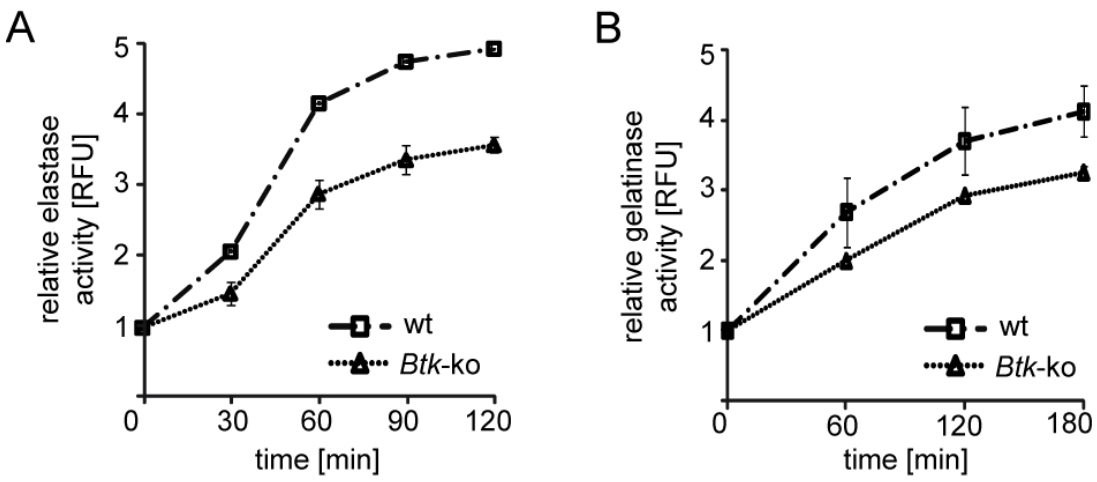


Figure 3.15 IC-induced degranulation of neutrophils lacking Btk.

The relative activity of released neutrophil elastase (A) and gelatinase (B) upon immune complex-induced degranulation of wild type (wt) or Btk-deficient (*Btk*-ko) neutrophils is presented. Data presented are the mean values (± SD).

3.4 Molecular Mechanisms Provoking Impaired Differentiation and Function of Btk-Deficient Neutrophils

After the discovery of considerable alterations in the granulopoiesis in Btk-deficient mice associated with maturation defects and functional impairments during an inflammatory response, we were interested in revealing the molecular mechanisms behind the defective granulopoiesis. First, we wondered whether Btk is activated in response to cytokines involved in granulopoiesis and monopoiesis such as GM-CSF, which we have used in the analyses of differentiation capacity downstream of GMP.

For this purpose, we have investigated the Btk phosphorylation status in response to stimulation with rmGM-CSF in MACS-isolated $CD11b^+$ myeloid cells from bone marrow of wild type mice. In these experiments, we found a significant phosphorylation of Btk at the position Y551 already after 5 min of stimulation with GM-CSF (Figure 3.16A). However, beside Btk phosphorylation different signaling events are induced upon GM-CSF receptor ligation. One of the earliest events in response to GM-CSF receptor engagement is the phosphorylation and activation of the protein signal transducer and activator of transcription 3 (Stat3) by Janus kinases, which results in activation of Stat3-dependent gene transcription. Additionally, GM-CSF receptor ligation promotes the activation of the phopsphoinositide-3-kinase (PI3K) pathway associated with several further events such as Akt phosphorylation and activation.

To analyze the signaling events downstream of the GM-CSF receptor, we have used erythrocytes-depleted bone marrow cells of wild type as well as Btk-deficient mice that were enriched for myeloid cells up to 60 % and treated with rmGM-CSF over a time course of 20 min. The subsequent evaluation for the phosphorylation status of downstream effectors of the GM-CSF receptor demonstrated no changes in the Stat3 phosphorylation at position Y705 and Stat3 expression in Btk-deficient myeloid cells. Whereas, the phosphorylation of the regulatory subunit p85 of PI3K at position Y458, Akt at position T308 and glycogen synthase kinase-3β (GSK-3β) at position S9 was clearly reduced in myeloid cells lacking Btk in comparison to wild type myeloid cells. Furthermore, these data were confirmed using enriched myeloid bone marrow cells obtained from *Xid*-mice.

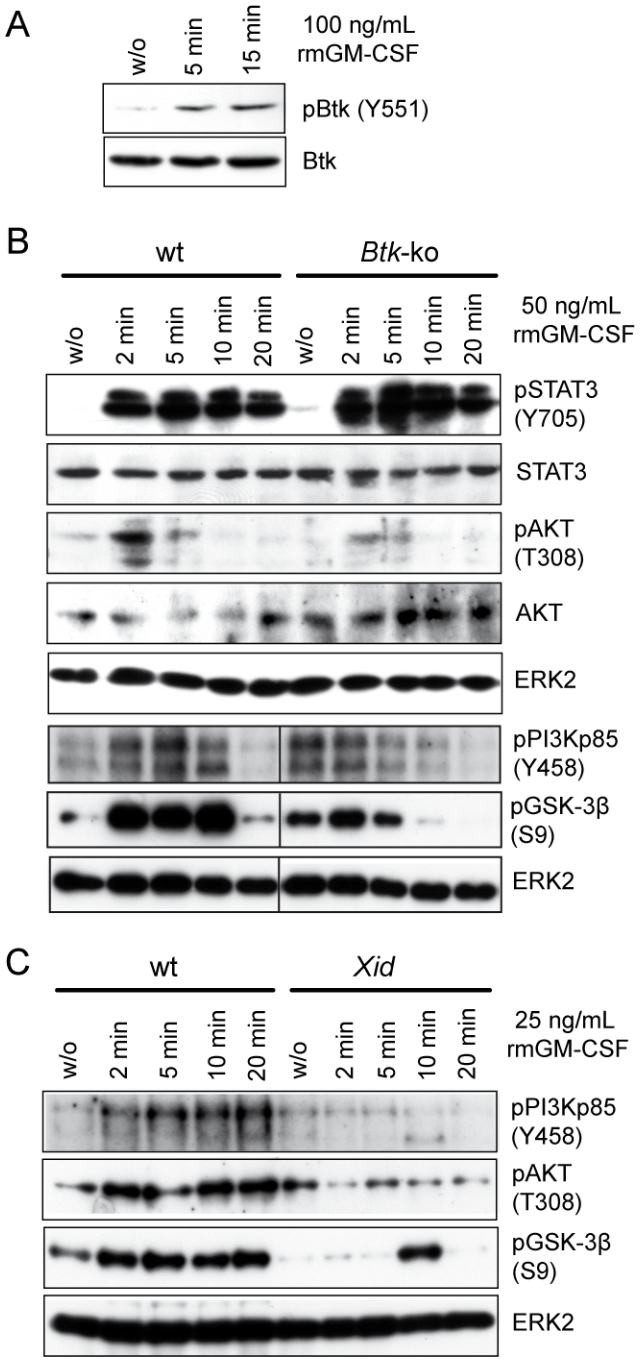


Figure 3.16 Involvement of Btk in GM-CSF-mediated signaling.

(A) MACS-sorted CD11b$^+$ cells from the bone marrow of wild type were treated for the indicated time with GM-CSF. Cells were analyzed for the phosphorylation of Btk at position Y551 (Btk pY551). Afterwards the membrane was stripped and analyzed for the amount of total Btk protein (Btk). (B, C) Erythrocytes-depleted bone marrow cells of wild type (wt) or Btk-deficient (*Btk*-ko) mice or *Xid*-mice were treated for the indicated time with GM-CSF. Cells were analyzed for the phosphorylation of Stat3 (pStat3), Akt (pAkt), PI3K (pPI3Kp85) and GSK-3β (pGSK-3β□ as well as for the total amounts of Stat3, Akt and Erk2 proteins.

The isolated myeloid cells were again stimulated with GM-CSF in a kinetic for up to 20 min and bared similar alterations in the phosphorylation status of PI3K, Akt and GSK-3β (Figure 3.16B,C).

The importance of PI3K-dependent signaling events for myelopoiesis was demonstrated in a study recently published, where the influence of PI3K inhibition on the G-CSF-induced myelopoiesis in human $CD34^+$ hematopoietic progenitors was analyzed. In this experimental setup, a decreased differentiation of progenitor cells into neutrophils or monocytes that was accompanied with a reduced expression of lactoferrin and CD14 was found [178]. Moreover, it could be demonstrated that PI3K activation led to Akt activation that in turn inactivated GSK-3β by phosphorylation. Finally, the inactivation of GSK-3β resulted in a dephosphorylation of the transcription factor C/EBPα, which is known to be the master transcription factor for myeloid differentiation. An earlier study has already revealed that activated GSK-3β phosphorylates C/EBPα at position T222/226 [179]. In addition, the authors could show the need of dephosphorylated C/EBPα for proper induction of lineage-specific gene transcription.

Taken together, these data prompted us to investigate the expression level of C/EBPα and C/EBPα-dependent genes in Btk-deficient whole bone marrow cells in comparison to wild type whole bone marrow cells. Indeed, analysis of *Cebpa* RNA level by quantitative PCR as well as of C/EBPα protein expression by immunoblot demonstrated a significant lower expression of C/EBPα in myeloid cells lacking Btk (Figure 3.17A,B). In line with these findings, the expression of several known C/EBPα target genes important for myelopoiesis, such as *Sfpi1* (PU.1), *Csf1r* (M-CSFR) or *Csf2ra* (GM-CSFRα) was drastically reduced in neutrophils isolated from bone marrow of Btk-deficient mice (Figure 3.17C). Further evidence for attenuated C/EBPα function provided the finding that the RNA expression level of *Cebpb* (C/EBPβ) was downregulated in Btk-deficient neutrophils, since C/EBPβ is another important transcription factor necessary for neutrophil maturation and granule protein expression and a target of C/EBPα (Figure 3.17D).

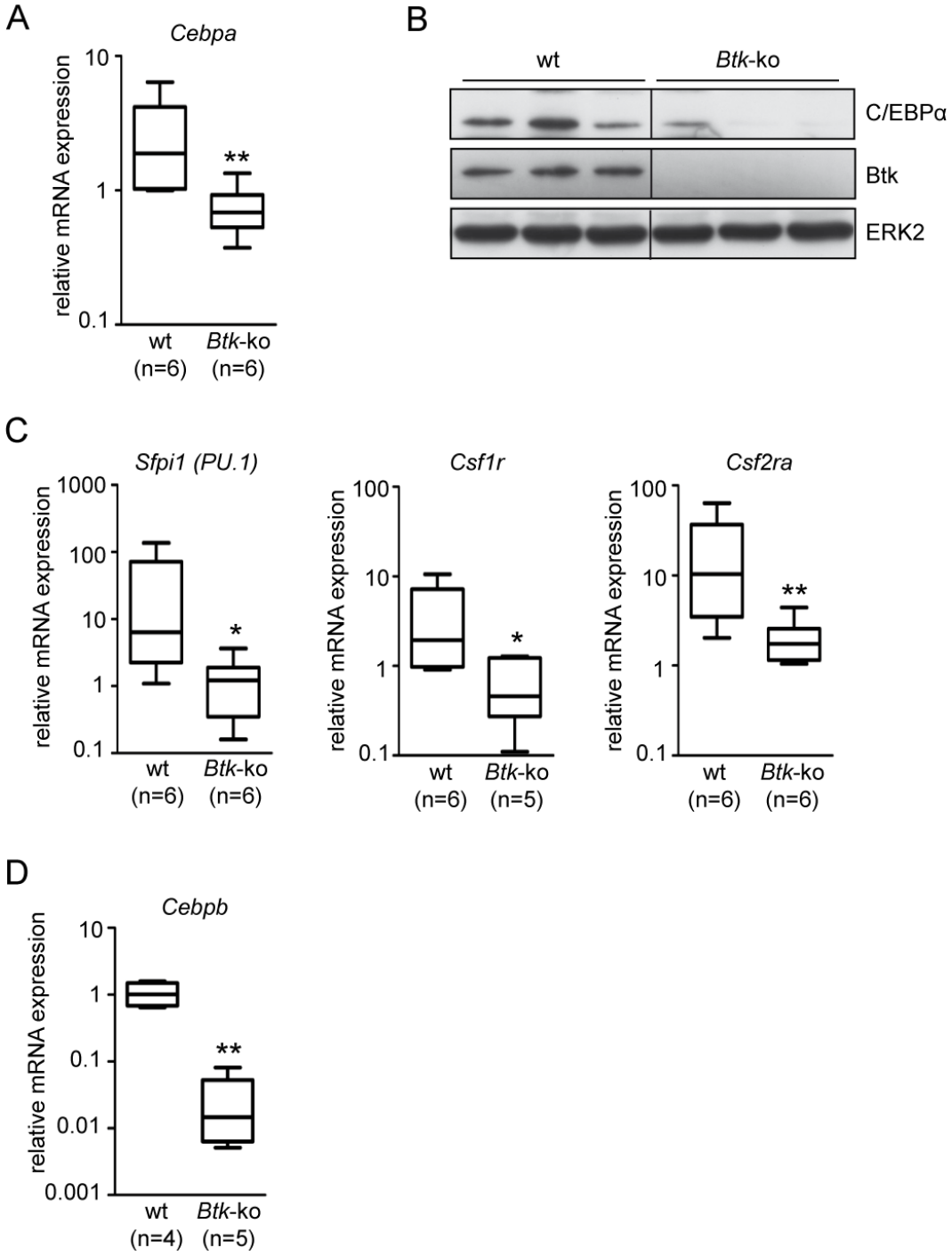


Figure 3.17 Expression of lineage-determining transcription factors and lineage-specific genes in the bone marrow of Btk-deficient mice.

(A) Total bone marrow cells were isolated from wild-type (wt) and Btk-deficient (*Btk*-ko) mice and analyzed for the mRNA expression of C/EBPα (*Cebpa*) relative to the expression of *Gapdh*. (B) Bone marrow cells of mice were analyzed for the expression of C/EBPα. Antibody against Btk was used as a control for the genotype of mice. An anti-Erk2 antibody serves as loading control. (C) Total bone marrow cells were isolated and analyzed for the mRNA expression of PU.1 (*Sfpi1*), M-CSFR (*Csf1r*) and GM-CSFRα (*Csf2ra*) relative to the expression of *Gapdh*. (D) Bone marrow derived neutrophils were

isolated mice and analyzed for the expression of C/EBPβ (*Cebpb*) relative to the expression of β-actin (*Actb*). Data presented are mean values (±SD). *P ≤ 0.05, **P ≤ 0.005. *n* represents the number of biological replicates.

To gain more insight into the molecular network that guides myelopoiesis and particularly the involvement of Btk, we have also analyzed the expression of Btk in the different myeloid progenitor populations that were isolated from bone marrow of Btk-deficient and wild type mice by FACS. The highest Btk expression was detected in CMP, but considerable amounts of Btk were also detectable in MEP and GMP.

In parallel, we assessed the expression levels of *Cebpa*, *Sfpi1* and *Gata1*, which are the master transcription factors for granulocyte, monocyte and megakaryocyte-erythrocyte development, respectively. Interestingly, we were not able to detect gross alterations between wild type and Btk-deficient CMP or GMP with respect to *Cebpa* or *Sfpi1* expression. However, the RNA expression level of known C/EBPα targets such as *Csf2ra* or *Csf3r* (G-CSFR) was reduced in Btk-deficient GMP. Moreover, the neutrophil-specific genes neutrophil elastase (*Elane*) and myeloperoxidase (*Mpo*) are also reduced in their expression levels in GMP lacking Btk (Figure 3.18).

In summary, these findings clearly demonstrated the activation of Btk upon GM-CSF receptor ligation and an impaired activation of the PI3K pathway in the absence of a functional Btk. Furthermore, expression and transcriptional activity of the transcription factor C/EBPα indispensable for granulocyte lineage commitment was reduced in Btk-deficient bone marrow cells. This was shown by the decrease of C/EBPα in whole bone marrow extracts and the lowered expression of C/EBPα target genes.

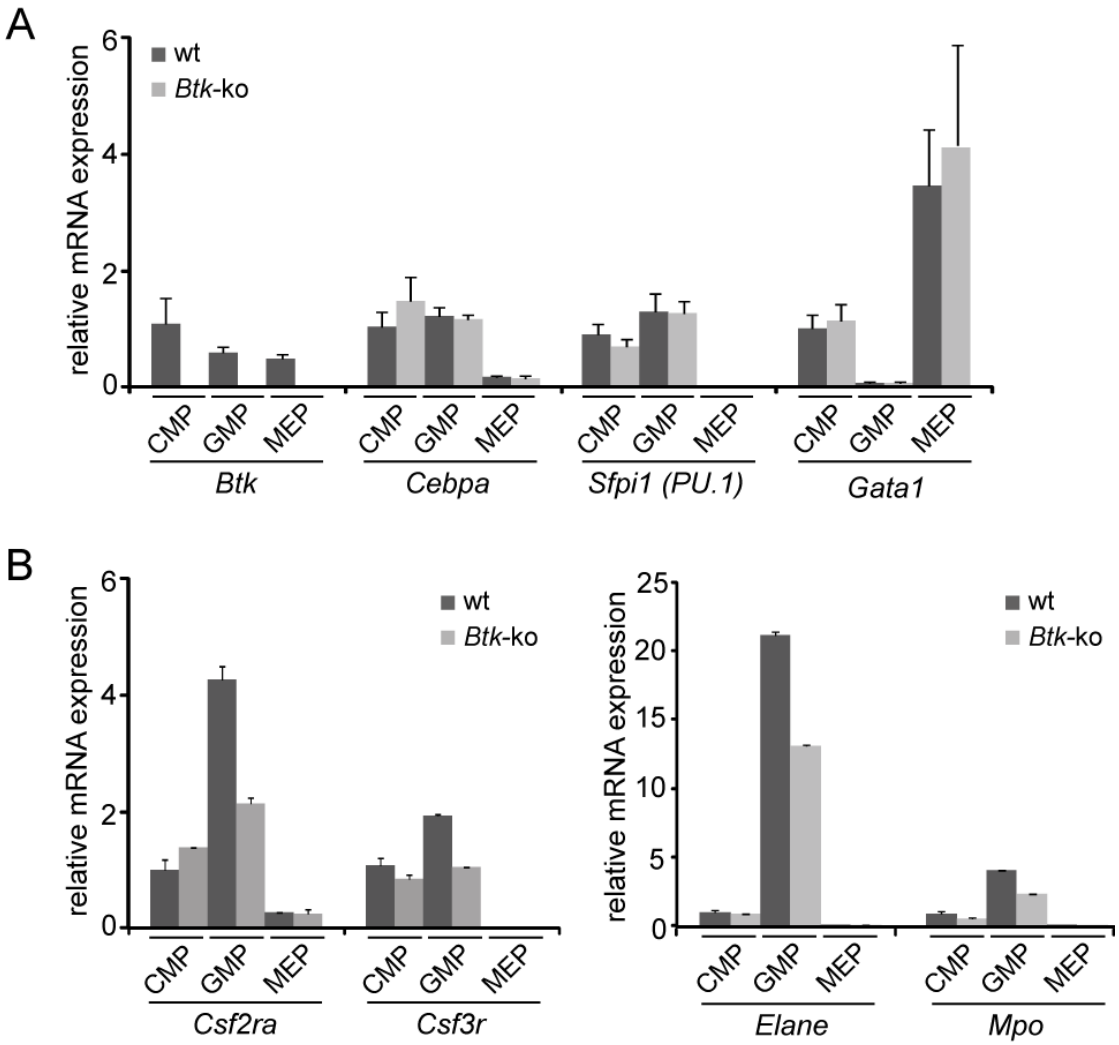


Figure 3.18 Influence of Btk in myeloid progenitors on expression of transcription factors important for early granulopoiesis.

CMP, GMP and MEP were isolated from the bone marrow wild type (wt) and Btk-deficient (*Btk*-ko) mice by FACS. (A) RNA was prepared and analyzed for the mRNA expression of Btk (*Btk*), and the lineage-determining transcription factors C/EBPα (*Cebpa*), PU.1 (*Sfpi1*), GATA1 (*Gata1*) by quantitative PCR relative to the expression of *Hprt*. (B) Additionally, mRNA expression of lineage-specific target genes of C/EBPα like GM-CSFRα (*Csf2ra*), G-CSFR (*Csf3r*), elastase (*Elane*) and myeloperoxidase (*Mpo*) was analyzed by quantitative PCR relative to the expression of *Hprt*. Data presented are mean values (±SD).

3.5 Cell-Autonomous Effects of Btk in Neutrophils

The results presented so far suggest a cell-autonomous effect of Btk in myelopoiesis. However, the Btk-deficient animal model is compromised by the systemic deletion of the *Btk* gene. For that reason, other explanations for the altered granulopoiesis in Btk-deficient mice are possible and should be taken in concern. For example, the known B cell defect could interfere with the regulation of hematopoiesis. Otherwise, alterations within the hematopoietic niche could influence the outcome of hematopoiesis towards an increase in granulopoiesis.

To investigate whether Btk plays a cell-intrinsic role in neutrophil development and function, syngeneic bone marrow transplantations were performed. The Btk-deficient mice as well as the wild type littermates used in the present work express the CD45.2 allelic form of the hematopoietic cell-specific protein tyrosine phosphatase CD45, which can be distinguished by a specific antibody from the allelic form CD45.1. Therefore, we used lethally irradiated wild type mice expressing the allelic form CD45.1 as recipient mice and transplanted 5 million isolated nucleated bone marrow cells from wild type or Btk-deficient $CD45.2^+$ mice by intravenous injection. Moreover, we used wild type $CD45.1^+$ bone marrow cells and $CD45.2^+$ Btk-deficient bone marrow cells and mixed them in 3 different ratios prior to transplantation into lethally irradiated $CD45.1^+$ wild type mice to generate chimeras. The competitive reconstitution of the hematopoietic system with wild type and Btk-deficient bone marrow simultaneously would allow distinguishing between a cell-intrinsic effect of Btk in neutrophils and a cell-extrinsic effect of Btk due to alterations in the B cell compartment or in the hematopoietic niche.

First, we investigated the success of the transplantation and reconstitution of the hematopoietic system 4 weeks after bone marrow transplantation with the help of blood analyses using the ABC and flow cytometry. The obtained white blood count revealed a reconstitution of the hematopoietic system in almost all mice with one exception were we could detect nearly no leukocytes in the blood. Interestingly, mice that had received Btk-deficient bone marrow showed clearly reduced numbers of white blood counts per µL blood due to lower numbers of B cells, similar to the B cell reduction described for Btk-deficient mice [8,9]. Furthermore, the B cell reduction could be corrected by increasing amount of wild type bone marrow cells transplanted into the recipient mice. With respect to the granulocyte population, we found a marked augmentation in the number of neutrophil

granulocytes in the blood when Btk-deficient bone marrow cells were transplanted. Moreover, increasing amounts of wild type bone marrow cells rescued the phenotype of enhanced granulocyte numbers in the blood (Figure 3.19).

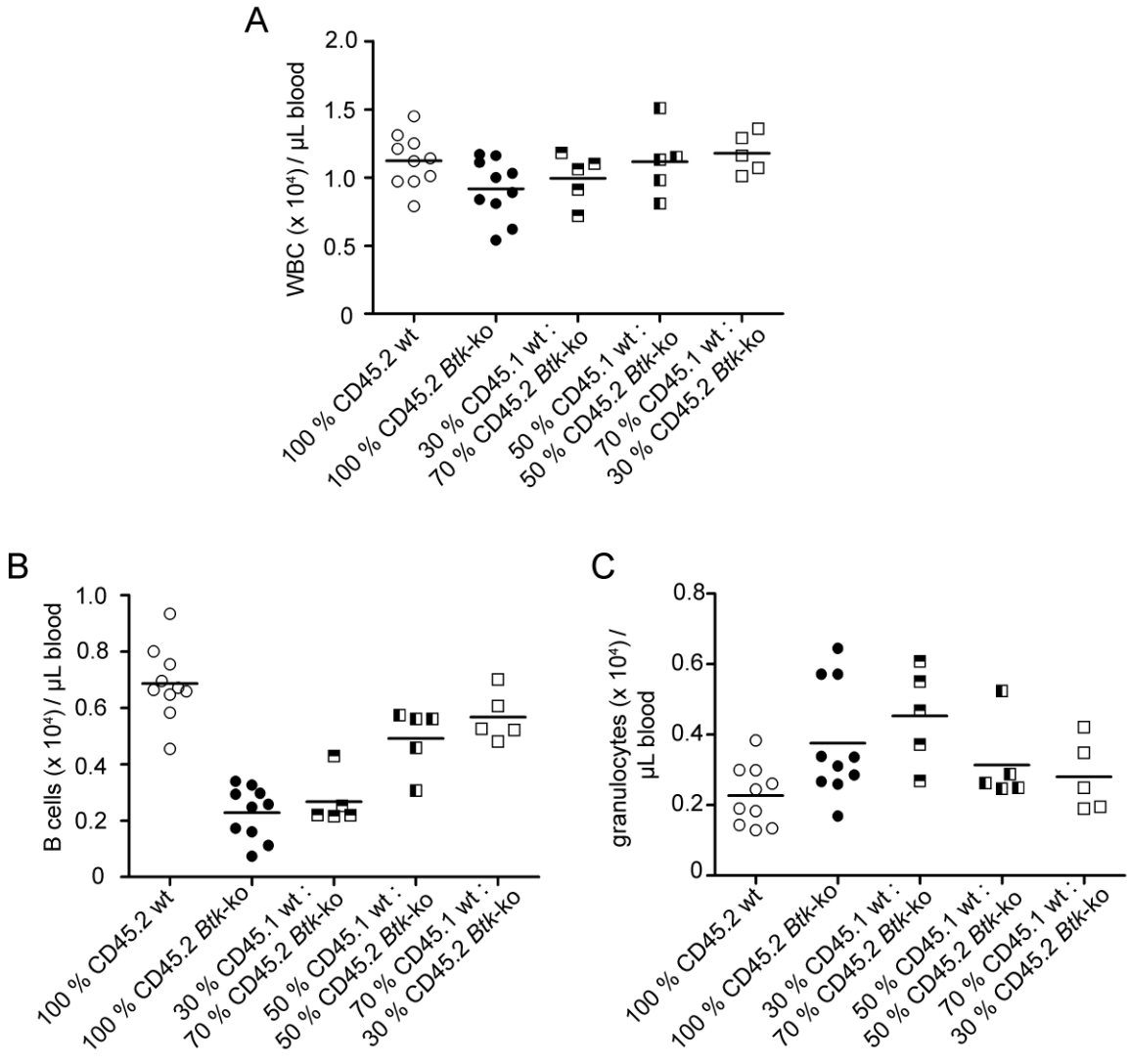


Figure 3.19 Generation of chimeric mice.

Chimeric mice were generated by transplantation of either CD45.2$^+$ wild type (wt) or Btk-deficient (*Btk*-ko) bone marrow cells into lethally irradiated CD45.1$^+$ recipient mice. Alternatively, lethally irradiated CD45.1$^+$ recipient mice received a mixture of wild type CD45.1$^+$ and Btk-deficient CD45.2$^+$ bone marrow cells in different ratios. Blood analyses were performed 4 weeks after transplantation by use of an Animal Blood Counter and flow cytometry. (A) White blood count (WBC) per µL blood is presented. (B) B cell numbers (B220$^+$ leukocytes) per µL blood are shown. (C) Neutrophil granulocytes (CD11b$^+$/Gr-1$^+$ leukocytes) per µL blood are presented.

Next, we analyzed the origin of the B cells and granulocytes in the blood with the help of the allelic differences in the CD45 marker to confirm the success of the reconstitution and to reveal developmental advantages of wild type or Btk-deficient bone marrow cells in both hematopoietic compartments.

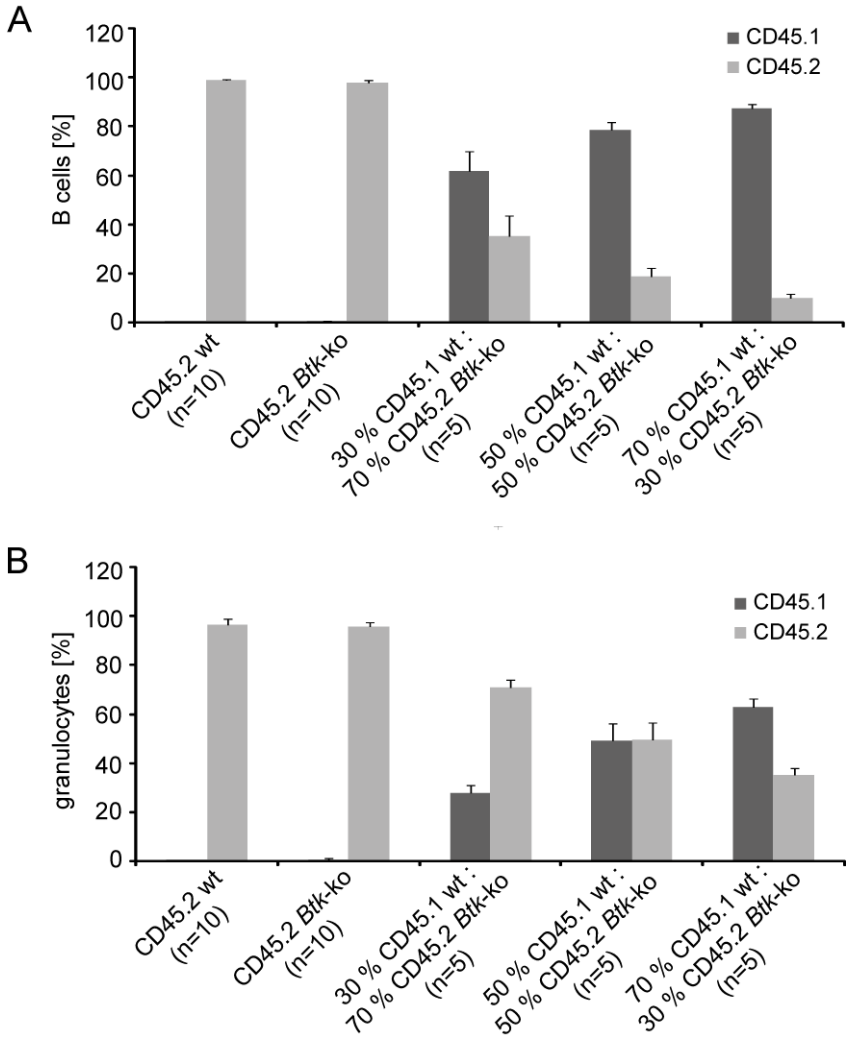


Figure 3.20 Origin of B cells and granulocytes in the blood of chimeric mice.
Chimeric mice were analyzed 4 weeks after transplantation by flow cytometry with regard to the origin of leukocytes in blood using antibodies against the different CD45 allelic forms. (A) Percentage of either wild type (wt) B cells and Btk-deficient (*Btk*-ko) $CD45.2^+$ B cells or wild type $CD45.1^+$ and Btk-deficient $CD45.2^+$ B cells of the whole B cell population ($B220^+$ leukocytes) in the blood is presented. (B) Percentage of either wild type and Btk-deficient $CD45.2^+$ granulocytes or wild type $CD45.1^+$ and Btk-deficient $CD45.2^+$ granulocytes of the whole granulocyte population ($CD11b^+/Gr\text{-}1^+$ leukocytes) in the blood is presented. Data presented are mean values (±SD). *n* represents the number of biological replicates.

The competitive transplantations were done either with 70 %, 50 % or 30 % Btk-deficient CD45.2$^+$ bone marrow cells together with increasing amounts of CD45.1$^+$ wild type bone marrow cells. But the obtained data regarding the B cell compartment clearly demonstrated an starting ratio of 60 % wild type CD45.1$^+$ B cells when only 30 % were transplanted, which increased up to 90 % wild type B cells in the 70 % wild type transplanted mice (Figure 3.20A). These data indicated obviously a developmental advantage of wild type B cells over Btk-deficient B cells in a competitive situation. Additionally, the enhanced amounts of wild type B cells were not able to change the extended granulopoiesis of Btk-deficient cells, which implies a cell-intrinsic effect of Btk in neutrophils.

Analyzing granulocyte populations, we could not detect a shift in the origin of the cells like for the B cell compartment, meaning that the ratios of the CD45 allelic forms expressed on the surface of the granulocytes in the blood perfectly matched the transplanted amounts of wild type CD45.1$^+$ and Btk-deficient CD45.2$^+$ bone marrow cells (Figure 3.20B).

After 6 weeks of reconstitution, we investigated the neutrophil granulocyte compartment in the bone marrow of transplanted mice concerning the maturation and the origin of neutrophils. Transplantation of wild type and Btk-deficient bone marrow cells at different ratios again revealed that the absence of Btk favored granulopoiesis. This is displayed by the increase in CD45.2$^+$ Btk-deficient granulocytes in the myeloid bone marrow compartment up to 90 % when only 70 % Btk-deficient bone marrow cells were transplanted, and the increase over 50 % when only 30 % Btk-deficient cells were injected (Figure 3.21A). Moreover, granulocytes lacking Btk exhibited again an immature phenotype determined by the expression level of the surface markers CD11b and Gr-1 on bone marrow granulocytes in the competitive transplantation experiments (Figure 3.21B). The significant augmentation in immature neutrophils in the context of Btk deficiency was even more emphasized in the mice that had received 100 % of Btk-deficient bone marrow cells in comparison to mice that had received 100 % wild type bone marrow (Figure 3.21C).

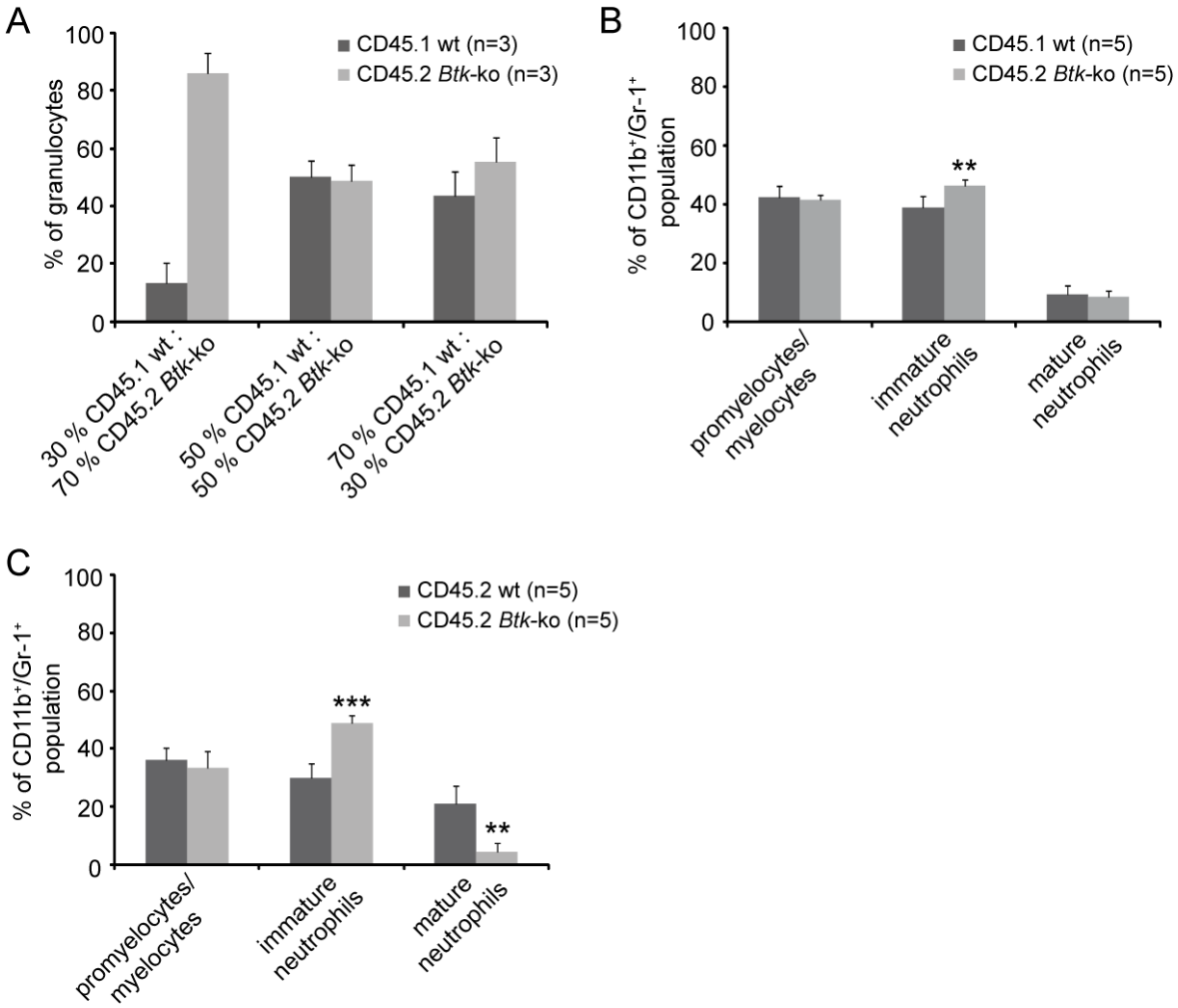


Figure 3.21 Analysis of the neutrophil granulocyte compartment in the bone marrow of chimeric mice.

The bone marrow of mixed bone marrow chimeras that received CD45.1$^+$ wild type (wt) and CD45.2$^+$ Btk-deficient (*Btk*-ko) bone marrow cells in different ratios was analyzed 6 weeks after transplantation by flow cytometry. (A) The reconstitution of the neutrophil compartment in mixed CD45.1/CD45.2 chimeric mice was examined concerning the origin of the neutrophils. (B) The maturation status of the CD11b$^+$/Gr-1$^+$ population in mixed CD45.1/CD45.2 chimeric mice is presented. (C) Granulocytes obtained from lethally irradiated CD45.1$^+$ recipient mice that received bone marrow from CD45.2$^+$ wild type or CD45.2$^+$ Btk-deficient mice were analyzed for their maturation status. Data presented are mean values (±SD). **P ≤ 0.005, ***P ≤ 0.0005. *n* represents the number of biological replicates.

Taken together, the data obtained from the transplantation experiments suggest a cell-intrinsic effect of Btk on the enhanced granulopoiesis that is associated with a hampered maturation and results in an immature phenotype of the Btk-deficient

neutrophils. To clarify further the maturation defect of neutrophil granulocytes in the mixed transplanted mice, we have sorted the bone marrow neutrophils with regard to the allelic form of the surface molecule CD45 by FACS and analyzed the expression of several genes important for granulocyte differentiation.

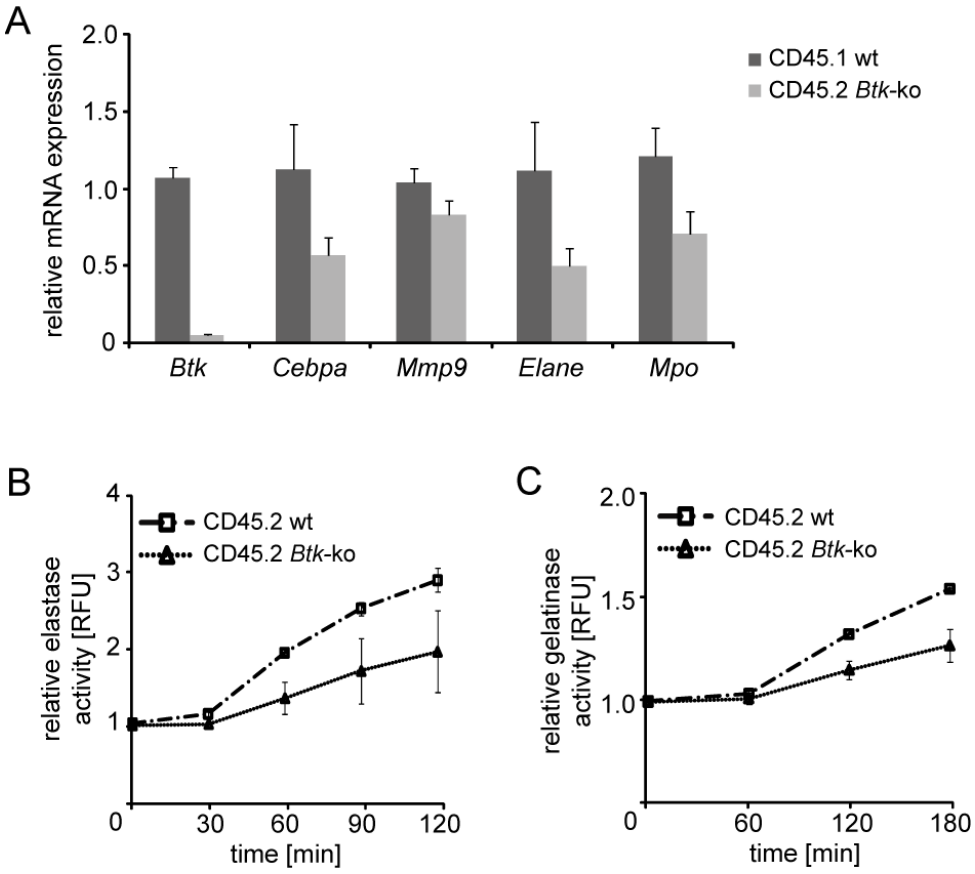


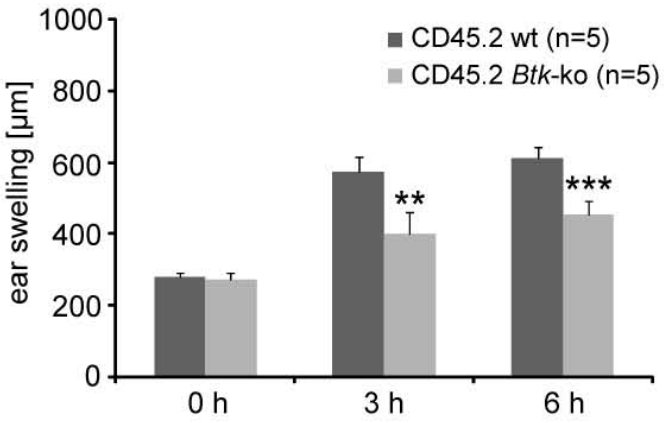
Figure 3.22 Expression and activity of granule proteins in neutrophils obtained from chimeric mice.

(A) CD45.1$^+$ wild type (wt) and CD45.2$^+$ Btk-deficient (*Btk*-ko) neutrophils were sorted by FACS out of the bone marrow of mixed chimeric mice 6 weeks after transplantation. Purified neutrophils were analyzed for the expression of Btk as control and for the transcription factor C/EBPα (*Cebpa*) as well as for the granule proteins gelatinase (*Mmp9*), neutrophil elastase (*Elane*) and myeloperoxidase (*Mpo*) by quantitative PCR relative to the expression of *Hprt1*. CD45.2$^+$ wild type and CD45.2$^+$ Btk-deficient neutrophil granulocytes were isolated from the bone marrow of CD45.1$^+$ recipient mice 6 weeks after transplantation and IC-induced release and activity of elastase (B) and gelatinase (C) was measured in a degranulation assay.

Once more, Btk-deficient neutrophils showed a decreased expression of the lineage-determining transcription factor C/EBPα and of several granule-specific genes such as gelatinase (*Mmp9*), neutrophil elastase (*Elane*) or myeloperoxidase (*Mpo*) (Figure 3.22A). Additionally, neutrophils isolated from bone marrow of mice that had received either wild type CD45.2$^+$ cells or Btk-deficient CD45.2$^+$ cells were used in an immune complex-induced degranulation assay and the release of elastase and gelatinase was measured. Similar to the previous findings, neutrophils lacking Btk exhibited a decreased release of elastase as well as gelatinase in response to IC-induced degranulation (Figure 3.22B,C).

Last but not least, we have examined mice that had received wild type or Btk-deficient CD45.2$^+$ bone marrow cells in the reverse passive Arthus reaction after a reconstitution time of 8 weeks. In line with former results using wild type and Btk-deficient mice, we observed a significant decrease in edema formation, determined by ear swelling, in mice lacking Btk in neutrophils (Figure 3.23A). Again, no tissue-infiltrating neutrophils could be observed in the immunofluorescence pictures obtained from the treated ears of mice that had received Btk-deficient bone marrow, whereas in mice that had received wild type bone marrow cells a considerable neutrophil infiltration into the tissue of the treated ear was detectable (Figure 3.23B). Moreover, the reverse passive Arthus reaction in reconstituted mice further allowed dissecting the contribution of Btk-deficient mast cells to the decreased induction of the inflammatory response in mice lacking Btk, since mast cells are long living cells and reside in skin for several months after lethal irradiation of mice [180]. On the other site, reconstitution of skin mast cells after bone marrow transplantation takes up to 6 months [180]. Former studies using Btk-deficient mast cells have demonstrated the need for Btk in the FcεR-mediated mast cell activation [181] and FcR signaling as well as mast cells in general are essential for initiation of the Arthus reaction [182,183]. To exclude any contribution of functionally impaired Btk-deficient mast cells to the reduced signs of inflammation in Btk-deficient mice after eliciting the reverse passive Arthus reaction, mast cell numbers and origin were analyzed histological 8 hours after eliciting IC-mediated immune response. Staining of mast cells in the tissue was performed with toluidin on 5 µm paraffin-sections of the ears and revealed equal numbers of residing mast cells in the ear tissue of wild type and Btk-deficient reconstituted mice (Figure 3.23C).

A



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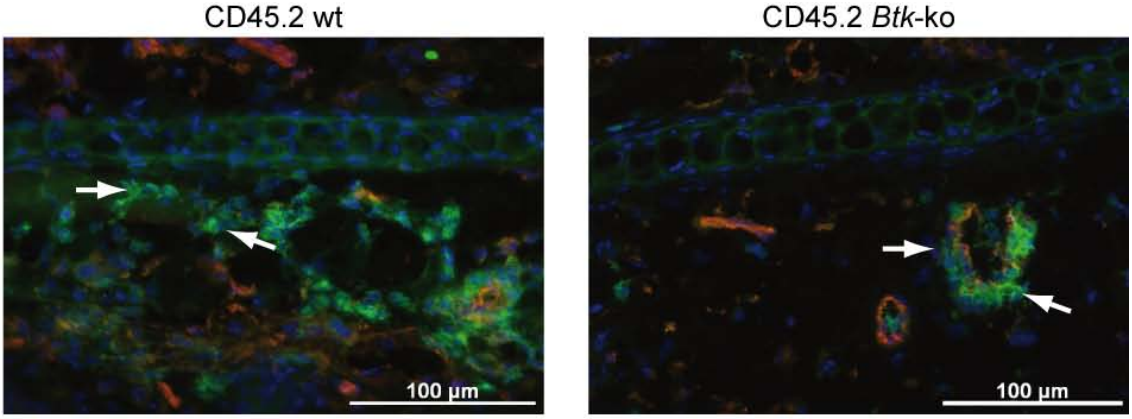

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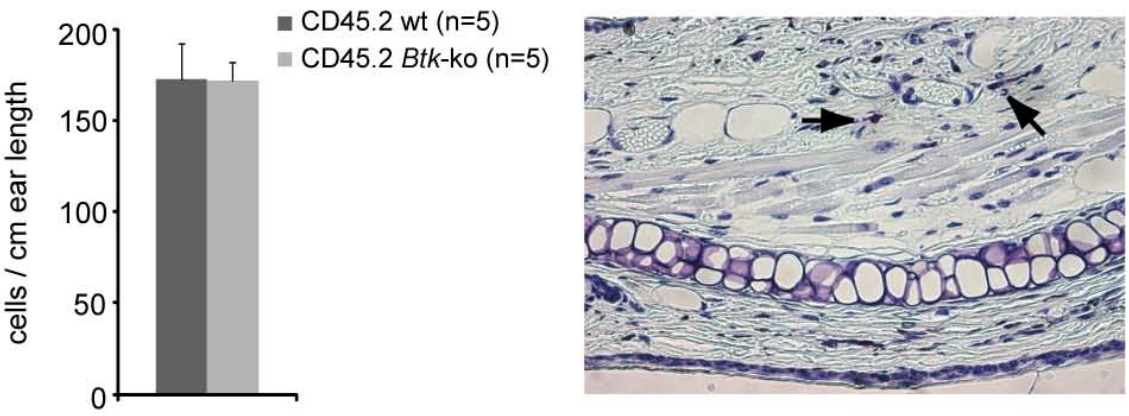

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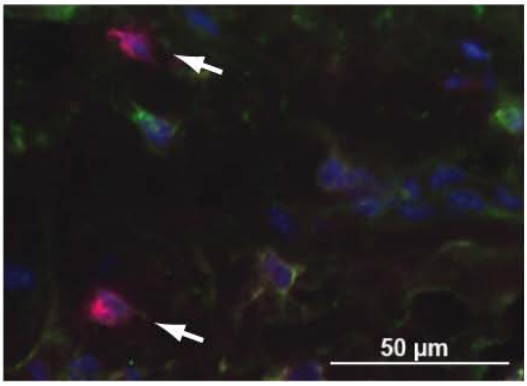

donor	CD45.2 wt	CD45.2 *Btk*-ko
Kit⁺ cells analyzed	68	111
Kit⁺ / CD45.2⁺	2	3
n	5	5

Figure 3.23 Reverse passive Arthus reaction in chimeric mice.
Lethally irradiated CD45.1$^+$ mice that had received bone marrow from CD45.2$^+$ wild type (wt) or Btk-deficient mice (*Btk*-ko) were investigated in the IC-mediated Arthus reaction (A) for ear swelling as a sign of tissue damage and edema formation. Treated ears were harvested 8 hours after elicitation of the immune response and analyzed by immunofluorescence (B) for neutrophil infiltration with anti-Gr-1 for neutrophils (green), anti-CD31 for endothelial cells of the blood vessels (red) and DAPI (blue). Arrowheads indicate the position of neutrophils. (C) In parallel, a half of the treated ears was analyzed by toluidin-staining for the presence of mast cells. (D) To determine the origin of mast cells, ear slides were analyzed by staining with anti-CD45.2 (green) for donor cells, anti-Kit (red) for mast cells and DAPI (blue) for nucleic DNA. A representative image taken from an ear of a lethally irradiated CD45.1$^+$ mouse that had received CD45.2$^+$ Btk-deficient bone marrow cells is presented. Data presented are mean values (±SD). **P ≤ 0.005, ***P ≤ 0.0005. *n* represents the number of biological replicates.

For determination of the mast cell origin, 5 µm cryo-sections were stained with anti-Kit antibody specific for mast cells in skin tissue and anti-CD45.2 antibody that is specific for donor cells. The obtained data clearly showed that the mast cells were to 97 % of host origin meaning skin mast cells had remained Btk wild type (Figure 3.23D). Therefore, the reduction in the inflammatory response found in mice that had received Btk-deficient bone marrow cells was mainly depended on the impaired neutrophil function, and not on deficiencies in mast cell functions caused by defective Btk.
In summary, these experiments demonstrated that the defect observed in Btk-deficient neutrophils is not impacted by extrinsic factors such as the bone marrow microenvironment or by the comprised B cell compartment. Because we observed a severely impaired Arthus reaction in the presence of Btk-deficient neutrophils and Btk-proficient mast cells, we conclude that Btk-deficient neutrophils are not able to mount an efficient IC-induced immune response *in vivo*.

3.6 Influence of Cytokine Treatment on Granulopoiesis in Btk-Deficient Mice

In the previous experiments we could show that myelopoiesis in mice lacking Btk expression is significantly altered towards an enhanced granulopoiesis accompanied with a retarded terminal differentiation of neutrophils. Moreover, this defect in differentiation and maturation is due to cell-intrinsic mechanisms of the granulocytes and caused by the loss of Btk function in myeloid precursors as well as myeloid cells. Therefore we wondered whether external differentiation cues like G-CSF or GM-CSF, which are well known to stimulate granulopoiesis and myelopoiesis downstream of myeloid progenitors, could induce the terminal differentiation and maturation of neutrophils in Btk-deficient mice. Especially, G-CSF as the major cytokine regulator of granulocytes might influence granulopoiesis under Btk-deficient conditions, since G-CSF not only stimulates the proliferation of neutrophil precursors but also the terminal differentiation of neutrophils [184]. Moreover, deletion of the murine G-CSF gene revealed important physiological roles for this cytokine in normal and emergency granulopoiesis. Upon infection or inflammatory conditions, G-CSF level in the serum elevates substantially and leads to stimulation of granulocyte proliferation. The G-CSF receptor is expressed on all cells of the granulocyte lineage, but increasing amounts of molecules can be detected during maturation, with highest expression in mature neutrophils [185]. Hence, the induced release of G-CSF into the serum after activation of the inflammatory response elicits the proliferation and maturation of granulocyte precursor towards mature neutrophils, accompanied with the increased release of neutrophils into the blood stream [186]. Thus, the influence of this pro-maturation effect of G-CSF after G-CSF administration in Btk-deficient mice was analyzed. Additionally, we have also examined the effect of GM-CSF on granulopoiesis in mice, since our previous *in vitro* experiments already revealed alterations in the outcome of GM-CSF-induced myeloid development downstream of the GMP upon Btk-deficient conditions. Therefore, we wondered whether similar results could be obtained or whether the maturation phenotype of Btk-deficient neutrophils could be rescued upon systemic GM-CSF administration in mice. Finally, we extended the analysis for LPS to examine the potential effects of TLR-ligation on a mimicked “emergency” myelopoiesis *in vivo* in mice lacking Btk.

First, we evaluated the effect of the both CSF and LPS on myelopoiesis *in vitro* by culturing whole bone marrow cells in the presence of rmG-CSF, rmGM-CSF or LPS for 72 h. After this time of incubation, the amount of granulocytes ($CD11b^+/Gr\text{-}1^+$) was determined by flow cytometry. An increase in granulocytes was observed when wild type as well as in Btk-deficient bone marrow cells where cultivated in the presence of G-CSF as well as GM-CSF.

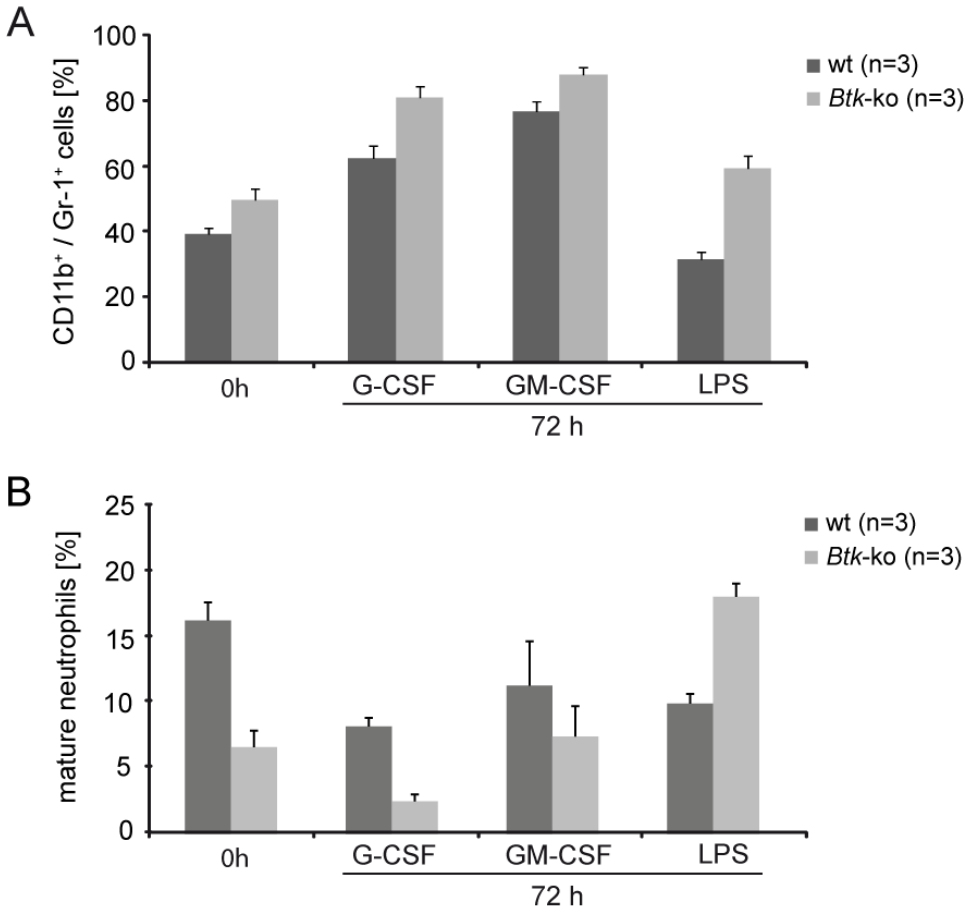

Figure 3.24 Differentiation of Btk-deficient bone marrow cells *in vitro*.
Bone marrow cells obtained from wild type (wt) or Btk-deficient mice (*Btk*-ko) were treated *in vitro* (A) with rmG-CSF, rmGM-CSF (25 ng/mL both) or LPS (10 µg/mL) for 72 h. The development of $CD11b^+/Gr\text{-}1^+$ neutrophils was monitored by flow cytometry. (B) The degree of maturation after 72 h of G-CSF administration was analyzed by the level of CD11b and Gr-1 expression. The percentage of cells in the matured state relative to overall $CD11b^+/Gr\text{-}1^+$ population is shown. Data presented are mean values (±SD). *n* represents the number of biological replicates.

In contrast, in the case of LPS treatment, an enhanced granulopoiesis was obtained only in Btk-deficient bone marrow cells, but not in wild type bone marrow

cells. Additionally, we detected again an obvious enhancement of neutrophil proliferation under Btk-deficient conditions in comparison to wild type bone marrow cells after G-CSF as well as GM-CSF administration that was again associated with an immature phenotype of the obtained neutrophils (Figure 3.24). Interestingly, for LPS-induced granulopoiesis, we found even an enhanced maturation of Btk-deficient neutrophils, probably due to the massive induction of granulocyte development by LPS in Btk-deficient bone marrow cells.

Next, we asked whether the granulocyte differentiation and maturation is also changed, when one of the CSF or LPS is administrated *in vivo*. For that purpose, G-CSF, GM-CSF or LPS was injected intravenously and after 36 h blood and bone marrow of treated mice was analyzed with regard to neutrophil numbers and maturation as well as myeloid progenitor number. Similar to published data [184], *in vivo* administration of G-CSF resulted in a comparable neutrophilia in wild type and Btk-deficient mice indicating a unchanged release of neutrophils from the bone marrow under Btk-deficient conditions. In contrast to the nearly equal neutrophil numbers in peripheral blood after G-CSF administration, we obtained a substantial increase in bone marrow neutrophil numbers in wild type mice but not in mice lacking Btk. Moreover, the analysis of the maturation status of the obtained bone marrow neutrophils in Btk-deficient mice revealed an obvious increase in promyelocytes and myelocytes at the expanse of immature as well as mature neutrophils. Accordingly, a clear decrease in the GMP number was observed upon G-CSF treatment in the bone marrow of Btk-deficient mice in comparison to wild type mice.

Surprisingly, administration of GM-CSF revealed no alterations in neutrophil numbers in peripheral blood or in the myeloid compartment of the bone marrow neither in wild type mice nor in Btk-deficient mice, may be due to an insufficient induction of myelopoiesis. Similar to GM-CSF treatment, LPS did not significantly change the numbers of circulating or bone marrow neutrophils in comparison to untreated control mice in Btk-deficient and wild type mice. Regarding the neutrophil maturation status, we also found no obvious differences between wild type and Btk-deficient mice after *in vivo* LPS treatment (Figure 3.25). Notably, the overall increase in the granulocyte population under Btk-deficient conditions remained after treatment of mice with any of the above-mentioned biological agents.

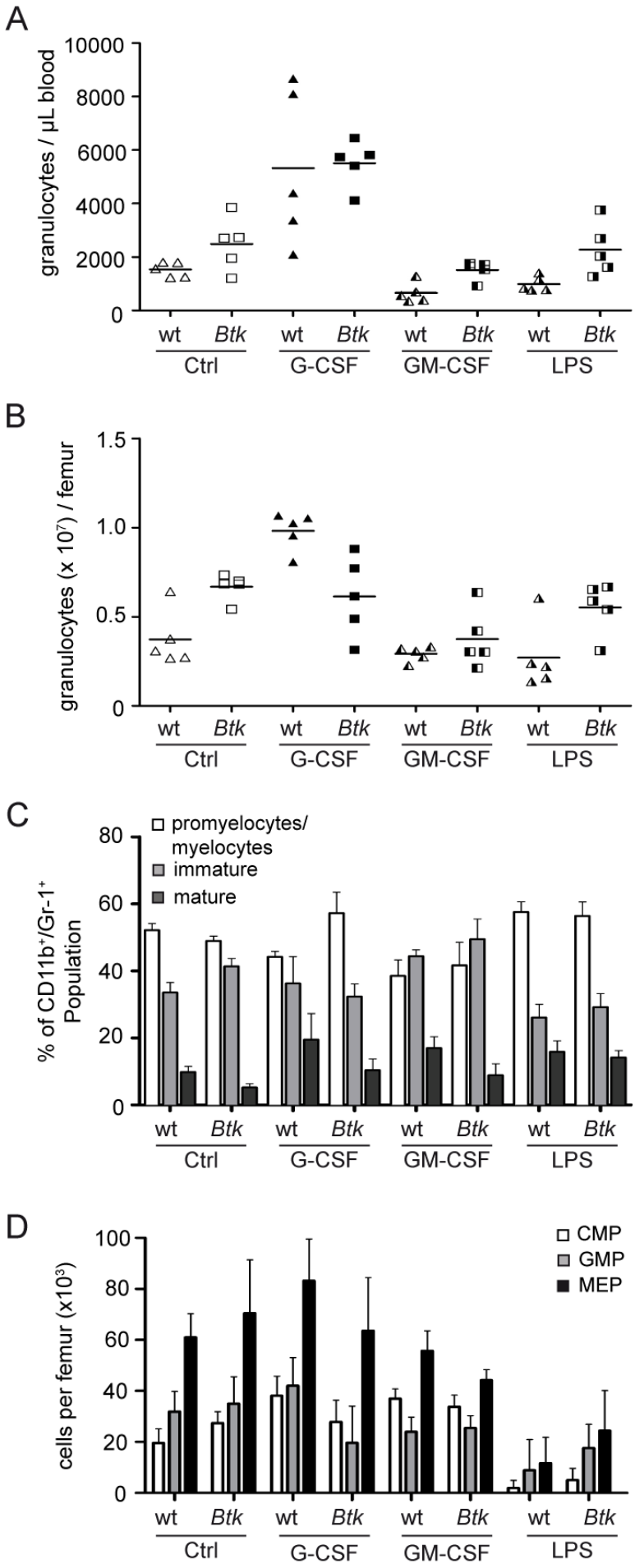
A
granulocytes / µL blood
10000
8000
6000
4000
2000
0
wt
Btk
Ctrl
G-CSF
GM-CSF
LPS
B
granulocytes (x 10⁷) / femur
1.5
1.0
0.5
0
C
promyelocytes/ myelocytes
immature
mature
% of CD11b⁺/Gr-1⁺ Population
80
60
40
20
D
CMP
GMP
MEP
cells per femur (x10³)
100
80
60
40
20

Figure 3.25 Rescue of the developmental phenotype of myeloid precursors and neutrophils in Btk-deficient mice in response to CSF or LPS treatment.
Wild type (wt) and Btk-deficient (*Btk*) animals were left untreated (Ctrl) or treated with rmG-CSF, rmGM-CSF or LPS (25 µg/kg body weight). (A) After 36 hours, the blood of mice was taken and analyzed for absolute numbers of granulocytes. Finally, mice were sacrificed and the bone marrow was analyzed for absolute numbers of neutrophil granulocytes per femur (B) and their maturation status (C). Changes in the myeloid precursor cell compartments were analyzed by flow cytometry (D). Data presented are mean values (±SD). At least four biological replicates per group were analyzed.

In addition, the myeloid progenitor compartment showed a severe decrease in cell numbers in wild type as well as Btk-deficient mice, with no obvious differences in the progenitor ratios (Figure 3.25).
Taken together, the retrieval of external differentiation cues that partially resembles infection or inflammation could not rescue the differentiation and maturation defects observed in neutrophils lacking Btk expression. The administration of G-CSF was able to enhance the neutrophil numbers in circulation shortly by the increased release of immature neutrophils from the bone marrow. Nevertheless, the terminal differentiation was still affected and the G-CSF-induced proliferation of neutrophils probably led to an exhaustion of the developing myeloid compartment in the bone marrow, since the myeloid progenitor numbers were reduced in G-CSF-treated animals in comparison to G-CSF-treated wild type animals as well as to untreated Btk-deficient animals.
Furthermore, the analysis of mRNA expression in sorted GMP after 36 h of G-CSF or LPS administration by quantitative PCR revealed again a decrease in neutrophil-specific genes like *Cebpa*, *Mmp9* and *Elane* (Figure 3.26).

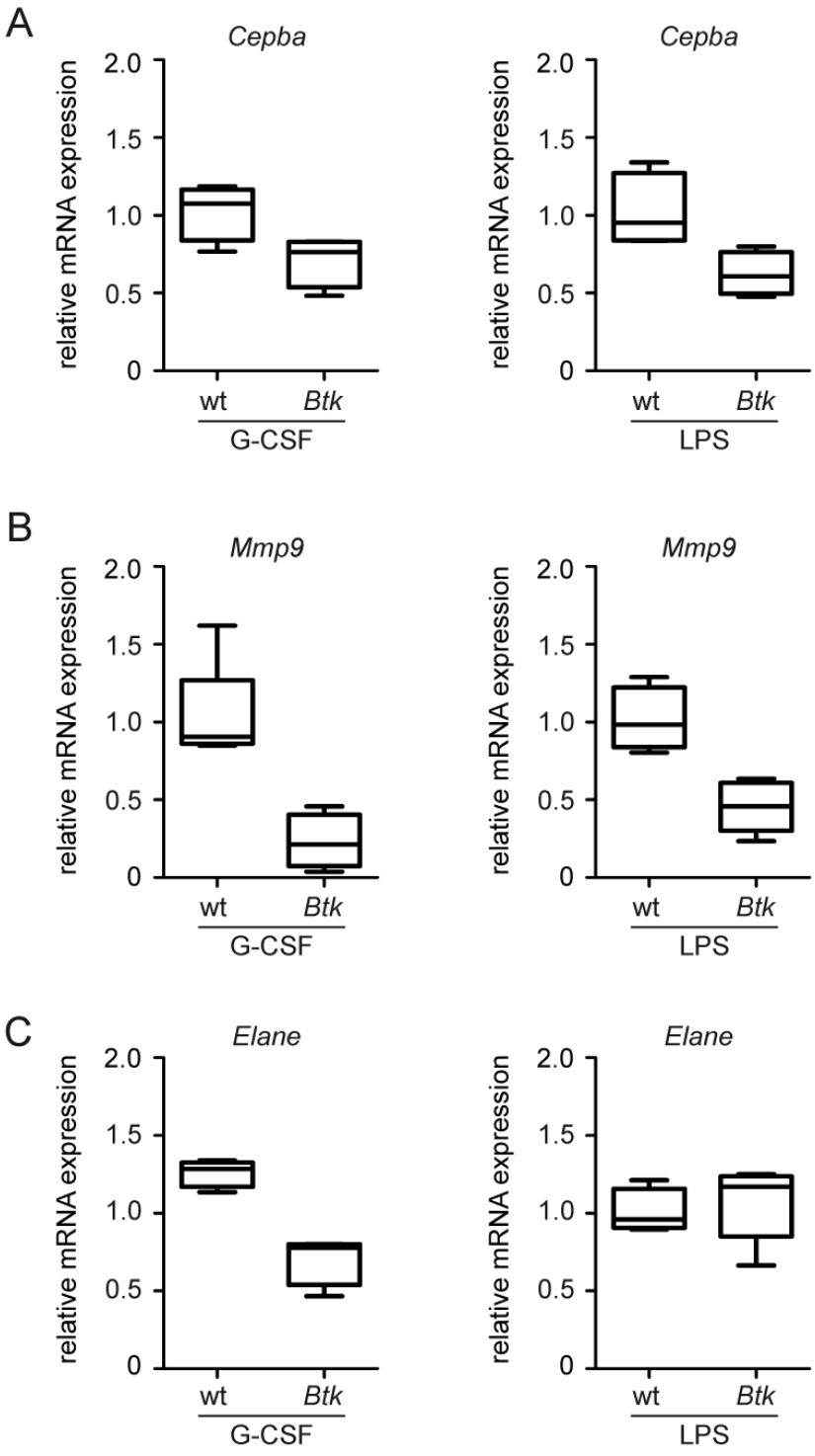

Figure 3.26 Expression of lineage-specific genes in GMP after cytokine treatment. GMP were isolated by FACS from wild type (wt) and Btk-deficient mice (*Btk*) 36 hours after the injection of rmG-CSF or LPS and analyzed for the expression of (A) C/EBPα (*Cebpa*), (B) gelatinase (*Mmp9*) and (C) neutrophil elastase (*Elane*) relative to the expression of *Hprt1*. At least four biological replicates per group were analyzed.

4 Discussion

4.1 Btk Function in Myeloid Cells

Almost two decades ago, for the first time Bruton tyrosine kinase was described as a new member of the Tec kinase family, and as the cause of the human primary immunodeficiency XLA [2]. Subsequent extensive research demonstrated the central functions of Btk for BCR-mediated signaling, which are essential for proper B cell development behind the pre-B cell stage. Dysfunction of Btk accounts for the severe B cell defects in XLA-patients characterized by the almost complete absence of circulating B cells as well as immunoglobulins [24,27,36,37,187].

Owing to the fact that Btk is not only expressed in B cells but also in cells of the myeloid lineage, several studies addressed the question whether Btk is additional important for the innate immunity. However, with regard to the potential roles of Btk in other myeloid cells such as macrophages, dendritic cells or neutrophils, a plethora of controversial results were published mainly concerning the involvement of Btk in TLR-signaling. In detail, it was demonstrated that Btk interacts with the Toll/interleukin-1 receptor (TIR) domain of several TLR leading to Btk activation via phosphorylation as well as its participation in NFκB activation [188]. Moreover, Btk associates specifically with MyD88 or MyD88 adapter-like (Mal), TRIF and IL-1 receptor-associated kinase 1 (IRAK-1) [188] and mediates the phosphorylation of Mal during TLR2 and TLR4 signal transduction [189]. Additionally, Btk together with PI3K are members of the TLR2 multiprotein-complex that mediate TLR2-induced NFκB activation [190]. Recently, a remarkable study described an association of intracellular MHC class II molecules with Btk via the costimulatory molecule CD40 in endosomes that maintains Btk activation thereby supporting the recruitment to the adaptor molecules MyD88 and TRIF, which finally promotes the TLR-triggered innate immune responses [191]. However, whether the observed TLR signaling alterations caused by the Btk deficiency in dendritic cells or macrophages are of physiological relevance is discussed quite controversial. Some studies demonstrated lower production of IL-6, IL-10 or TNFα [192,193] and others did not find changes in cytokine secretion upon activation [194,195]. In the same line of controversy are the results regarding T cell activation via Btk-deficient dendritic cells. One study demonstrated that Btk-deficient murine dendritic cells exhibit a more mature phenotype and a stronger T cell-stimulatory ability due to

decreased Stat3 phosphorylation and decreased IL-10 production [196]. Otherwise, the analysis of dendritic cells from XLA-patients concerning differentiation, maturation and antigen-presenting cell function revealed no differences between dendritic cells from XLA-patients and healthy donors [194]. Taken together these contradictory results illustrate the remaining lack of knowledge regarding the function of Btk in myeloid cells, particularly in the interplay with other innate or adaptive immune cells. Moreover, very little is known regarding the function of Btk during the development of myeloid cells, although Btk is already expressed during very early stages of hematopoietic differentiation.

Therefore, we investigated the influence of Btk on myeloid cell development and demonstrated that Btk is not only involved in TLR-dependent signaling in myeloid cells, but also in myeloid cell development. The analysis of the myeloid differentiation *in vivo* and *in vitro* revealed significant alterations in myelopoiesis in Btk-deficient mice. In detail, we could show that GMP isolated from the bone marrow of Btk-deficient mice preferentially developed into granulocytes at the expense of monocytes or undifferentiated cells when cultivated in the presence of GM-CSF or TLR-ligands as differentiation cues. The enhanced granulopoiesis *in vitro* could be confirmed by the evaluation of the bone marrow compartment of Btk-mutant mice, where we found an elevation in granulocyte and also in erythrocyte numbers. The enhancement in erythrocyte numbers obtained in our analyses could be caused by Btk-mediated alterations in erythroid differentiation, as the essential role of Btk for the regulation of signaling events downstream of the erythropoietin receptor is described [197]. The expanded populations of both granulocytes and erythrocytes caused an increase of total cell number in the bone marrow of Btk-mutant mice, but in the blood the numbers of granulocytes and erythrocytes were not significantly altered in comparison to wild type littermates. These data are at odds with results published previously by Mangla and colleagues, where reduced numbers of neutrophils in the bone marrow as well as in the peripheral blood were found when analyzing *Xid*-mice [198]. Low numbers of mice that were analyzed in the previous study possibly caused the discrepancy in the obtained results. In our studies, we also obtained in some animals reduced numbers of granulocytes, but by analyzing large cohorts of animals these differences were negated. Furthermore, the authors defined granulocytes in the bone marrow compartment as cells expressing high levels of the surface marker

Gr-1, which display only the matured population of granulocytes. However, with respect to the terminal differentiation of granulocytes in the bone marrow we found a substantial maturation defect in granulocytes obtained from Btk-deficient mice, although the generation of granulocytes was enhanced in these mice. This immature phenotype of granulocytes was not only defined by the expression levels of CD11b and Gr-1, but also by an inefficient development of granules as well as the reduced expression of granule proteins, like myeloperoxidase, neutrophil elastase, lactoferrin or gelatinase.

4.2 Altered Myelopoiesis in Btk-Deficient Mice

The process of cell fate decisions during hematopoietic differentiation is mainly regulated by the expression and function of lineage-determining transcription factors. With respect to the lineage decision between monocytes and granulocytes, the transcription factors C/EBPα and PU.1 are necessary for the lineage-specific gene expression as well as for the repression of lineage-foreign genes. Particularly, C/EBPα is indispensable for granulocyte and monocyte cell differentiation as demonstrated by the MxCre induced excision of *Cebpa* causing a complete abrogation of granulocyte as well as monocyte development that is due to the block in CMP to GMP transition [68]. Moreover, Keeshan and colleagues have showed that the transcriptional activity of C/EBPα is required for the induction of granulocyte differentiation [199].

Although the expression of C/EBPα was comparable in wild type and Btk-deficient CMP and GMP, the C/EBPα expression in the whole bone marrow that includes beside the early myeloid progenitors developing immature and mature neutrophils was clearly reduced at RNA as well as at protein level in the case of Btk-deficiency. Additionally, the analysis of C/EBPα target gene expression in GMP and whole bone marrow cells revealed a substantial decrease in several important C/EBPα target genes like myeloperoxidase or neutrophil elastase in Btk-deficient mice. Therefore, the obtained data suggest a significant contribution of Btk to the activation of C/EBPα transcriptional activity and subsequently to the forced expression of C/EBPα target genes in hematopoietic progenitor and precursor cells.

However, C/EBPα is not only essential for granulocyte development but also for monocyte differentiation as shown by the transduction of murine bone marrow

cells with C/EBPα-estradiol receptor (ER) constructs prior to culture with estradiol together with cytokines inducing myeloid development. In these experiments, the forced expression of C/EBPα led to increased monopoiesis compared to empty vector controls via increased transcription of the Ets-family transcription factor PU.1. Moreover, PU.1 in turn activated the transcription of monocyte-specific genes like *CD14* and *Csf3r* and repressed the transcription of granulocyte-specific genes [200]. Other publications further emphasized the importance of C/EBPα for transcriptional activation of PU.1 via binding to the PU.1 promoter and its distal enhancer [201-203].

Accordingly, we observed a reduced RNA level of PU.1 and a decreased RNA expression level of the PU.1 target *Csf3r* as well as *Csf2ra* in bone marrow cells of Btk-deficient mice. These results further support the proposed reduction in transcriptional activity of C/EBPα in cells lacking functional Btk. Furthermore, the transcription factor PU.1 is indispensable for monocyte differentiation as demonstrated by using mice, where PU.1 was excised in hematopoietic cells using the inducible MxCre-system. Those mice displayed an enhanced granulopoiesis and an absence of CD11b and F4/80 expression [119]. In addition, PU.1 expression per se is necessary but not sufficient to induce monocyte development, the level of PU.1 expression is important for the lineage decision between granulocytes and monocytes. Another study revealed an increase in granulocyte progenitors and a diminution of monocyte progenitors in PU.1 haplo-insufficient mice in comparison to wild type mice that could be rescued by the restored expression of PU.1. Moreover, the inducible expression of PU.1-ER in a PU.1-deficient progenitor cell line directed monocyte differentiation only in the presence of a high dose of 4-hydroxy-tamoxifen, whereas a low dose induced preferentially granulocyte differentiation [204].

These data emphasize the importance of sufficient induction of PU.1 for proper monocyte development and demonstrate that abrogated monopoiesis results always in augmented granulopoiesis. Moreover, the favored granulopoiesis upon PU.1 deficiency suggests that granulocyte development is the basal differentiation program, which is repressed after induction of monopoiesis probably via cytokine signaling. Therefore, in addition to the alterations in the GM-CSF-induced differentiation of Btk-deficient GMP the reduced levels of PU.1 and the subsequently diminished expression of PU.1 target genes like *Csf3r* and *Csf2ra*

could contribute to the enhanced granulopoiesis and decreased monopoiesis observed in Btk-deficient mice.

The decreased function of C/EBPα in Btk-deficient mice was further supported by the *in vitro* differentiation experiments using isolated GMP. Upon GM-CSF induced myeloid differentiation, our data revealed an almost two-fold reduction of colony-forming units under Btk-deficient condition, which could be caused by the reduced activity of C/EBPα in these mice. Since, functional C/EBPα is necessary for the appropriate transition from CMP to GMP [68], an impaired transcriptional activity could lead to expression of GMP-specific lineage surface markers, but not necessarily to the full activation of the lineage-specific gene program.

Zhang and colleagues showed that C/EBPα is also involved in the promotion of myeloid differentiation by repression of the gene Bmi-1, which is a prerequisite for HSC self-renewal and repopulating activity [68]. Hence, deficiency in C/EBPα activity is associated with increased HSC repopulating capacity as well as self-renewal that could be explain the elevated cell numbers within the LSK-fraction of Btk-deficient bone marrow cells, especially with regard to the multipotent progenitor population. Additionally, the *in vivo* BrdU-incorporation assay revealed an enhanced proliferation of cells within the LSK-fraction of bone marrow cells lacking Btk expression. However, the differences in proliferative ability are low and possibly compromised by the experimental setup, where BrdU was administrated during four consecutive days and yielded to over 80 % of BrdU-positive cells. Probably, shorter administration of BrdU would generate more reliable results concerning the HSC self-renewal capacity of Btk-deficient HSC in comparison to wild type HSC.

Another function of C/EBPα in myeloid development was highlighted by two studies demonstrating that the induction of growth arrest of differentiating granulocytes is mediated via direct repression of E2F-dependent transcription by C/EBPα [205] and that E2F repression-deficient C/EBPα failed to support granulocyte differentiation [206]. Particularly, the c-Myc gene that is essential for the induction of myeloid differentiation is repressed by C/EBPα binding via an E2F site [207]. Therefore, the enhanced proliferation of Btk-deficient granulocytes in the *in vitro* differentiation, displayed by the up to three-fold increased cell number per colony and the elevated BrdU-incorporation in Btk-deficient promyelocytes and myelocytes, could be also related to the decreased activity of C/EBPα.

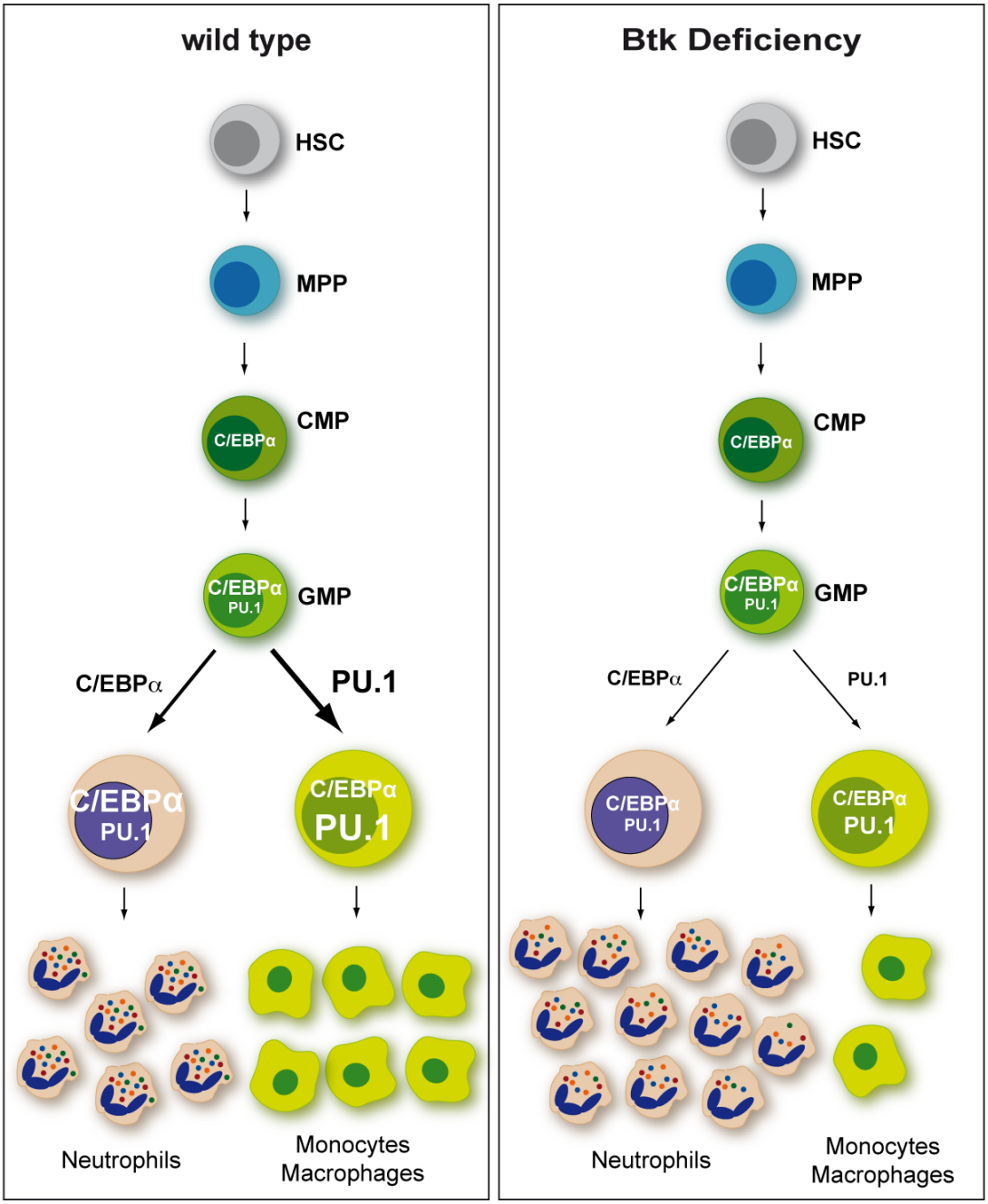

Figure 4.1 Proposed model for de-regulation of myelopoiesis in Btk-deficient mice. The master transcription factor C/EBPα is needed for the proper induction of granulocyte lineage differentiation. Additionally, C/EBPα is necessary for an efficient induction of PU.1, which represents the master transcription factor of the monocyte lineage. Loss of Btk function leads to the reduced expression and activation of C/EBPα that in turn leads to a diminished induction of *PU.1* transcription and therefore to a decreased monopoiesis, accompanied by an increased granulopoiesis.

In summary, the increased granulopoiesis at the expanse of monopoiesis found in Btk-deficient mice could be attributed to the diminished expression and activity of the lineage-determining transcription factor C/EBPα, which is indispensable for the proper induction of monopoiesis via activation of PU.1 transcription (Figure 4.1). Moreover, also the TLR2 or TLR4-induced myeloid differentiation of Btk-deficient GMP led also to an enhanced granulopoiesis at the expense of monocytes and macrophages. As TLR-induced myeloid differentiation of myeloid cells obviates the need for growth factors like colony-stimulating factors [89], it is likely that a developmental machinery is induced similar to that one activated upon GM-CSF treatment.

4.3 Btk-Dependent Activation of C/EBPα

Indeed, the detailed examination of the GM-CSF receptor-mediated signaling cascade demonstrated that Btk was phosphorylated upon GM-CSF receptor engagement in wild type bone marrow cells. Similar results were published for the analysis of the M-CSFR signaling pathway in macrophages, where the phosphorylation of Btk at position Y223 was detected [208]. Additionally, we could show that Btk was involved in proper activation of the PI3K signaling pathway necessary for Akt activation and GSK-3β repression. In support of our findings, Buitenhuis and colleagues have highlighted the influence of Akt activity on lineage choice during myelopoiesis. Precisely, the authors showed the need of PI3K as well as Akt activation for the phosphorylation of GSK-3β at position S9, which is required for its subsequent inhibition. This in turn facilitates the dephosphorylation of C/EBPα at position T222/226 being a prerequisite for full activation of C/EBPα-dependent gene transcription during differentiation towards granulocytes [178]. Therefore, the alterations in PI3K-dependent signaling obtained in Btk-deficient bone marrow cells provided a reliable mechanism for the reduced transcriptional activity of C/EBPα found in Btk-deficient hematopoietic cells.

Interestingly, at the basis of two publications that have demonstrated the activation of the PI3K-Akt pathway upon TLR2 [209] as well as TLR4 engagement [210] and together with the well described participation of Btk in TLR2 and TLR4 signaling, one might speculate that Btk is also involved in the PI3K activation in response to TLR-induced myeloid differentiation. However, it has never been shown before

that Btk-deficiency is associated with a decrease in PI3K activation in myeloid cells.

Most studies proposed a Btk-independent activation of PI3K after BCR in B cells, since PIP_3-formation seems to be a prerequisite for inducible Btk recruitment to the membrane that in turn is necessary for Btk activation. With regard to myeloid cells, Btk function in TLR- or GM-CSF-dependent PI3K activation has not been analyzed. Almost 10 years ago, a novel B cell adaptor for PI3K was discovered and named BCAP. Okada and colleagues demonstrated that upon BCR engagement in B cells BCAP recruits the regulatory subunit p85 of the PI3K-complex to the membrane, which is mandatory for full PI3K activation. In addition, the authors identified Syk and Btk as protein tyrosine kinases mediating BCAP phosphorylation after BCR ligation. Moreover, the extent of p85 association with BCAP was correlated with the BCAP tyrosine phosphorylation status [34]. A further support for a possible link between Btk and PI3K mediated by BCAP in myeloid cells is provided by a publication of Matsumura and colleagues that demonstrated the expression of BCAP in monocytes as well as the phosphorylation of BCAP after TLR4 activation [211].

Therefore, a potential mechanism for Btk-mediated activation of the PI3K signaling upon GM-CSFR or TLR stimulation could be the following: Btk is activated after GM-CSFR or TLR engagement and phosphorylates BCAP that in turn recruits the regulatory subunit p85 of PI3K, which is necessary for the activation of PI3K as well as for the subsequent activation of Akt leading finally to the repression of GSK-3β followed by the de-repression/activation of C/EBPα (Figure 4.2).

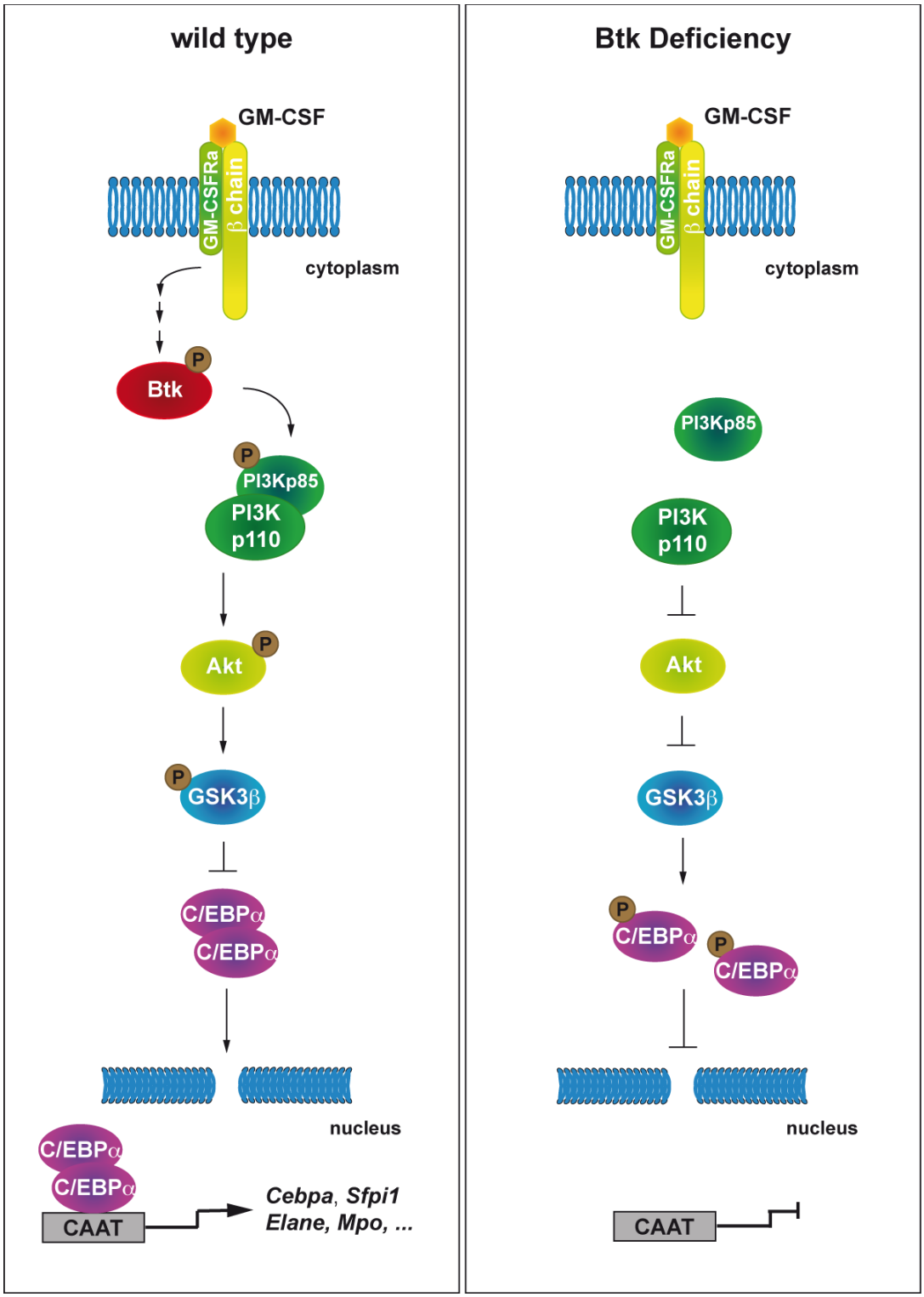

Figure 4.2 Proposed model for Btk-dependent activation of C/EBPα.

The proposed signaling cascade depicted in the scheme is based on the in the present work demonstrated phosphorylation of Btk upon GM-CSF receptor engagement. Moreover, the Btk-dependent phosphorylation of PI3Kp85, Akt and GSK-3β was demonstrated in the present study. The dephosphorylation of C/EBPα by GSK-3β inhibition and its impact on gene transcription is described elsewhere.

4.4 Impaired Immune Function of Btk-Deficient Neutrophils

Our study revealed not only significant alterations in myeloid differentiation accompanied with increased granulopoiesis, but also severe functional defects of Btk-deficient neutrophil granulocytes. In detail, using the reverse passive Arthus reaction as a model of an acute inflammatory response, we observed less vascular destruction as evidenced by less edema formation and reduced amounts of petechiae and hemorrhage in Btk-deficient mice. Moreover, the numbers of infiltrating neutrophils at the site of inflammation was obviously decreased in mice lacking Btk expression. We showed also that Btk-deficient neutrophils released significantly reduced levels of elastase and gelatinase after immune complex-induced degranulation. Additionally, analyses of mRNA expression levels of primary, secondary and tertiary granule proteins demonstrated that Btk is necessary for the sufficient expression of elastase, gelatinase and other bactericidal proteins, like myeloperoxidase and lactoferrin precursor. Particularly, the matrix-metalloproteinase gelatinase and the serine protease neutrophil elastase are assumed to be important for the breakdown of the basement membrane as well as the extracellular matrix [212] and needed for the recruitment of neutrophils into the site of inflammation [213,214]. Furthermore, an increasing body of evidence indicates that these enzymes can also modulate many biological functions like chemokine and cytokine activity as well as alter specific cell-surface receptors functions. Several studies have shown that gelatinase and elastase are able to cleave cell-surface adhesion molecules, such as ICAM-1, VCAM-1 or E-cadherin to facilitate transmigration of neutrophils [215-217]. Raptis and colleagues have demonstrated that immune complex-activated neutrophils lacking elastase and cathepsin G failed to undergo cytoskeletal reorganization, although they adhered normally to IC-coated surfaces [218]. Another study revealed the ability of gelatinase to potentiate IL-8 activity by amino-terminal processing [219]. In addition, Liu and colleagues have shown that gelatinase degrades the serine protease inhibitor serpin-α1 and subsequently stimulates the proteolytic activity of serine proteases [220]. Therefore, the reduced expression and release of granule proteins, especially gelatinase and elastase, could cause the decreased infiltration of neutrophils observed during the Arthus reaction in Btk-deficient mice. But certainly, other factors could also contribute to the detected inefficient immune response. For example, the impaired recruitment of neutrophils to the side of inflammation,

as previously shown in *Xid*-mice, where the recruitment of *Xid*-neutrophils into the peritoneum upon glycogen or thioglycollate-induced peritonitis was significantly reduced [198]. Moreover, Mueller and colleagues could show that Btk is necessary for the E-selectin mediated slow rolling of neutrophils during the activation phase of the leukocyte adhesion cascade [221]. However, in our experiments we could detect Btk-deficient neutrophils attached to the blood vessel endothelium as well as neutrophils that were able to transmigrate into the perivascular space. Hence, already mentioned factors such as reduced enzyme expression and consequent degranulation effects most likely contribute to the observed phenotype in the Arthus reaction using Btk-deficient mice.

Presumably, the impaired expression of granule proteins is associated with the hampered terminal differentiation observed in neutrophils lacking Btk, which was defined by the expression level of CD11b and Gr-1 and the decreased numbers of granules per neutrophil. The transcription factor C/EBPα not only regulates early myelopoiesis and lineage decision, but it is also indispensable for terminal differentiation of neutrophils via upregulation of transcription factors, which are necessary for the maturation of neutrophils in bone marrow. Particularly, the C/EBPα target gene *PU.1* is involved in the terminal differentiation of neutrophils shown by retroviral transduction studies of PU.1 in a PU.1 null myeloid cell line, where PU.1 was needed for the expression of the NADPH oxidase component gp91^{phox} as well as for the secondary granule genes *Mmp8* and *Mmp9* [222].

In addition, the transcription factor C/EBPβ is highly upregulated in late stages of neutrophil maturation [223], but especially after cytokine- or infection-induced granulopoiesis *in vitro* as well as *in vivo*. Cytokines like GM-CSF, G-CSF and IL-3 are responsible for the upregulation of C/EBPβ in neutrophils and C/EBPβ-deficient progenitors failed to generate efficient granulopoiesis in response to signals that mimic an acute infection [118]. Here, we have demonstrated that Btk is involved in GM-CSF-mediated signaling of neutrophil granulocytes and that C/EBPα, PU.1 and C/EBPβ expression levels are significantly reduced in Btk-deficient bone marrow cells. Moreover, the reduced expression of these transcription factors led to a reduced expression of granule proteins, which are necessary for proper immune function of neutrophils, and a decreased terminal differentiation of Btk-deficient neutrophils. These data indicate that Btk plays an

important role for the development, maturation and function of neutrophil granulocytes.

However, the analyses of Btk-deficient neutrophils not only revealed severe defects under steady-state conditions, but also during emergency circumstances. In a normal and healthy organism the number of mature granulocytes is maintained at a constant amount in steady-state conditions, but the hematopoietic system is able to supply even more granulocytes during acute infections. The concentrations of the cytokine G-CSF in serum are increased during infections and induce granulocytosis [118]. In the case of Btk-deficiency, the administration of G-CSF was able to promote the increase of circulating neutrophils, but the analysis of the bone marrow did not reveal an enhanced proliferation of differentiating granulocytes. Indeed, the number of CMP as well as GMP was clearly reduced in the bone marrow of Btk-deficient mice. Additionally, the maturation of the granulocytes was again blocked and the expression of granulocyte-specific genes was decreased in GMP of mice lacking Btk expression. Therefore, the neutrophil immune functions are probably even more hampered under conditions of an acute infection when Btk is absent, because of the exhaustion of the myeloid bone marrow compartment.

4.5 Neutrophil Function of XLA Patients

Based on the significant impairment in granulopoiesis of Btk-deficient mice elucidated in the present work, it is of particular interest to emblaze the maturation as well as the immune function of neutrophils in patients suffering from XLA. Unfortunately, there is only one study published dealing with this question. In these examinations – although the data suffer from small patient and control cohorts – the authors analyzed the shedding of surface CD62L, respiratory burst, apoptosis and MAP kinase phosphorylation after TLR4 as well as TLR7/8 activation and no obvious differences between XLA neutrophils and healthy donor neutrophils could be detected [224].

Nevertheless, neutropenia is described as a characteristic feature of XLA in association with severe infections in 10 to 30 % of the XLA patients [225-228]. Additionally, bone marrow evaluations of XLA patients with severe neutropenia revealed in 75 % of analyzed cases a profound maturation arrest at the stage of promyelocytes and myelocytes [229-231].

These data provide a reliable clue for cell-intrinsic neutrophil dysfunction in patients suffering from XLA that is comparable to that found by us in Btk-deficient mice. Moreover, the impaired neutrophils possibly contribute beside the B cell defect to the severity of bacterial infections reported in these cases. Since, neutropenia in XLA patients has been correlated with mutations within the *BTK* gene that cause the loss of Btk expression or function [229], it is likely that the type of *BTK* gene mutation may be associate with the degree of impaired granulopoiesis in those patients.

Our data indicate that Btk-deficient neutrophils are severely functional impaired during an acute inflammatory response that is induced by either IC or LPS. Therefore in the future, on the one hand the function of XLA neutrophils should be reassessed under conditions mimicking an acute infection and on the other hand the maturation status of neutrophils as well as the expression of granule proteins should be analyzed in detail. Neutrophils have indispensable functions with regard to the clearance of bacterial infections and impaired neutrophil maturation as well as function could severely affect immunity against bacteria in XLA patients even upon IVIG administration.

4.6 Conclusions

The tyrosine kinase Btk is mainly implicated in BCR-dependent signaling and B cell development, but there is also an increasing body of evidence for indispensable functions of Btk in myeloid cells. In our analysis of Btk function in developing myeloid cells, we could show that proper Btk function is necessary for the regulation of lineage decision between granulocyte and monocyte development in the bone marrow. Upon GM-CSF-mediated myeloid differentiation, Btk is phosphorylated and possibly contribute to activation of C/EBPα activity via stimulation of the PI3K/Akt/GSK-3β pathway, which is severely compromised in Btk-deficient neutrophils. Also, the C/EBPα expression was reduced in Btk-deficient bone marrow cells. C/EBPα activity is indispensable for the suitable induction of monopoiesis by the upregulation of PU.1 transcription, but also for sufficient activation of granulocyte-specific genes and terminal differentiation of granulocytes. In line with previous as well as our findings at the molecular level concerning the GM-CSF-signaling as well as C/EBPα expression in Btk-deficient neutrophils, we observed an increase in granulopoiesis in Btk-deficient animals

yielding in immature neutrophils with decreased granule numbers and diminished expression of granule proteins. Additionally, the Btk-deficient neutrophils were severely functional impaired upon an acute inflammation induced by different stimuli.

Thus, our data provide for the first time substantial evidence for the indispensable functions of Btk during myeloid development. Moreover, we could show that Btk-deficient neutrophils are hampered in their immune function probably due to the decreased expression of granule proteins and the severe granulocyte differentiation defects. As neutrophil maturation defects in XLA patients were also found, a reliable hint for similar Btk-dependent mechanisms in humans is provided. However, to a large extent the contribution of Btk for human myelopoiesis remains unsolved and has to be addressed in future. In addition, the detailed signaling cascade activated upon GM-CSF receptor ligation as well as the position of Btk in that signaling pathway remains to be proven.

Eventually, future research will continue to shed light on the specific functions of Btk in development and function of myeloid cells. Particularly, the crosstalk with immune cells of the innate as well as of the adaptive immune system should be evaluated in detail, to gain insight in processes associated with acute infection but also with chronic inflammation or autoimmunity.

5 Abbreviations

Ab	antibody
ABC	animal blood counter
AKT(PKB)	protein kinase B
AMV	avian myeloblastosis virus
AP-1	activating protein-1
APC	allophycocyanin
APS	ammonium persulfate
β-ME	b-mercaptoethanol
BAM11	Btk-associated molecule 11
BaP	basophil progenitor
BAP135	Btk-associated protein of 135 kDa
BCAP	B cell adaptor for PI3K
BCP	B cell progenitor
BCR	B cell receptor
BH	Btk homology
BLNK	B cell linker protein
BLyS	B-lymphocyte stimulator
Bmx	bone marrow kinase gene on the X chromosome
bp	base pair
BrdU	5-bromo-2'-deoxyuridine
BSA	bovine serum albumin
Btk	Bruton tyrosine kinase
Btk-ko	Btk-deficient
C	cysteine
c-Cbl	cellular Casitas B-lineage lymphoma
C/EBP	CAAT-enhancer binding protein
CAR	CXCL12-abundant reticular
cDNA	complementary DNA
CDP	common dendritic cell progenitor
CFU	colony forming unit
CFU-G	granulocyte-CFU
CFU-GM	granulocyte/monocyte-CFU
CFU-M	monocyte/macrophage-CFU
CLP	common lymphoid progenitor

CMP	common myeloid progenitor
CXCL	CXC-ligand
Cy	cyanin
d	days
DAG	diacylglycerol
DAP12	NDAX activation protein of 12 kDa
DAPI	4',6-diamidino-2-phenylindole
DC	dendritic cell
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleoside triphosphate
EBF	early B cell factor
EDTA	ethylenediamine tetraacetic acid
EGTA	ethyleneglycol-bis-tetraacetic acid
Elane	neutrophil elastase
ELISA	enzyme-linked immunoabsorbent assay
EoP	eosinophil progenitor
EP	erythroid progenitor
EPO	erythropoetin
EPOR	erythropoetin receptor
ERK	extracellular regulated kinase
ERM	ezrin, radixin and moesin
ESAM	endothelial cell-selective adhesion molecule
ESL-1	E-selectin ligand-1
EWS	Ewing sarcoma breakpoint region 1
FACS	Fluorescence-activated cell sorting
FADD	Fas associated via death domain
FAK	focal adhesion kinase
FcεR	Fcε receptor
FcγR	Fcγ receptor
FcRg	γ chain of Fc-receptors
FCS	fetal calf serum
FITC	fluorescein-isothiocyanate
Flit3-L	Flit3-ligand
Flt3	fms-related tyrosine kinase 3
fMLP	formyl-peptide

fMLP-R	formyl-peptide receptor
g	gravity
G-CSF	granulocyte-colony stimulating factor
G-CSFR	G-CSF receptor
GATA	GATA-binding protein
GEF	guanidine nucleotide exchange factor
Gfi-1	growth factor independent-1
GM-CSF	granulocyte-macrophage-colony stimlulating factor
GM-CSFR	GM-CSF receptor
GMLP	granulocyte-monocyte-lymphoid progenitor
GPCR	G-protein coupled receptors
GRK2	G protein-coupled receptor kinase 2
GSK-3β	glycogen synthase kinase 3β
GTP	guanosine triphosphate
HBSS	Hank`s balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hr	hours
HSC	hematopoietic stem cell
IC	immune complex
ICAM-1	intercellular adhesion molecule-1
ICAM-2	intercellular adhesion molecule-2
Ig	immunoglobulin
IL	interleukin
IL-R	interleukin receptor
IMDM	Iscove's modified Dulbecco's medium
IP_3	inositol-3,4,5-triphosphate
IRF	interferon regulatory factor
ITAM	immunoreceptor tyrosine-based activation motifs
Itk	IL-2 inducible T cell kinase
JAK	Janus kinase
JAM	junctional adhesion molecule
JNK1	c-jun NH_2-terminal kinase 1
kb	kilo base pairs
kDa	Kilo Dalton
LFA-1	lymphocyte function-associated antigen-1
LMPP	lymphoid-primed multpotent progenitor
LPS	lipopolysaccharide

LSK	lineage-Sca1-Kit
LT-HSC	long-term repopulating hematopoietic stem cell
LTB4	leucotriene B4
Ltf	lactoferrin
M-CSF	macrophage-colony stimulating factor
M-CSFR	M-CSF receptor
Mac-1/CD11b	integrin aM
MACS	magnetic-activated cell sorting
MAPK	mitogen-activated protein kinase
MCP	mast cell progenitor
mDC	myeloid dendritic cell
MDP	monocyte-dendritic cell progenitor
MEP	megakaryocyte-erythrocyte progenitor
mg	milligram
min	minutes
MKP	megakaryocyte progenitor
mm	millimeter
mM	millimolar
Mmp	matrix-metalloproteinase
Mpo	myeloperoxidase
MPP	multipotent progenitor
mRNA	messenger RNA
MW	molecular weight
Myc	v-myc myelocytomatosis viral oncogene homolog
MyD88	myeloid differentiation primary response gene 88
NDM	non-fat dried milk
NETs	neutrophil extracellular traps
NFκB	nuclear factor κB
NK cell	natural killer cell
nm	nanometer
nt	nucleotid
PAGE	polyacrylamide gel electrophoresis
Pam_3CSK_4	Synthetic triacylated lipoprotein
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pDC	plasmacytoid dendritic cell
PE	phycoerythrin

PECAM-1	platelet endothelial cell adhesion molecule-1
PEST	rich in proline, glutamic acid, serine, and threonine
pg	picogram
PH	pleckstrin homology
PI	propidium iodide
PI3K	phosphoinositide-3-kinase
PIP_2	phosphatidylinositol-4,5-bisphosphate
PIP_3	phosphatidylinositol-3,4,5-triphosphate
PIP5K	phosphatidylinositol-4-phosphate 5-kinase
PKC	protein kinase C
PLC	phospholipase C
PMA	phorbol-12-myristate-13-acetate
PMN	polymorph-nucleated cell
pmol	picomole
PRR	proline-rich regions
PSGL-1	P-selectin ligand-1
R	arginine
rad	radiation-absorbed dose
RIPA	radio immunoprecipitation assay
Rlk/Txk	resting lymphocyte kinase/T and X cell expressed kinase
RNA	ribonucleic acid
RNase	ribonuclease
ROS	reactive oxygen species
rpm	rounds per minute
RPMI	Roswell Park Memorial Institute medium
RT	reverse transcriptase
s	second
S	serine
SCF	stem cell factor
SCN	severe congenital neutropenia
SCN	severe congenital neutropenia
SD	standard deviation
SDS	sodium dodecyl sulfate
SGD	specific granule deficiency
SH	Src homology
Slam	signaling lymphocyte activation molecule
SLP-65	SH2 domain–containing leukocyte protein of 65 kD

ST-HSC	short-term repopulating hematopoietic stem cell
STAT5a	signal transducer and activator of transcription 5a
T	threonine
TAE	Tris acetate EDTA
TBS	Tris-buffered saline
TC	tissue culture
TE	Tris EDTA
Tec	tyrosine kinase expressed in hepatocellular carcinoma
TEM	transmission electron microscopy
TEMED	tetramethylethylenediamine
TFII-I	general transcription factor II-I
TGFβ	transforming growth factor β
TH	Tec homology
TLR	Toll-like receptor
TNFα	tumor necrosis factor α
TNK	T cell natural killer cell progenitor
TPO	thrombopoietin
Tris	Tris-amino-methane
tRNA	transfer RNA
v-SNARE	N-ethylmaleimide-sensitive factor attachment protein receptors
v/v	volume/volume
VAMP-2	of vesicle-associated membrane protein-2
WASP	Wiskott-Aldrich syndrome protein
Wnt	wingless type
wt	wild type
Xid	X-linked immunodeficiency
XLA	X-linked agammaglobulinemia
Y	tyrosine
µg	microgram
µL	microliter
µM	micromolar

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Erklärung

Ich versichere hiermit, dass ich die Arbeit selbständig angefertigt habe und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt sowie die wörtlich oder inhaltlich übernommenen Stellen als solche kenntlich gemacht habe.

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