Original Article
MyD88 is involved in myeloid as well as lymphoid hematopoiesis independent of the presence of a pathogen

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Abstract: MyD88 was originally described as a primary response gene up-regulated during myeloid differentiation after IL-6 induction. Later, MyD88 was shown to be a key molecule necessary for IL1, IL18 and Toll-like receptor signaling. Since these receptors recognize abundantly produced cytokines during infection or molecular patterns of pathogens, MyD88 itself was suggested to be an important regulator of the first line of defense against invading pathogens, including the differentiation and maturation of myeloid cells. Here we describe that MyD88 is important for early and late hematopoietic events that occur independently of antigen under steady-state conditions. In MyD88-deficient mice the earliest alteration in hematopoiesis was found at the level of long-term hematopoietic stem cells. Moreover, we found that MyD88 influences not only the development of the myeloid lineage but also the differentiation of B cells. The B cell defect observed in Btk-deficient mice is further enhanced when both molecules, Btk and MyD88, are not expressed. Therefore, MyD88 affects myeloid as well as lymphoid hematopoiesis. Since Btk and MyD88 deficiencies influence differentially myeloid and lymphoid development, both molecules seem to act in different signaling pathways important for appropriate developmental events during myelo- and lymphopoiesis.

Keywords: MyD88, Btk, hematopoiesis, myelopoiesis, lymphopoiesis

Introduction

MyD88 was first described as a gene that is up-regulated upon treatment of M1D+ myeloblasts by IL6 to induce terminal myeloid differentiation of these cells. Accordingly MyD stands for myeloid differentiation primary response gene and 88 refers to the number within a list of up-regulated genes [1]. Later it was shown that MyD88 is the mammalian homologue of *Drosophila* Tube that recruits Interleukin1 receptor-associated kinase (IRAK) to the IL1 receptor complex, the prerequisite for activation of NF-κB [2-4]. Indeed, targeted disruption of the MyD88 gene results in the loss of IL1- and IL18-mediated signal transduction [5] at which both receptors are structural related. Additionally, MyD88 has been identified as an adaptor molecule necessary for Toll-like receptor (TLR) signaling [6] that becomes activated upon recognition of pathogen associated molecular patterns (PAMPs). Therefore, MyD88 is important for terminal differentiation of myeloid cells induced by IL6 as well as for recognition of pathogens by TLR, both indispensable for the appropriate function of the innate immune system.

Beside its role for terminal differentiation of myeloid cells MyD88 plays also a crucial role in early hematopoiesis, since the MyD88-dependent activation of TLR by pathogenic patterns influences the development of hematopoietic stem cells (HSC) as well as of differentiated myeloid and lymphoid precursors [7]. Nagai and colleagues showed that the stimulation of HSC and multipotent progenitors by TLR2 or TLR4 ligands increases their proliferation. Moreover, stimulation of common myeloid progenitors as well as granulocyte/macrophage progenitors by the above-mentioned ligands leads to their differentiation, dispensable of the presence of the appropriate growth factor macrophage colony-stimulating factor (M-CSF) or granulocytemacrophage colony-stimulating factor (GM-CSF), respectively. Most strikingly, stim-

ulation of common lymphoid progenitors with TLR ligands alters their differentiation program towards the development of myeloid dendritic cells (mDC).

However, non-myeloid cells, like B and T cells, also express TLR promoting their differentiation and function. Innate-like B1 cells and marginal zone B cells (MZB), which act independently of T cell help, express a different TLR repertoire than adaptive follicular B cells that need T cell help for production of high-affinity antibodies and generation of memory B cells. The dual stimulation of the B cell receptor (BCR) and TLR rapidly alters B cell migration as well as antibody responses and cytokine secretion (for review see [8]). On T cells, also expressing significant levels of TLR, TLR2, TLR3, TLR5 and TLR9 act as co-stimulatory receptors of the engaged T cell receptor (TCR) of effector T cells leading to increased proliferation and cytokine production. In contrast, the ligation of TLR2, TLR5 and TLR8 on naturally occurring regulatory T cells (nTregs) influences their suppressive activity (for review see [9]).

MyD88 is not only important for the signaling of IL1 receptor or TLR family members, as recent studies demonstrate that MyD88 is also required for the signaling via the transmembrane activator and CAML (calcium modulator and cyclophilin ligand) interactor (TACI). Activation of TACI by innate immune mediator BAFF (activated by B cell activating factor) or proliferation-inducing ligand (APRIL) triggers class-switch recombination in B cells via MyD88 [10, 11]. Although TACI lacks a TLR/interleukin1 receptor (TIR) domain, necessary for the recruitment of MyD88 to the TLR or IL-1 receptor complex, MyD88 binds to a conserved motif within the intracellular domain of TACI leading finally to activation of NF-κB via a TLR-like MyD88-IRAK1-IRAK4-TRAF6-TAK1 pathway [10].

TLR and MyD88 are not exclusively expressed in immune competent cells. The necessity of these signaling molecules was also shown, for example, for neuronal cells, where MyD88 is required for nerve growth factor (NGF) induced activation of the p75neurotrophin receptor [12]. Moreover, TLR and MyD88 are important for differentiation and proliferation of adult neuronal stem cells [13]. As adult neurogenesis occurs in the absence of pathogens typically

responsible for activation of TLR, endogenous TLR ligands are discussed such as heat-shock proteins, extracellular matrix proteins, oxidative-modified lipids or cellular breakdown products [14-18] as well as other soluble mediators.

Additionally, MyD88-dependent TLR signaling is important for the function of mesenchymal stem cells (MSC) [19]. Beside their ability to differentiate into osteocytes, adipocytes, chondrocytes, myocytes, tenocytes and myocardiocytes, MSC also differentiate into hematopoietic supportive stroma [20]. Therefore, the TLR/MyD88 signaling pathway may affect the development of immune cells also indirectly via alterations in hematopoietic stroma cell function.

Changes in the activity of MyD88 are associated with different types of hematopoietic as well as non-hematopoietic malignancies. Recent studies revealed, that mutations in the *MYD88* gene leading to expression of a constitutive active version of MYD88 cause a considerable percentage of B cell lymphomas [21, 22]. Interestingly, in most cases a single amino acid substitution within the TIR domain of MYD88 (L256P) is responsible for the spontaneous formation of a signaling complex composed of MYD88, IRAK1 and IRAK4 followed by an enhanced activation of NF-κB [21].

Our previous studies indicate that Bruton's tyrosine kinase (Btk), a cytoplasmic non-receptor tyrosine kinase of the Tec family, associates with MyD88 in B cells as well as myeloid cells and is implicated in TLR/MyD88 signaling [23]. Btk was first identified as an essential kinase for BCR signaling and B cell function. Mutations along the *BTK* genomic locus cause X-linked agammaglobulinemia (XLA) in humans, associated with a B cell differentiation defect at the stage of pro B cells. Consequently, the number of peripheral B cells is severely reduced resulting in an almost complete loss of primary and secondary immunoglobulins [24]. Therefore, affected individuals are highly susceptible to bacterial infections [25, 26]. A similar B cell defect - X-linked immunodeficiency (*Xid*) - was observed in mice bearing *Btk* mutations, although this defect is somewhat milder than that in humans [27-29]. Beside in B cells, Btk is expressed in a variety of hematopoietic cells, including myeloid cells [24] in which Btk has been shown to be involved in TLR/MyD88 sig-

naling [23, 30-35]. However, other investigations came to different conclusions [36-38]. Hence, the impact of the potentially impaired TLR function for the overall immunodeficiency of XLA is still an issue for investigation.

To get more insights in the role of Btk for MyD88-dependent TLR signaling, we generated Btk/MyD88 double deficient (DKO) mice. Unexpectedly, these mice showed significant smaller spleens than single mutant mice, caused by a severe reduction of B cell as well as myeloid cell compartment. This observation prompted us to investigate the role of Btk and particularly MyD88 for early, antigen-independent hematopoiesis.

Materials and methods

Mice

Knockout mice for Btk [28] and MyD88 [5] have been described previously. Btk- as well as MyD88-single knockout mice, double-deficient mice and wild type littermates (all at the C57BL/6 background) were used at age of 8 to 12 weeks. All animal experiments were in accordance with institutional guidelines and German animal protection laws.

BrdU-incorporation

The APC-BrdU-Flow-Kit (BD Pharmingen) was used for analysis of BrdU-incorporation into bone marrow cells. After a single intraperitoneal injection of BrdU solution (1 mg/6g of body weight), BrdU was also administered at 1 mg/ml with drinking water for 2 days.

Flow cytometry

Flow cytometry was performed on a FACSCanto™ II, the data were analyzed with FACSDiva 6.2 software (BD). The following antibodies were purchased from eBioscience: anti-CD8a (53-6.7), anti-CD11b (M1/70), anti-CD16/32 (93), anti-CD34 (RAM34), anti-CD45R/B220 (RA3-6B2), anti-CD117 (ACK2), anti-F4/80 (BM8), anti-Gr-1 (RB6-8C5) anti-IL-7Ra (A7R34), anti-MHC class II (M5/114.15.2), anti-Sca1 (D7); from BD Pharmingen: anti-CD11b (M1/70), anti-CD11c (HL3), anti-CD21/CD35 (7G6), anti-CD23 (B3B4), anti-CD24 (M1/69), anti-CD43 (S7), anti-CD45R/B220 (RA3-6B2), anti-IgD (11-

26c.2a), anti-IgM (R6-60.2); from BioLegend: anti-CD45 (30-F11), anti-CD48 (HM48-1), anti-CD150 (TCF15-12F12.2), anti-Ly6G (1A8); from Miltenyi Biotec: anti-CD3e (145-2C11), anti-CD19 (6D5), anti-Gr-1 (RB6-8C5), anti-Ly6C (1G7.G10), anti-Ter119 (Ter-119).

Isolation of hematopoietic progenitor cells

Bone marrow cells were enriched for lineage-negative cells using antibodies against CD11b, CD19, Terr119 and rat-IgG-Dynabeads (Invitrogen). For sorting of GMP, lineage-depleted cells were blocked with mouse IgG (Jackson ImmunoResearch) and stained with lineage markers (anti-CD3e, anti-CD4, anti-CD8a, anti-CD45R, anti-Gr-1, anti-CD19, anti-CD11b, anti-Ter119) as well as anti-IL-7Ra, anti-Kit, anti-Sca1, anti-FcgRII/III and anti-CD34. Cells were sorted on a FACSAriaII (BD).

In vitro colony-forming assays

Cell sorter-purified progenitors were placed in MethoCult M3231 supplemented with 50 ng/mL SCF, 10 ng/mL IL-3 and 25 ng/mL GM-CSF (Stem Cell Technologies). At day 6 colonies were counted. To avoid massive cell death, colonies were analyzed phenotypically after 8 days of culture [39].

Cytospins of CFU

Cytospins (Cytospin3 Cytocentrifuge, Thermo Shandon) were stained by Pappenheim and analyzed in a blinded way using the Leica Microscope DM IRB.

Immunofluorescence staining and histology

Spleens were embedded in paraffin automatically with a TP1020 tissue processor (Leica) and cutting in 3 μm sections was done with a Microm 355S (Thermo Scientific). Before staining sections were rehydrated. Immunofluorescence stainings were performed using anti-IgD-FITC (BD Pharmingen), anti-IgM-PE (Southern Biotech) or anti-CD169-FITC (MOMA-1) (AbD Serotec). Slides were analyzed with a fluorescence microscope (Axiovert 200M; Carl Zeiss) equipped with AxioCam MR3 (Carl Zeiss) and AxioVision software (version 2.2.5). Hematoxylin-Eosin staining was done according to standard protocols. Slides were analysed with a Leica Microscope DM IRB equipped with
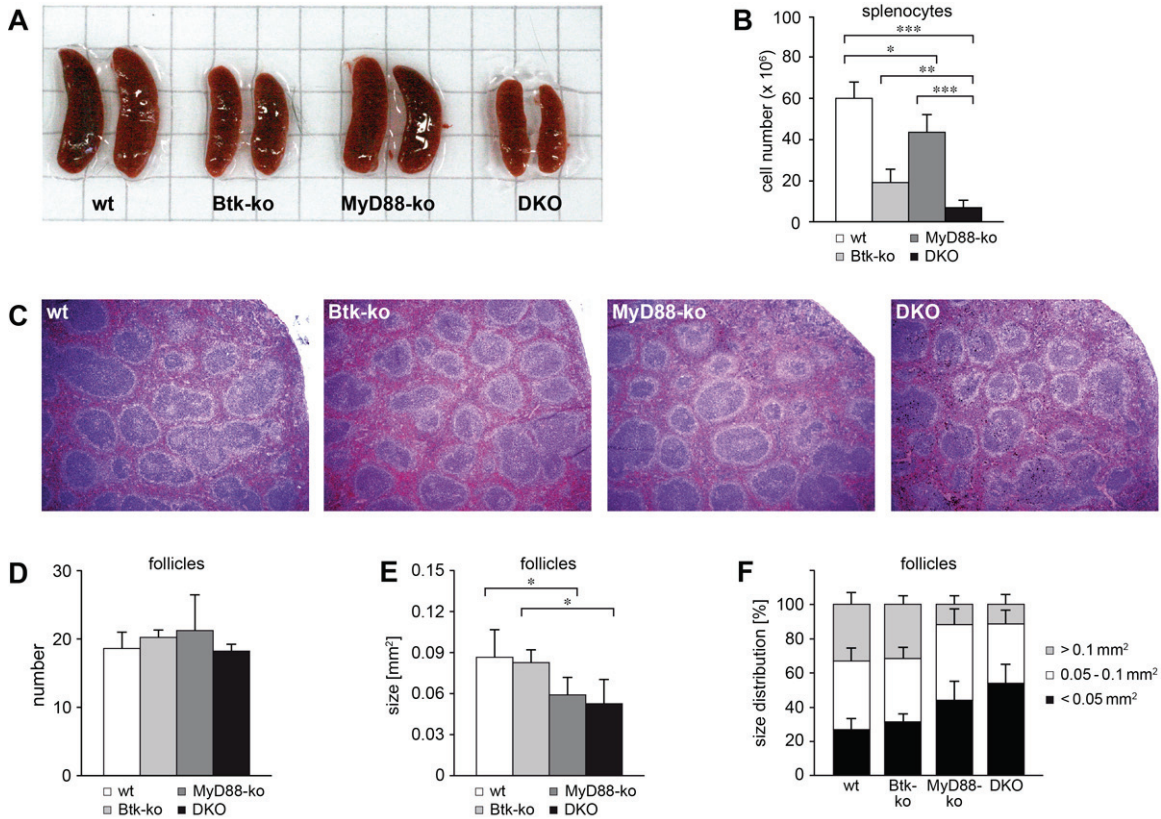
Figure 1. Altered architecture of the spleen in MyD88-deficient mice. The macroscopic phenotype of spleens isolated from wild type (wt), Btk- (Btk-ko) or MyD88-single mutant mice (MyD88-ko) as well as double-deficient mice (DKO) was determined by (A) photography and (B) analysis of cell number using a ViCell XR Cell Viability Analyzer after lysis of erythrocytes. Data presented are mean values (±SD) (n=11). C: Spleens of each genotype were embedded in paraffin and processed for HE-staining. Parameters of follicle architecture like (D) follicle number, (E) follicle size and (F) follicle size distribution were analyzed using light microscopy and ImageJ software. Data presented in D and E are mean values (±SD) (n=5). Data presented in F are mean values (±SEM) (n=5). *$P \leq 0.05$; **$P \leq 0.005$; ***$P \leq 0.005$. n represents the number of biological replicates.

ResPag C14 (Jenoptik) at 5-fold magnification and Openlab software 4.0.4.

Results

Changes of splenic architecture in MyD88-deficient mice

Recently, we identified Btk as a MyD88-interacting protein essential for MyD88-dependent TLR signaling [23]. In order to investigate the consequences of deletion of Btk and MyD88 for the signal transduction in several types of immune cells in more detail, we generated Btk/MyD88 double deficient mice (DKO) and analyzed them together with wild type littermates and Btk-ko as well as MyD88-ko single mutant mice. Beside the known defects in hematopoiesis found in Btk-ko mice leading to severe changes in spleen size, we found that loss of MyD88 also leads to an apparent reduction in spleen dimension. Additionally, the spleens of DKO mice are significantly smaller in comparison to the spleens of respective single mutant mice. (**Figure 1A**). These macroscopic observations are in accordance with the reduced cellularity of spleens determined for each analyzed genotype (**Figure 1B**). Furthermore, Hematoxylin-Eosin stainings of individual spleen sections revealed that MyD88-deficiency additionally leads to alterations in splenic microarchitecture (**Figure 1C**). In detail, whereas the number of splenic follicles is comparable between each genotype analyzed, the size of individual follicles is considerable reduced in MyD88-deficient spleens in comparison to the wild type situation (**Figure 1D-F**). Moreover, in spleens of DKO mice this effect is further enhanced.
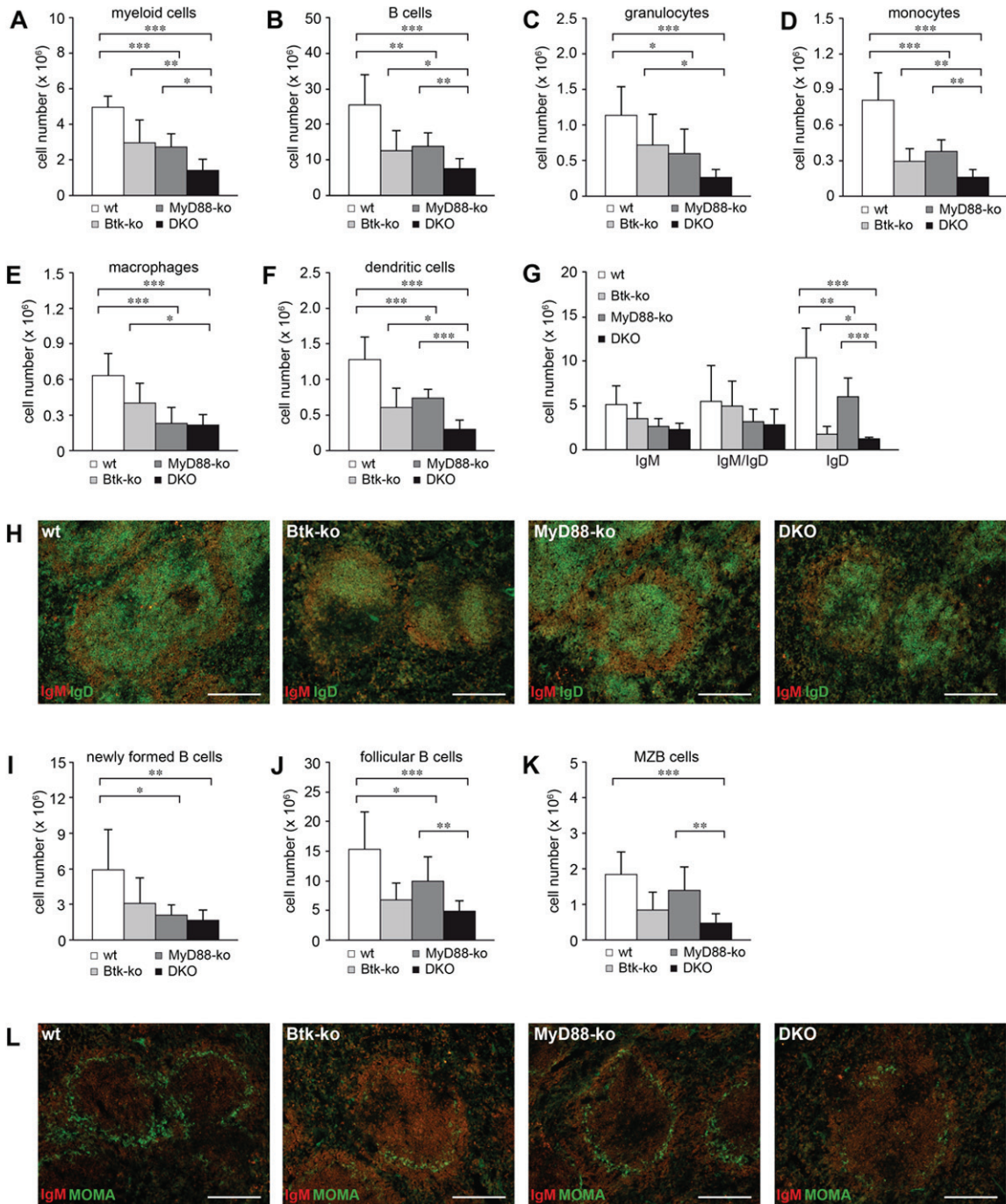
Figure 2. Reduced myeloid and lymphoid cell populations in spleen of MyD88-deficient mice. Spleens of wild type (wt), Btk-deficient (Btk-ko), MyD88-deficient mice (MyD88-ko) and double-deficient mice (DKO) were analyzed by flow cytometry for the frequency of myeloid and lymphoid cell subpopulations. A-G, I-K: Cell suspensions of spleens from mice of each genotype were counted using a ViCell XR Cell Viability Analyzer after lysis of erythrocytes and stained for (A) myeloid cells (CD11b$^+$), and (B) B cells (B220$^+$) as well as different myeloid subpopulations like (C) granulocytes (CD11b$^+$/Ly6G$^+$), (D) monocytes (CD11b$^+$/Ly6C$^+$) (E) macrophages (CD11b$^+$/F4/80$^+$) and (F) dendritic cells (CD11b$^+$/CD11c$^+$/MHC II$^+$) (n=11 for wt, Btk-ko, DKO; n=7 for MyD88-ko). G: B cells were stained for IgM as well as IgD surface expression (B220$^+$/IgM/IgD) and the peripheral B cell subpopulations were analyzed as follows: (I) newly formed B cells (CD21^{low}/CD23^{low}), (J) follicular B cells (CD21^{int}/CD23^{high}) and (K) MZB cells (CD21^{high}/CD23^{low}) (n=10). Data presented are mean values (±SD). Additionally, spleens of each genotype were analyzed by immunofluorescence staining for IgM and IgD (H) as well as for metallophilic macrophages (MOMA) mainly found in the marginal zone of splenic follicles (L). *$P \leq 0.05$; **$P \leq 0.005$; ***$P \leq 0.0005$. n represents the number of biological replicates.

MyD88-deficiency affects the number of splenic myeloid as well as lymphoid cells

Next, flow cytometry analyses were performed to investigate which cell population is responsible for reduced spleen size and disturbed splenic microarchitecture in MyD88-deficient mice. These data revealed that the number of myeloid cells (CD45$^+$/CD11b$^+$) as well as the number of B cells (CD45$^+$/B220$^+$) is significantly reduced in the absence of MyD88 similarly to the decreased levels of myeloid cells and B cells found in Btk-deficient mice (**Figure 2A** and **2B**).

The myeloid compartment was further characterized to identify monocytes (CD11b/$^+$Ly6C$^+$), macrophages (CD11b$^+$/F4/80$^+$/MHCII$^+$), neutrophils (CD11b$^+$/Ly6G$^+$) and myeloid dendritic cells (CD11b$^+$/CD11c$^+$/MHCII^{high}) and these entire cell populations are significantly reduced when MyD88 is not expressed (**Figure 2C-F**). Also, the Btk deletion affects the size of most myeloid cell populations as described previously [40]. However, deletion of both genes, *Btk* and *MyD88*, leads to a further decrease in myeloid cells numbers, with most significant reduction in monocyte as well as dendritic cell populations and to a lesser extent in granulocytes, whereas the number of splenic macrophages is similar to that identified in MyD88-deficient animals.

To investigate the peripheral B cell development, splenic B220$^+$ B cells were stained for the expression of IgM and IgD. Here, the loss of MyD88 mainly affects the final antigen-independent maturation to IgD$^+$ B cells, since this population is clearly reduced in numbers although the percentage of MyD88$^{-/-}$ IgD$^+$ B cells relative to the entire B220$^+$ B cell population is similar to wild type levels (**Figure 2G** and data not shown). This effect is less pronounced in comparison to that found in Btk-ko animals. However, in DKO mice the population of IgD$^+$ B cells is significantly smaller than in both single knock out animals. Additionally, immunofluorescence staining of spleens for IgM and IgD support the significant reduction of IgD$^+$ B cells found by flow cytometry in single knockout mice that is further enhanced in spleens of double-deficient mice (**Figure 2H**). Together these data indicate that MyD88 has an important impact on final peripheral B cell development.

Staining of B220$^+$ B cells with anti-CD23 and anti-CD21 antibodies allows distinguishing three peripheral B cell subpopulations: newly formed B cells that left the bone marrow and entered peripheral lymphoid organs (CD21^{low}/CD23^{low}), follicular B cells (CD21^{int}/CD23^{high}) and MZB cells (CD21^{high}/CD23^{low}) (**Figure 2I-K**). Flow cytometry analysis revealed that MyD88-deletion leads to a strong reduction of newly formed B cells comparable to the reduction found in Btk-ko mice. Also, the follicular B cell number is significantly reduced in the absence of MyD88. In contrast, the number of MZB cells is only slightly reduced when MyD88 is not expressed. However, deletion of Btk and MyD88 together leads to a further reduction of the follicular B cell and MZB cell number in comparison to each mutant alone. Again, the significant reduction in MZB cells in double-deficient mice was also supported by the use of immunofluorescence staining of marginal metallophilic macrophages, which only reside in the marginal zone. These staining revealed a significant reduction of marginal metallophilic macrophages and therefore of the marginal zone in Btk-ko animals. This effect is further enhanced in double-deficient animals (**Figure 2L**).

Additionally, the T splenic cell compartment including the CD4$^+$ and CD8$^+$ T cell subpopulations are slightly reduced when MyD88 is deleted. However, the B to T cell ratio is not altered in comparison to the wild type situation (data not shown).

MyD88 influences the development of myeloid and lymphoid cells in the bone marrow

Adult hematopoiesis occurs mainly in the bone marrow. Therefore, we analyzed differentiating bone marrow cell populations. In contrast to Btk-deficient mice, where we observed in the past an increase in bone marrow cell numbers due to increased erythro- and granulopoiesis [40], in MyD88-deficient mice the number of bone marrow cells is slightly but significantly reduced (**Figure 3A**). The decreased cell number of MyD88-single mutant mice is largely caused by the reduction of myeloid as well as erythroid cell number (**Figure 3B-D**). However, in the case of double mutant mice the lower myeloid and erythroid cell numbers of MyD88-deficient mice are not compensated by the phenotype of Btk-single mutant mice, in which the
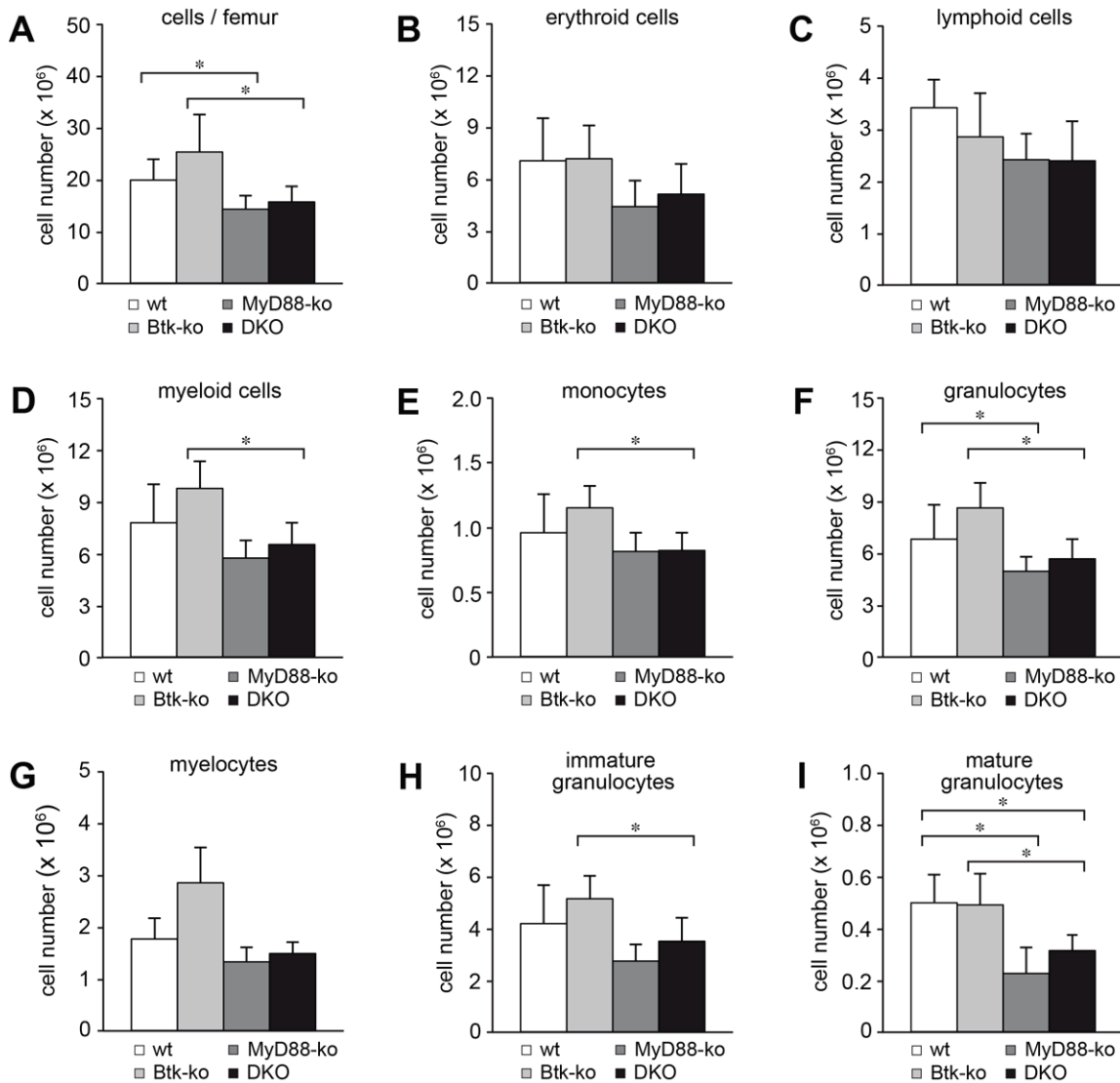
Figure 3. Decreased myeloid cell populations in the bone marrow of MyD88-deficient mice. Bone marrow cells were isolated from femurs of wild type (wt), Btk-deficient (Btk-ko), MyD88-deficient (MyD88-ko) as well as double-deficient mice (DKO) and analyzed by flow cytometry for the frequency of hematopoietic cell lineages and myeloid subpopulations. A: Cell numbers of whole bone marrow cells per femur were determined using a ViCell XR Cell Viability Analyzer and cells were stained for (B) erythroid lineage (Ter119$^+$), (C) lymphoid lineage (B220$^+$) as well as (D) myeloid lineage (CD11b$^+$). The myeloid subpopulations were further analyzed by staining for (E) monocytes (CD11b$^+$/Ly6C$^+$) and (F) granulocytes (CD11b$^+$/Ly6G$^+$). Additionally, the maturation status of granulocytes in the bone marrow can be distinguished by the expression level of CD11b and Ly6G. The different maturation stages were (G) myelocytes (CD11b$^+$/Ly6G^{low}), (H) immature granulocytes (CD11b^{med}/Ly6G^{high}) and (I) mature granulocytes (CD11b^{high}/Ly6G^{high}). Data presented are mean values (±SD) (n=5). *$P \le 0.05$. n represents the number of biological replicates.

myeloid cell number is increased in comparison to wild type littermates.

Further analysis of the myeloid cell lineage revealed that the decrease in myeloid cell number of MyD88-deficient mice is caused rather by reduction of granulocytes than by reduction of monocytes (**Figure 3E** and **3F**). Additionally,

the expression level of the surface markers CD11b and Ly6G allow to subdivide granulocytes in the bone marrow into several maturation stages like myelocytes, immature and mature granulocytes [40, 41]. In contrast to Btk-deficient mice, that showed a clear increase only of the myelocyte stage, MyD88-deficient mice displayed a decrease of all granulocyte
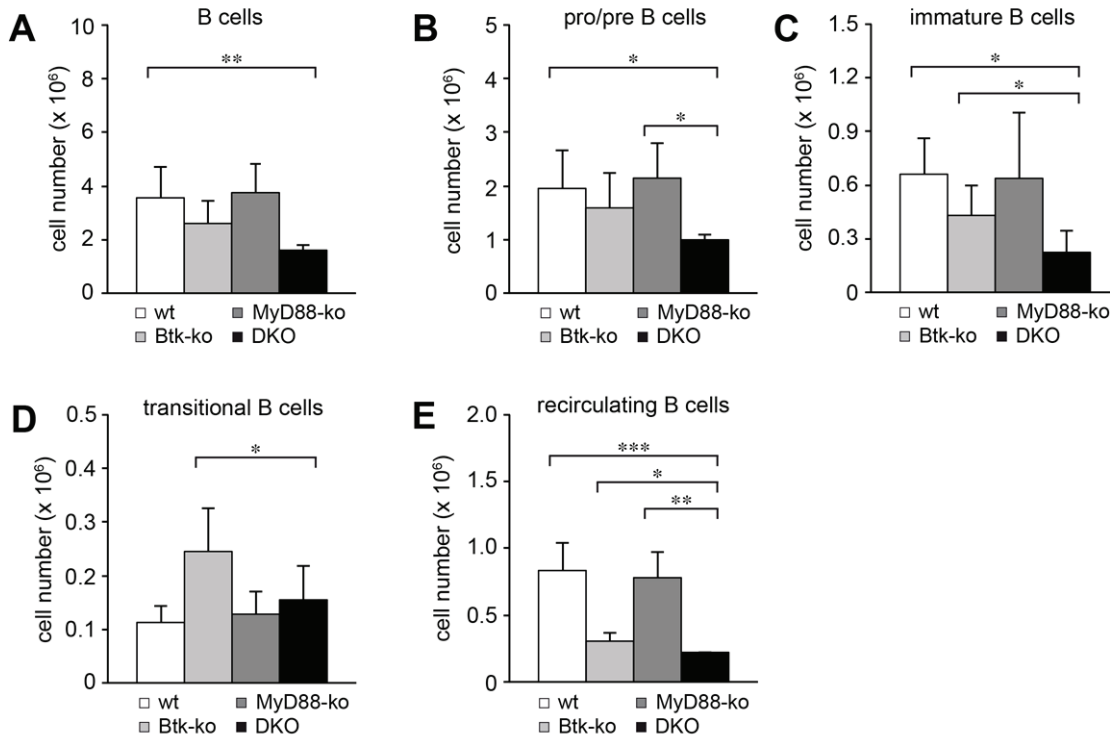
Figure 4. Apparent reduction of lymphoid cell populations in the bone marrow of Btk/MyD88-deficient mice. Bone marrow cells were isolated from femurs of wild type (wt), Btk-deficient (Btk-ko), MyD88-deficient (MyD88-ko) as well as double-deficient mice (DKO) and analyzed by flow cytometry for the frequency of B cell subpopulations. Cell numbers of whole bone marrow cells per femur were determined using a ViCell XR Cell Viability Analyzer and cells were stained for (A) entire B cell population (B220$^+$), (B) pro/pre B cells (B220^{med}/IgM$^-$), (C) immature B cells (B220^{med}/IgM^{med}), (D) transitional B cells (B220$^+$/IgM^{high}) as well as (E) recirculating B cells (B220^{high}/IgM$^+$). Data presented are mean values (±SD) (n=6). *$P \leq 0.05$; **$P \leq 0.005$; ***$P \leq 0.0005$. n represents the number of biological replicates.

maturation stages with most significant reduction of the mature granulocyte compartment (**Figure 3G-I**). Similar to the overall reduction of myeloid cells in bone marrow of DKO mice and MyD88-ko mice, the decrease in all granulocyte populations found in MyD88-single mutant mice is barely compensated by the additional loss of Btk.

Although the bone marrow B cell number is not significantly altered in MyD88-deficient animals, in DKO mice there is a considerable reduction of bone marrow B cells in comparison to single mutant and wild type mice indicating that MyD88 also influences bone marrow B cell development (**Figure 4A**). Detailed analysis of the different B cell developmental stages occurring in bone marrow, revealed no significant alterations between wild type and MyD88-deficient mice similar to the overall B cell number. However, the double-deficient mice have a

substantial decrease in the pro/pre B cell number (B220$^+$/IgM$^-$) and the immature B cell number (B220$^+$/IgM^{int}) in comparison to wild type and single-mutant mice (**Figure 4B** and **4C**). The last maturation step of B cell development in the bone marrow displays the transitional B cell population (B220$^+$/IgM^{high}) that is not altered in MyD88- or double-deficient mice, but the increase in transitional B cell numbers found in Btk-deficient mice is reverted in DKO mice almost back to wild type levels (**Figure 4D**). Nevertheless, the most significant alteration of bone marrow B cell populations could be detected in the population of recirculating B cells (B220^{high}/IgM$^+$), which is tremendously decreased in double-deficient mice in comparison to wild type as well as MyD88-ko littermates and even in relation to the recirculating B cell number of Btk-ko mice (**Figure 4E**). Therefore, the overall reduction in B cell numbers found in DKO animals is due to a decrease
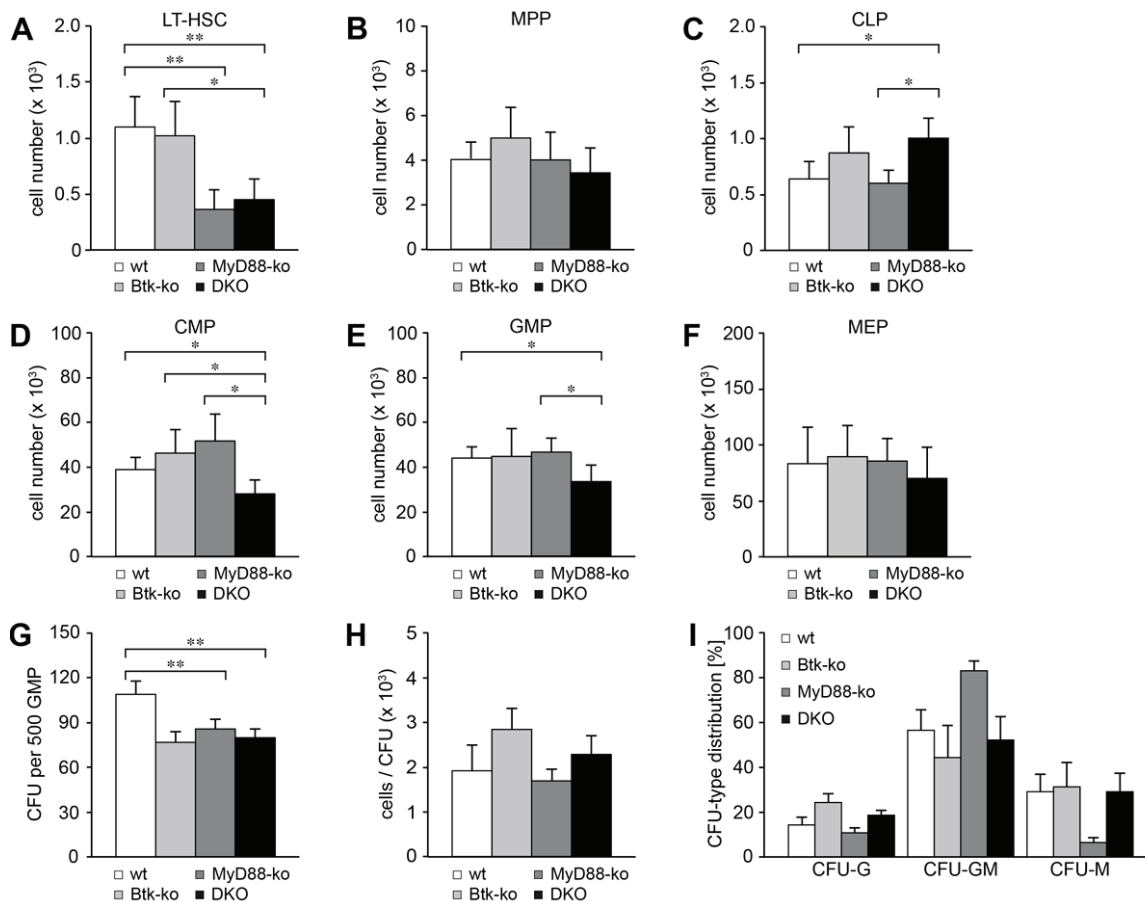
Figure 5. Disturbed hematopoiesis in the bone marrow of MyD88-deficent mice. Bone marrow cells obtained from femurs of wild type (wt), Btk-ko, MyD88-ko and Btk/MyD88-deficient mice (DKO) were counted with a ViCell XR Cell Viability Analyzer and analyzed by flow cytometry to distinguish (A) LT-HSC (lin- Sca1+ Kit+ CD48- CD150+), (B) multipotent progenitors (lin- Sca1+ Kit+ CD48- CD150-), (C) common lymphoid progenitors (lin- Sca1med Kitmed IL-7Ra+), (D) common myeloid progenitors (lin- Sca1- Kit+ CD34+ CD16med), (E) granulocyte/macrophage progenitor (lin- Sca1- Kit+ CD34+ CD16high) and (F) megakaryocyte/erythrocyte progenitor (lin- Sca1- Kit+ CD34- CD16-). Data presented are mean values (±SD) (n=6). To analyze myeloid differentiation potential of granulocyte-macrophage progenitors in vitro, 500 GMP were sorted out of individual mice from each genotype and seeded in methylcellulose media supplemented with SCF, IL3 and GM-CSF. G: After 6 days in culture colony-forming units (CFU) were counted. Data presented are the mean values (±SD) (n=5). H: The cell number per CFU was calculated. I: Twenty to thirty individual CFU per biological replicate were processed for cytospins plus 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 granulocytes, CFU-GM=the content of macrophages and granulocytes was in between 20 to 80%. The data are presented as mean values (±SEM) (n=5). $*P \leq 0.05$, $**P \leq 0.005$. n represents the number of biological replicates.

of cell numbers at almost all stages of B cell development, but most significantly related to a disturbed peripheral B cell development.

MyD88 contributes to early hematopoiesis in the bone marrow

Before occurrence of myeloid or lymphoid lineage-restricted cells during hematopoietic differentiation processes, first hematopoietic stem cells have to generate lineage-restricted progenitor cells in the bone marrow.

For detailed investigation of defects in myeloid as well as lymphoid cell populations observed in single and double knockout mice, the hematopoietic stem and progenitor cell compartment in the bone marrow was analyzed. In contrast to Btk-deficient mice, where we found no difference in long-term hematopoietic stem cell (LT-HSC; lin-/Sca1+/Kit+/CD48-/CD150+) population in comparison to wild type littermates, the number of LT-HSC is drastically reduced in MyD88-/- mice. Also, in double-deficient animals the decrease in LT-HSC population is visi-
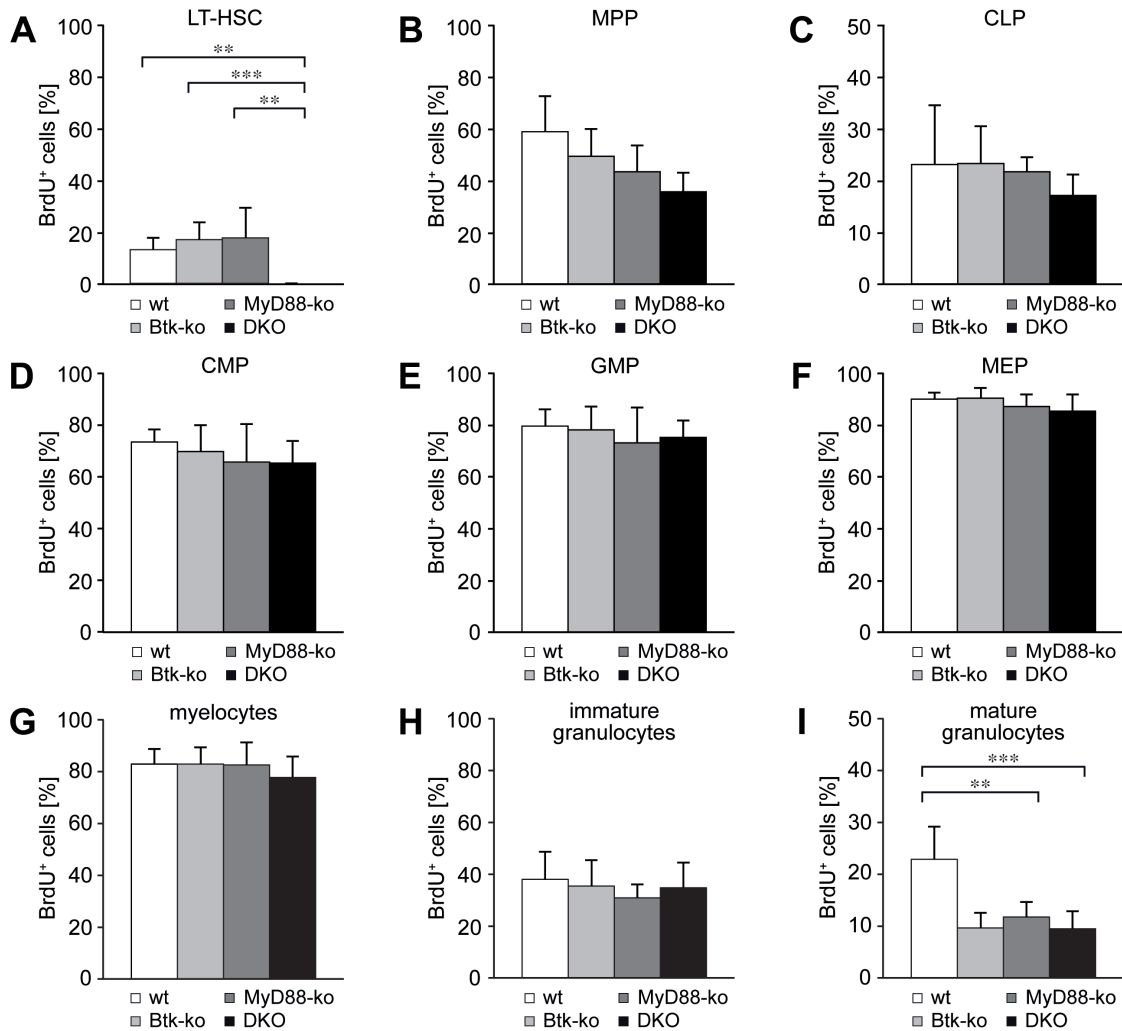
Figure 6. Reduced proliferation of LT-HSC in the bone marrow of MyD88-deficient mice. Proliferation of hematopoi-etic stem and progenitor cell population as well as granulocyte subpopulations was determined by use of BrdU-in-corporation. Bone marrow cells obtained from femurs of wild type (wt), Btk-ko, MyD88-ko and Btk/MyD88-deficient mice (DKO) after 2 consecutive days of BrdU administration were analyzed by flow cytometry for percentage of BrdU$^+$ cells. A: LT-HSC (lin$^-$ Sca1$^+$ Kit$^+$ CD48$^-$ CD150$^+$), (B) multipotent progenitors (lin$^-$ Sca1$^+$ Kit$^+$ CD48$^-$ CD150$^-$), (C) common lymphoid progenitors (lin$^-$ Sca1^{med} Kit^{med} IL-7Ra$^+$), (D) common myeloid progenitors (lin$^-$ Sca1$^-$ Kit$^+$ CD34$^+$ CD16^{med}), (E) granulocyte-macrophage progenitor (lin$^-$ Sca1$^-$ Kit$^+$ CD34$^+$ CD16^{high}) and (F) megakaryocyte-erythrocyte progenitor (lin$^-$ Sca1$^-$ Kit$^+$ CD34$^-$ CD16$^-$). The analyzed granulocyte maturation stages were (G) myelocytes (CD11b$^+$/Ly6G^{low}), (H) immature granulocytes (CD11b^{med}/Ly6G^{high}) and (I) mature granulocytes (CD11b^{high}/Ly6G^{high}). Data pre-sented are mean values (±SD) (n=6). $**P \le 0.005$; $***P \le 0.0005$. n represents the number of biological repli-cates.

ble (**Figure 5A**). However, the more differentiat-ed multi-potent progenitor population (MPP; lin$^-$/Sca1$^+$/Kit$^+$/CD48$^-$/CD150$^-$) shows no alter-ations, neither in the single-mutant mice, nor in the double-deficient mice (**Figure 5B**). Under steady-state conditions during antigen-inde-pendent hematopoiesis lineage-restriction appears at the level of common lymphoid pro-genitor (CLP; (lin$^-$/Sca1^{int}/Kit^{int}/IL-7Rα$^+$) or com-mon myeloid progenitor (CMP; lin$^-$/Sca1$^-$/Kit$^+$/CD34$^+$/CD16^{low}). The loss of Btk considerable

elevated the cell number of CLP, while in the case of MyD88 deficiency no alterations were observed (**Figure 5C**). Interestingly, the CMP cell number is slightly enhanced when Btk or MyD88 are not expressed, but significantly reduced in comparison to the single mutant or wild type littermates when both, MyD88 and Btk, are deleted. Moreover, the cell number of the more restricted granulocyte/macrophage progenitor (GMP; lin$^-$/Sca1$^-$/Kit$^+$/CD34$^+$/CD16^{high}), which develops from the CMP, is also

decreased in double-deficient mice but not in single-mutant mice, whereas the megakaryocyte/erythrocyte progenitor population (MEP; (lin$^-$/Sca1$^-$/Kit$^+$/CD34^{low}/CD16$^-$) is not altered (**Figure 5D-F**).

To dissect, if the decreased myeloid cell number in DKO mice is either due to the reduced number of myeloid progenitors or to a decreased differentiation potential of the progenitor population, in vitro differentiation assays were performed. For this purpose GMP were purified from the bone marrow of mice and used in colony-forming unit (CFU) assays in the presence of IL-3 and GM-CSF to induce myeloid differentiation. These analyses revealed no considerable reduction in the amount of CFU obtained from double-deficient GMP in comparison to both single-mutant GMP. In accordance with previously published data, Btk-ko GMP show a significant reduction in CFU numbers that is also found for MyD88-ko GMP. In contrast to the increased proliferation of Btk-deficient GMP no significant alterations in proliferative potential of MyD88-deficient or double-deficient GMP was observed (**Figure 5G and 5H**). However, examination of individual MyD88-ko CFU revealed a considerable shift of the composition of CFU from M-CFU and G-CFU towards mixed GM-CFU. In contrast, Btk-deficient CFU show a considerable increase in G-CFU at the expense of mixed GM-CFU as published previously (**Figure 5I**) [40]. However, the loss of both, MyD88 and Btk reverts the phenotype towards almost wild type distribution of CFU.

The analyses of proliferation of the different hematopoietic stem and progenitor cell populations using BrdU incorporation demonstrated only for LT-HSC a significant down-regulation of proliferation in the case of double-mutant mice in comparison to wild type or even single-mutant littermates (**Figure 6A**). All other stem and progenitor populations showed no considerable alterations in their proliferative potential, similar to the more differentiated myeloid populations of myelocytes and of immature granulocytes (**Figure 6B-H**). However, the proliferation of mature granulocytes is significantly reduced in MyD88-deficient animals and MyD88/Btk-mutant animals compared with wild type animals (**Figure 6I**). Therefore, the reduced myeloid as well lymphoid development in the bone marrow of mice after deletion of

MyD88 is mainly due to defects in differentiation, whereas the limited proliferation has only a minor impact on the overall phenotype described here.

Discussion

For the first time, we provide a comprehensive analysis of the phenotype of MyD88-deficient mice without challenging the mice with pathogens or pathogen-associated pattern molecules. The main finding of this study is that deletion of MyD88 not only influences myeloid differentiation after TLR ligation or cytokine administration, but has a much broader effect on myeloid as well as lymphoid cell development. In contrast to previous studies [5, 42], where no alterations in myeloid cell development have been shown, we demonstrate a significant reduction in terminal differentiation of granulocytes in the bone marrow independent from a defined TLR or interleukin stimulus. Moreover, the entire myeloid cell compartment in the spleen is drastically decreased in mice after the loss of MyD88. The combination of MyD88 and Btk deletion even leads to a more pronounced reduction in peripheral myeloid cell populations, especially monocytes and dendritic cells, but also granulocytes. Beside the disturbed myeloid development in MyD88-deficient mice, we could also show a severe antigen-independent reduction of the splenic B cell population that compromises almost all B cell subpopulations, particularly the IgD$^+$ B cells, newly formed B cells and follicular B cells. Additionally, the combined deletion of Btk, an important factor of B cell development, and MyD88 results in more pronounced defects in B cell differentiation already at the pro/pre B cell and immature B cell stage in the bone marrow. Also early hematopoietic cell populations like the long-term HSC are significantly reduced in MyD88-deficient as well as in double-mutant mice.

Adult hematopoiesis, the differentiation of LT-HSC towards lineage-restricted progenitors and finally the development of mature myeloid as well as lymphoid cells, depends on both intrinsic regulating factors like lineage-determining transcription factors and extrinsic factors like cytokines or direct cellular interaction with stroma cells of the bone marrow niche or similar structures in secondary lymphoid organs. Until now, the regulation of such a com-

plex process and the impact of single components toward the outcome of hematopoiesis are not fully understood.

The function of the adaptor molecule MyD88 is well described for TLR signaling pathways, where it is necessary for the signal transduction from the receptors towards the transcription factors of the NF-κB family. The different members of the TLR family are mainly expressed on myeloid cells but also on B cells. In both cell types we found significant differences in terminal differentiation after MyD88 deletion. However, TLR signaling is part of the defense mechanisms of the immune system during infection or injury, but should be not activated under antigen-independent, steady-state hematopoiesis. Based on our findings one could speculate that TLR ligation may also occur during homeostatic conditions, where no exogenous pathogen-associate molecular patterns are present. Several recent studies provide evidence for endogenous TLR ligands released from damaged tissues like breakdown products of the extracellular matrix [17, 43, 44], heat-shock proteins [45], or oxidized lipids [46]. These agents are able to induce TLR signaling without the presence of pathogens. Moreover, Rolls and colleagues have demonstrated that TLR4 activation increases the proliferation and differentiation towards neurons of neuronal progenitor cells, whereas TLR2 deficiency results in an enhanced differentiation of NPC into astrocytes [13]. These results as well argue for the existence of endogenous TLR ligands, since neuronal development normally occurs in the absence of pathogens.

Another possible explanation for the evident influence of MyD88 on antigen-independent hematopoiesis would be the existence of a homeostatic, basal TLR signaling induced for example by commensal intestine microbes. In the last few years, several publications could show that failed restriction of basal TLR signaling drives the development of chronic inflammation via dendritic cells [47], chronic myeloid cell activation [48] or autoimmunity and B cell hyperresponsiveness [49] without exogenous stimulation. Hence, this findings support the existence of a homeostatic TLR signaling induced by commensal microbes or endogenous ligands, since deletion of MyD88 prevents the induction of chronic inflammation. Additionally, Nagai and colleagues demonstrat-

ed that TLR are already expressed on HSC as well as progenitor cells and that TLR ligation induces a robust myeloid differentiation independent of the presence of colony-stimulating factors or interleukins [7]. Therefore, the reduced development of mature granulocytes in the bone marrow of MyD88-deficient mice could be due to disturbed TLR signaling after endogenous ligand ligation or basal, homeostatic activation via commensal microbes. The significant reduction of MyD88-deficient as well as double deficient LT-HSC supports additionally the possible importance of a basal TLR signaling for early hematopoietic development.

In contrast to granulocytes and monocytes, which differentiate in the bone marrow, development of dendritic cells as well as macrophages mainly occurs in peripheral tissues, where they originate from a common progenitor [50, 51]. Also monocytes are able to differentiate into macrophages or dendritic cell during inflammatory responses [52-54]. Thus, the decreased cell number of macrophages, dendritic cells and monocytes in the spleen could be dependent on a reduced terminal differentiation potential of MyD88-deficient myeloid progenitors due to endogenous ligand-induced or basal TLR signaling. Interestingly, the combination of MyD88 and Btk deletion augments the phenotype of MyD88 deficiency especially in the case of granulocytes, monocytes and dendritic cells. In line with these findings, we and others already demonstrated the significant influence of Btk deficiency on the outcome of myeloid differentiation, particularly on granulocyte differentiation [31, 40] but also on TLR-induced maturation of dendritic cells or macrophages [23, 33, 55]. The here presented data reveal a synergistic effect of MyD88 plus Btk deletion on the decreased cell number of differentiated granulocytes, monocytes and dendritic cells, but also on early myeloid progenitors like CMP and GMP, which argues for the association of MyD88 and Btk with different pathways or with separate parts of the TLR signaling. Accordingly, we could demonstrate previously the involvement of Btk in GM-CSF signaling pathway in myeloid precursors that later develop into granulocytes, monocytes or dendritic cells [40].

In addition, with regard to the diverse effects of MyD88 deficiency on myeloid cell development as well as on terminal B cell development, the

existence of other signaling pathways than TLR signaling, where MyD88 is involved, cannot be excluded. Mamidipudi and colleagues have already demonstrated the recruitment of MyD88 at the NGF receptor p75 after NGF ligation that finally leads to activation of NF-κB [12]. Moreover, MyD88 supports RAS-mediated tumorigenesis through the constitutive activation of pro-mitogenic MAPK signaling probably by steric inhibition of the dephosphorylation of ERK by its phosphatase MPK3 [56]. The recruitment of MyD88 to TACI after ligation of APRIL or BAFF during class switch recombination in B cells is a further example of TLR-independent functions of MyD88 [10]. Interestingly, the authors demonstrated a TIR domain-independent binding of MyD88 to TACI, which allows the speculation about signaling pathways not yet identified, where MyD88 can be implied via TIR-dependent or -independent interaction.

The involvement of MyD88 after TACI activation by APRIL and BAFF during class switch recombination in B cells offers a further explanation for our findings, especially with regard to the B cell phenotype. Previous studies of deletion mutants for APRIL and BAFF revealed a significant effect of BAFF on B cell maturation in the spleen, since BAFF deficiency leads to reduced splenic B cell numbers, particularly due to the reduction of follicular B cells and MZB cells. Additionally, the bone marrow B cell numbers are normal, but the recirculating B cells are absent [57]. In contrast to the phenotype of BAFF deletion, the APRIL deficiency does not result in an obvious alteration within the B cell compartment [58]. The different outcome of APRIL and BAFF deletion is probably due to the differences in their receptor binding properties, because BAFF not only interacts with TACI, but also with the BAFF receptor [59] as well as with the B cell maturation antigen (BCMA). In contrast, APRIL only binds to TACI and BCMA [60]. The analyses of receptor deletion mutants demonstrated that BAFF-R deficiency results in a similar phenotype like BAFF deletion [61, 62], while BCMA deficiency affects only the survival of long-lived bone marrow plasma cells [63]. The TACI deletion leads to an increased B cell accumulation with a marked splenomegaly and enlarged secondary lymphoid organs [64]. Indeed, our results concerning the B cell compartment of MyD88- and Btk-deficient mice show a striking similarity to the BAFF/BAFF-R

deletion phenotype with decreased follicular B cells and MZB cells in the spleen as well as significant reduced recirculating B cells in the bone marrow. The Btk deficiency has definitely a major impact on the B cell phenotype due to the absent BCR signaling. However, the deletion of MyD88 also leads to a significant reduction of follicular B cells as well as IgD+ B cells, and the combination of Btk and MyD88 deficiency further decreases the B cell number. Thus, the described B cell phenotype of MyD88/Btk double deficient mice could be due to an interaction of MyD88, Btk or both with not yet identified signaling pathways that are active during B cell maturation in the spleen. Additionally, the significant reduction of innate immune cells in the spleen of double deficient mice could also cause the altered B cell maturation, since myeloid cells such as monocytes, dendritic cells and granulocytes are the main source of BAFF and APRIL [65].

Moreover, in the analyzed mouse model the global MyD88 deletion could cause the described variations in the myeloid as well as in the lymphoid differentiation also via an exocrine pathway. Pevsner-Fischer and colleagues have demonstrated that mesenchymal stem cells express functional TLR and that MyD88-deficient mesenchymal stem cells show a reduced differentiation capacity compared with wild type cells [19]. The stem cell niche mainly consists of mesodermal cell lineages, which develop from mesenchymal stem cells, and regulates the hematopoietic differentiation in the bone marrow by cytokine secretion as well as by cellular interaction. Also the follicular dendritic cells of the spleen, which are important regulators of splenic B cell maturation, are the putative progeny of mesenchymal stem cells [66]. Therefore, the MyD88-deficient mesenchymal stem cells could also be responsible for the alterations in the myeloid and lymphoid cell development described here. Taken together, the role of MyD88 for steady-state myeloid and lymphoid hematopoiesis is complex and needs further investigation including the analyses of signaling pathways in which MyD88 might be involved during the process of blood cell development.

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