Elucidating the role of Btg1 and Btg2 in hematopoietic homeostasis

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Elucidating the role of Btg1 and Btg2 in hematopoietic homeostasis

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ABSTRACT

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Hematopoietic homeostasis refers to the maintenance of various progenitor cells in the hematopoietic system at a certain ratio and is disrupted by activation in response to stress stimuli such as chemotherapy intervention. Progenitor cells of various lineages that exist in heamtopoiesis can be explained by the intrinsic net work driven by transcription factors and the potential for multilineage differentiation depending on the bone marrow microenvironment (a.k.a. bone marrow niche). Despite recent advances in the understanding of hematopoietic homeostasis, the regulatory mechanisms mediated by cell intrinsic factors still remain a challenge. Btg (B cell translocation gene)1 and Btg2 are important regulators of early hematopoiesis and function as cell intrinsic self-maintenance factors that regulate cell growth and quiescence. Here, immunophenotyping was performed under steady-state and 5-FU-induced stress conditions to investigate the major immunophenotypes of Btg1 and Btg2-mediated functions. The effect on Btg1/2-mediated regulation of hematopoietic homeostasis was demonstrated using flow cytometry, in vitro colony forming unit (CFU) assays, and complete blood count (CBC) test assays. Under steady-state conditions, the Btg1/2-deficient hematopoiesis showed upregulation of

common-myeloid progenitor (CMP) and granulocyte-macrophage progenitor (GMP), suggesting disruption of hematopoietic homeostasis toward myelopoiesis. Subsequent myeloid lineages, Neutrohil and Monocyte-Macrophage, significantly increased and decreased, respectively. Under 5-FU-induced stress conditions, common lymphoid progenitor (CLP) increased along with CMP and GMP, suggesting that Btg1/2 has a mediated effect on lymphopioesis as well as myelopoiesis. The neutrophil ratio was also upregulated, and monocyte-macrophage was decreased under stress conditions, suggesting a Btg1/2-mediated biphasic role. This study reveals Btg1/2-mediated hematopoietic homeostasis and suggests that it is an important regulator in maintaining myeloid lineage hematopoietic homeostasis.

Key words : hematopoietic homeostasis, Btg (B cell translocation gene)1, Btg2, Common myeloid progenitor (CMP), Granulocyte-Macrophage progenitor (GMP), myelopoiesis

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I. INTRODUCTION

Hematopoiesis is the process by which all blood cells are formed in the bone marrow[1].It's an extremely complex and tightly regulated process. Because mature blood cells have a finite lifespan, cells within bone marrow are constantly being replaced[2]. During this process, progenitor blood cells must be present in the right proportions, which is called hematopioetic homeostasis. By maintaining this hematopoietic homeostasis, our body produces blood cells between 10^{11} to 10^{12} every day in a typical steady-state condition[3,4]. However, under stressful conditions, such as infection or injury, this number can be significantly increased. These hematopoietic events originate from multipotent stem cells called hematopoietic stem cells (HSCs). During differentiation, HSC progeny undergo a wide range of maturation stages, forming multipotent stem cells and lineage-committed progenitor cells[5].

Hematopoietic lineage can be broadly classified into five major types: multipotent stem cell, multipotent progenitor cell, lineage-commiitted progenitor cell, lymphoid cell and myeloid cell[6]. Multipotent stem cells include long-term hematopoietic stem cell (LT-HSC), which are the fraction that maintains the strongest quiescent state and has high self-renewal activity. It is the most primitive form of hematopoietic stem cell and is characterized by the presence of only a very small amount in the bone marrow[7,8]. Multipotent progenitor cells include short-term hematopoietic stem cell (ST-HSC), LSK ($Lin^-Sca1^+c\text{-}kit^+$), LK ($Lin^-Sca1^-c\text{-}kit^+$), Multipotent Progenitor cell (MPP)4, MPP3, and MPP2. ST-HSCs are characterized by increased Flk-2 expression as LT-HSCs differenatiate[9]. The difference between LT-HSCs and ST-HSCs is the duraion period of fraction[10]. The LT-HSCs must survive for the duration of the organism in order to continuously replenish the hematopoietic system, whereas ST-HSCs or multipotent progenitors (MPPs) can only maintain hematopoieis in the short term[11]. LSK cells and LK cells are characterized by c-kit and Sca1 expression without the expression of lineage markers such as CD4, CD8, CD19[12,13]. Both MPP cells and lineage-committed progenitor cells are included in the LSK or LK cell fraction, and a higher purity of HSC can be isolated by combining it with CD150, which is a SLAM family marker[14,15]. It has been reported that LSK cells exist in a small amout range from 0.1% to 0.4% of bone marrow total nucleated cells (TNCs) and increase rapidly within 4 days upon 5-fluorouracil injection, participating in the repopulation of the blood pool[16-18]. The main difference between LSK cells and LK cells is the expression pattern of Sca1[19]. LK cells differentiate into downstream subgroups of common-myeloid progenitors (CMPs), megakaryocte-erythrocyte progenitors (MEPs), and granulocyte-macrophages progenitors (GMP), which are myeloid-biased progenitor cells[20,21]. Meanwhile, MPP is a group within the LSK cell fraction and can be divided into MPP4, MPP3, and MPP2 depending on the surface mocule expression pattern[22-24]. MPP4, also called LMPP, is a lymphoid-primed MPP, a population with a high potential for differentiation toward the lymphoid lineage rather than the meyloid lineage[25,26]. On the other hand, the MPP3 and MPP2 fractions can be classified into myeloid-biased lineages.

Multipotent progenitor cells show highly heterogeneous properties. Therefore, unlike CMP, MEP, and GMP fractions, MPP fractions are not classified as lineage-committed progenitor cells[27]. CMP, MEP, and GMP are lineage-committed progenitor cells, which are populations with a determined fate[28]. Unlike the MPP fraction, they do not have intercellular plasticity and differentiate into myeloid lineages such as RBC, platelet, neutrophil, and monocyte. There are five major types of fractions that exist in the bone marrow and produce peripheral blood (PB) output occurs in the proper situation. For this reason, the balance of hematopoietic homeostasis must be maintained at all times[29].

HSC can be functionally defined by four main factors: first, the quiescence of HSC; second, repopulation potential; third, self-renewal activity; and lastly, multi-lineage differentiation potential. Theses four features of HSCs should remain constant even in the presence of strong proliferative stress to maintain hematopoietic homeostasis.

Btg(B cell lymphoma translocation gene)1 and Btg2 are involved in these four features and are highly likely to be involved in hematopoietic homeostasis. Btg1 and Btg2 are a member of the B cell translocation gene/transducer of ERBB2 (BTG/TOB)family[30,31], which has been linked to a variety of celluar functions and exhibits antiproliferative process[31,32]. It can be hypothesized that it may play a central role in homeostasis in the context of suppressing excessive proliferation. Btg1 and Btg2 have been reported to play various physiological roles in previous studies using whole knockout and conditional knockout systems[33]. The knock-out study of Btg1 and Btg2 in bone marrow and spleen showed that B cell development was significantly impaired, resulting in a significant decrease in the frequency of mature B cells. This suggests that the Btg genes are significantly involved in early B cell development during hematopoieis in the bone marrow[34]. Additionally, recent research has shown that quiescent T cells were significantly reduced in the Btg1/2_CD4-cre system[35]. Btg1/2 have the ability to control the global down-regulation of mRNAs because they interact with the proteins PABP and CNOT complex, which bind to mRNAs in an unspecific manner[36]. This suggests that both Btg1 and Btg2 may function as cell intrinsic factors that maintain quiescence. Furthermore, this

feature of the Btg1 and Btg2 genes is likely to be involved in the homeostasis of hematopoietic stem cells that are tyring to maintain a quiescent state[37]. However, the correlation between Btg1 and Btg2 with hematopoietic homeostasis has not been revealed. This study therefore proposes a model wherein bone marrow maintains hematopoietic homeostasis, which together coordinates the output of the myeloid and lymphoid lineages with Btg1 and Btg2.

II. MATERIALS AND METHODS

1. Mice

Btg1 and Btg2 conditional knockout mice were generated by insertion of two loxp sites into Btg1/2 loci using the CRISPR/Cas9 genome editing system. $Btg1^{f/f}$ $Btg2^{f/f}$ mice were crossed with Flk1-cre mice to generate Double conditional knockout mice because theses two genes are functionally redundant. $Btg1^{f/f}$ $Btg2^{f/f}$ – Flk1-cre mice were crossed with $Btg1^{f/f}$ $Btg2^{f/f}$ mice to get littermate wild-type control mice group (WT : $Btg1^{f/f}$ $Btg2^{f/f}$) and double conditional KO mice group (DKO : $Btg1^{f/f}$ $Btg2^{f/f}$ – Flk1-cre) for experiments. All WT and DKO mice were used at 8-12 weeks of age unless otherwise noted. All mice required for this study were bred in specific-pathogen-free conditions in the animal facility of the Yonsei University College of Medicine Avison Biomedical Research Center (ABMRC) and all corresponding animal procedures and protocols were approved by the Institutional Animal Care and Use Committee of Yonsei University (IACUC).

2. Isolation of Total Nucleated Cells from Mouse Bone Marrow, Spleen and Thymus

Femur, Tibia, Spleen and Thymus were harvested into ice cold DPBS. Bone marrow (BM) cells were flushed by using 10mL syringe replaced with 26g needle from the Femur and Tibia and passed through 70μm filter (BD Falcon). The filtrate was centrifuged at 2000rpm for 5 min at RT and the pellet obtained was resuspended in 3mL 1X RBC lysis buffer (G-DEX™ IIb RBC Lysis Buffer) for 5min. This was stopped by addition of ice-cold DPBS with 2% FBS. Following centrifugation at

2000rpm after RBC lysis, the total nucleated cells were obtained for downstream analysis.

Spleen and Thymus were passed through a 70μm filter. The single cell suspension was centrifuged and resuspended in 5mL 1X RBC lysis buffer for 5min. This was stopped by addition of ice-cold DPBS with 2% FBS. Following centrifugation at 2000rpm for 5min after RBC lysis, the total nucleated cells were obtained for downstream analysis.

3. RNA isolation and relative quantitative real-time PCR

Total RNA was isolated from total Bone marrow cells and Splenocytes using the RNeasy micro kit (QIAGEN, 74004). Oligo dT primer should have been avoided for cDNA synthesis because Btg1/2 could shrink the length of poly A tail via process of deadenylation. Total RNAs were then reverse transcribed using random hexamer. RNA levels of Btg1 and Btg2 genes were determined by quantitative real-time polymerase chain reaction (qRT-PCR) on Applied Biosystems Quant Studio 3 Real-Time PCR System using Universal SYBR Green Master (ROX). Hprt, GAPDH, b-actin, Rpl18, Rpl7a was used to normalize the data. The list of primer sequence is shown in Table 1.

4. Flow cytometry

For Immunophenotyping of WT and DKO mice bone marrow, spleen and thymus, TNCs were obtained with IMDM supplemented with 2% FBS or ice cold DPBS

(Hyclone). TNCs were counted with an Automated LUNA Cell Counter (Thermo Fisher SCIENTIFIC). Cells were then stained with antibody cocktail diluted in FACS buffer, at the concentration of 50uL per 1 x 10^6 cells at 4℃ for 30 minutes. To identify Hematopoietic population in the bone marrow and spleen, Lineage positive (Lin^+) marker was conjugated with Biotin for negative selection and can be distinguished based on streptavidin-biotin interaction. The lineage cocktail for Hematopoietic Stem Progenitor cell population (LSK, LT-HSC, ST-HSC, MPP4, MPP3, MPP2, LK, MEP, CMP, GMP) consisting of biotin-conjugated anti-CD19, B220, CD3ε, CD4, Gr1, NK1.1, Ter119, CD11b, CD11c antibody. The lineage cocktail for common lymphoid progenitor cell population consisting of biotin-conjugated anti-CD4, CD8a, B220, Gr1, Ter119, CD3ε antibody. Obtained bone marrow cells, splenocytes and thymocytes were stained with APC-Cy7-anti-c-kit (2B8, BioLegend), PE-Cy7-anti-Sca1 (E13-161.7, BioLegend), APC-anti-CD150(SLAM) (W19132B, BioLegend), BV421-anti-Flt3 (A2F10, BioLegend) , FITC-anti-CD48 (HM48-1, BioLegend), Streptavidin-PerCP (BioLegend), BV421-anti-CD34 (RAM24, BD Biosciences), BV711-anti-FcγRII/III (2.4G2, BD Biosciences), PE-anti-IL-7Rα (A7R34, BioLegend), APC-anti-CD135 (A2F10, BioLegend), FITC-anti-Ly6D (49-H4, BioLegend), AF488-anti-CD11b (M1/70, BioLegend), AF700-anti-CD11b (M1/70), AF488-anti-Ly6g (1A8, BioLegend), APC-Cy7-anti-B220 (RA3-6B2, BioLegend), FITC-anti-CD3ε (145-2C11, BioLegend), APC-Cy7-anti-CD4 (RM4-5, BioLegend), AF700-anti-CD8 (53-6,7, BioLegend) antibody for 30-60min at 4℃. Then cells were washed with FACS buffer (DPBS with 2% FBS) for twice. All flow cytometric analysis in this study was performed on an LSR II (BD Biosciences) or an LSR Fortessa (BD Biosciences) or a Symphony A5 (BD Biosciences). Data were processed by using FlowJo software (version 10.0, BD Bioscience). The List of Hematopoietic Stem and Progenitor Cell population and mature Lymphoid, Myeloid lineage population's immunophenotypes are shown in Table 2.

5. Complete Blood Count (CBC) Test

Mice was anesthetized with 2% isoflurane, and 300-500uL peripheral blood was obtained with Heparinized Capillary tube (JD-S-135R) by retro-orbital venipuncture into BD microtainer EDTA coated tube. The collected blood was immediately used for analysis. Complete blood count (CBC) test was performed using automated hematology analyzer BC-5000vet (Mindray animal care, China).

6. Colony Forming Unit (CFU) assay

The CFU assay was performed to compare the capacity of Hematopoietic stem and progenitor cells to form Hematopoietic colonies between littermate wild-type control and DKO mice. 10 weeks of aged male mouse were used in CFU assay. 3 x 10^4 cells of bone marrow cells were transferred to 3mL MethoCult Media (STEMCELL Technologies, MethoCult™ GF M3434) containing SCF, EPO, IL-3 and IL-6. CFU-G, CFU-M, CFU-GM, CFU-GEMM, BFU-E were counted after 10-12 days of culture based on the criteria of number and morphology of the colonies according to manufacturer's instruction. The representative colonies were photographed using OLYMPUS IX71 Research Inverted Microscope and data recorded.

7. Drug administration

2,4-Dihydroxy-5-fluoropyrimidine (5-FU, Sigma-Aldrich, F6627-5G) was administrated for stress-induced Hematopoietic conditions resulting in the depletion

of actively proliferating hematopoietic cells. Both littermate control mice and DKO mice were injected with 5-FU via retro-orbital route with 150mg/kg of single dose. The analysis was performed on Days 10 after 5-FU administration.

8. Determination of Absolute Numbers of Hematopoietic stem cell

The frequency of cells in total events acquired by flow cytometric analysis was used to calculate absolute number of Hematopoietic lineage population. Total number of TNCs were counted by Automated LUNA Cell Counter.

9. Bone marrow derived macrophages (BMDM) culture

Macrophages were derived from the bone marrow of littermate control and Btg1/2 DKO mice. L929 cell culture supernatant was used as BMDM culture medium for differentiation of bone marrow cells to macrophages. L929 cell line was kindly provided by Seoul National University (SNU) and incubated in 175T cell culture flask for 7 days. Theses were incubated at 37℃ and 5% CO_2. L929 cell culture supernatant was collected and filtered by 0.22㎛ strainer and transfer into 15mL conical tube. Bone marrow cells were collected from the femur and tibia and single cell suspension was cultured in complete RPMI media (10% FBS, 1% L-Glutamine, 1% Penicillin-Streptomycin) with 30% L929 supernatant at 37℃ and 5% CO_2 for 7 days, changing the media on day 3 and 5. M0 BMDM was stained with AF700-anti-CD11b (M1/70) and AF488-anti-F4/80 (BM8) antibody for 30 minutes at 4℃ or 15 minutes at room temperature. Flow cytometry analysis was performed on BD LSR II (BD Biosciences).

10. Statistical analysis

Differences between groups were calculated by Unpaired Student's *t*-test or Mann-Whitney *U*-test with GraphPad Prism, Version 8.0 (GraphPad Software) unless otherwise specified. Statistical significance is displayed across figures using the following legend: $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$. Data are represented as mean ± SEM as indicated in the figure.

Table 1. List of primers used in this study

Gene	Direction	Nucleotide sequence (5' – 3')
Btg1	Forward	TGTCCTTCATCTCCAAGTTC
	Reverse	AGAGGATCCATCTTATGGTTG
Btg2	Forward	CTGACCGATCATTACAAACAC
	Reverse	AGACACTTCATAGGGATCAAC
Hprt	Forward	AGGGATTTGAATCACGTTTG
	Reverse	TTTACTGGCAACATCAACAG
GAPDH	Forward	AGGTCGGTGTGAACGGATTTG
	Reverse	TGTAGACCATGTAGTTGAGGTCA
β-actin	Forward	GATGTATGAAGGCTTTGGTC
	Reverse	TGTGCACTTTTATTGGTCTC
β2M	Forward	GTATGCTATCCAGAAAACCC
	Reverse	CTGAAGGACATATCTGACATC
Rpl18	Forward	CATCATGGGTGTTGACATTC
	Reverse	CTCATGAACAACCTCTTCAG
Rpl7a	Forward	CGTAGACCCCATTGAGCTGG
	Reverse	GCACCCTTGTCTTCCGAGTT

Table 2. List of Mouse Hematopoietic Stem and Progenitor Cell Immunophenotype

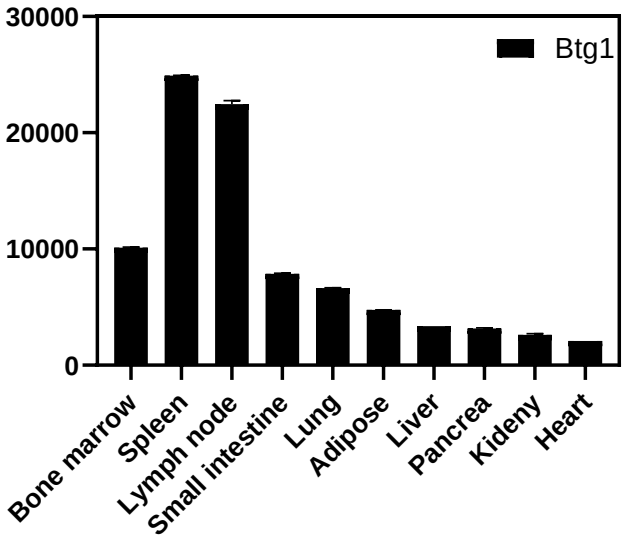
HSPC compartment	Fraction	Surface markers
Multipotent Stem Cells	LT-HSC	$Lin^-cKit^+SCA\text{-}1^+FLT3^-CD150^+CD48^-$
	ST-HSC	$Lin^-cKit^+SCA\text{-}1^+FLT3^-CD150^-CD48^-$
	LSK	$Lin^-cKit^+SCA\text{-}1^+$
	LK	$Lin^-cKit^+SCA\text{-}1^-$
	MPP4 (Lymphoid-Primed)	$Lin^-cKit^+SCA\text{-}1^+FLT3^+CD150^-$
Multipotent Progenitor Cells	MPP3 (Myeloid-biased)	$Lin^-cKit^+SCA\text{-}1^+FLT3^-CD150^-CD48^+$
	MPP2 (Myeloid-biased)	$Lin^-cKit^+SCA\text{-}1^+FLT3^-CD150^+CD48^+$
	CMP	$Lin^-cKit^+SCA\text{-}1^-CD34^{lo/int}CD16/32^{lo/int}$
Lineage Committed Progenitor Cells	MEP	$Lin^-cKit^+SCA\text{-}1^-CD34^{-/lo}CD16/32^{-/lo}$
	GMP	$Lin^-cKit^+SCA\text{-}1^-CD34^{int/+}CD16/32^{int/hi}$
	CLP	$Lin^-cKit^+IL7R^+FLT3^+Ly6D^{+/-}$
	Neutrophil	$CD11b^+Ly6g^+$
Myeloid Cell	Monocyte-Macrophage	$CD11b^+Ly6g^-$
	T cell	$CD3\varepsilon^+B220^-$
Lymphoid Cell	B cell	$CD3\varepsilon^-B220^+$

III. RESULTS

1. Btg1 and Btg2 are primarily expressed in the immune system, including bone marrow

A significant number of T cells, which play a central role in the adaptive immune system, remain in a quiescence state and remain in a poised state before encountering a specific antigen. The balance between quiescent T cells and effector T cells, that is, the homeostasis of the entire blood pool, must be maintained at all times to enable the body to carry out an optimal immune response to infection or injury. Previous studies have shown that among the BTG/TOB family, Btg1 and Btg2 have high expression levels in white blood cells and lymph nodes, and particularly high expression levels in quiescent naive T cells[35]. This suggests that in the immune system, Btg1/2 is a cell intrinsic maintenance factor that plays a central role in maintaining homeostasis by controlling the proliferation and differentiation of blood cells. Since hematopoiesis is predominantly carried out in the bone marrow[29], where Btg1/2 expression was validated, it is clear that this quiescent state and homeostasis are also connected to hematopoietic homeostasis. The expression level of Btg1 was highest in the secondary lymphoid organs (spleen and lymph node), and confirmed to be higher in the primary lymphoid organ, bone marrow, compared to other organs (Fig. 1A). On the other hand, Btg2 was confirmed to be expressed at a higher level than in the spleen and lymph nodes, suggesting that Btg2 plays a specific role in the bone marrow (Fig. 1B).

A



B

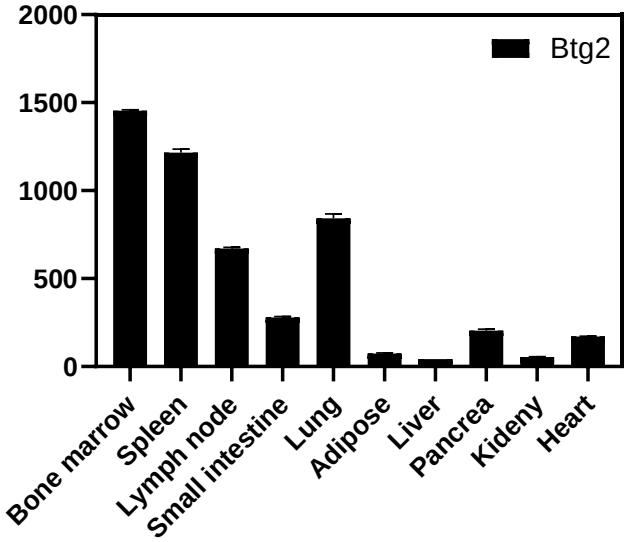


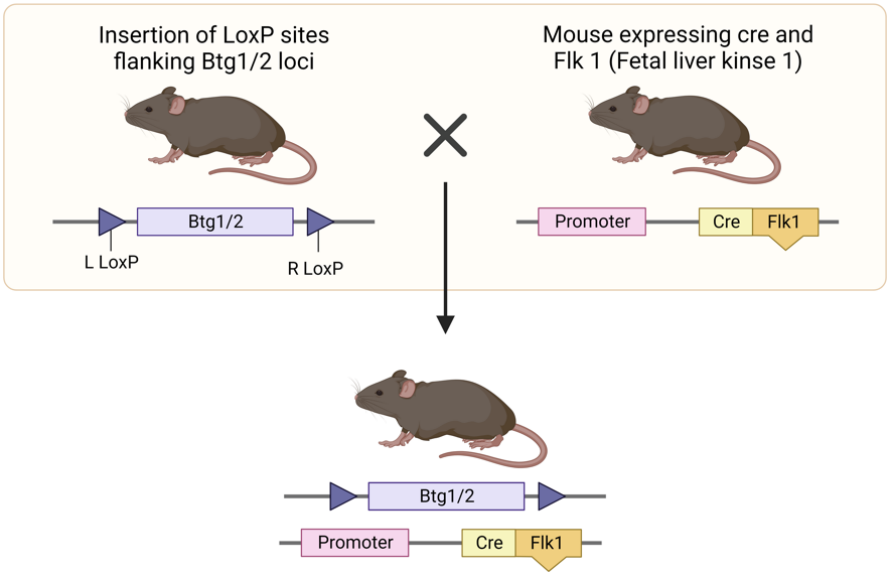
Figure 1. **Tissue-specific expression of Btg1 and Btg2 in multiple tissue.** **(A)** Tissue-specific expression of Btg1. High-throughtput gene expression profile is adopted from BioGPS dataset (GeneAtlas MOE430_1426083_a) **(B)** Tissue-specific expression of Btg2. High-throughput gene expression profile is adopted from BioGPS dataset (GeneAtlas MOE430_1448272_a).

2. The assessment of Btg1 and Btg2 double conditional knockouts

Since one of Btg1 and Btg2 loses its function, the other shows a compensatory mechanism in physiological condition[35], Double conditional knockout system was used to verify its function in vivo. Btg1/2 floxed mice were crossed with Flk1-cre mice, and its littermate was crossed with Btg1/2-Flk1-cre mice to obtain littermate controls for the experiment (Fig. 2A). During the development process, the first primitive LT-HSCs are derived from the mesoderm at 10.5 DPC (day post coitum)[38], and by 12.5 DPC, they migrate to the yolk sac and fetal liver and colonized for primitive hematopoiesis[39]. After 12.5 DPC, Flk1 (fetal liver kinase 1) binds to vascular endothelial growth factor (VEGF) to initiate vasculogenesis and hematopoiesis[40-42]. At this time, the first primitive hematopoiesis from LT-HSCs begins in the yolk sac and fetal liver. Btg1/2 is conditionally knocked out in all blood cell lineages derived from LT-HSC, starting at 12.5 DPC.

To elucidate the Btg1/2-Flk1-cre system, bone marrow cells were obtained by harvesting femur and tibia from littermate control mice and DKO mice, and RNA was extracted for qRT-PCR. Since a wide range of multipotent stem and progenitor cells exist in the bone marrow, the relative expression of Btg1 and Btg2 was normalized through a total of six genes (β2M, β-actin, GAPDH, Hprt, Rpl18, Rpl7a).

A



B

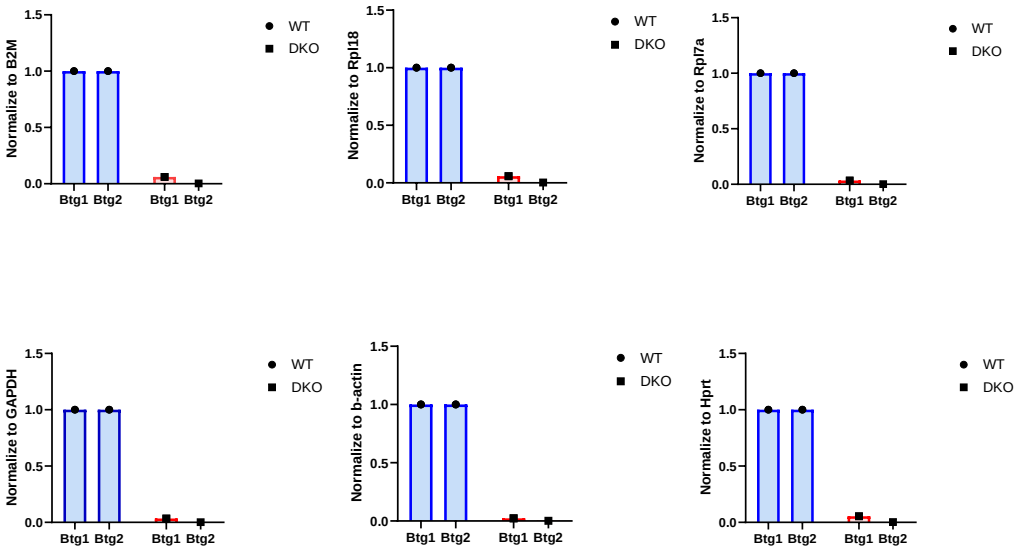


Figure 2. **Validation of Btg1 and Btg2 double conditional knockout in bone marrow.** **(A)** Generation of Btg1/2 double conditional knockout mice. Schematic illustration for generating Btg1/2 double conditional knockout mice in bone marrow. **(B)** Validation of Btg1/2 deletion in the Flk1-cre system. Each y-axis shows normalization genes for a total of 6 housekeeping genes (β2M, β-actin, GAPDH, Hprt, Rpl18, Rpl7a). The primer sequences are shown in Table 1.

3. Absence of Btg1 and Btg2 in normal hematopoiesis results in no significant change in most of multipotent stem cells

Multipotent stem and progenitor cells were analyzed by flow cytometry analysis to determine whether there were any changes in the multipotent stem and progenitor cell fraction (Table 2). Multipotent stem and progenitor cells are the group with the highest four functional characteristics of HSC: quiescence, repopulation potential, self-renewal activity, and multilineage differentiation potential. Because of these four functional properties, HSC is sustainable throughout an individual's lifespan. HSPC (Hematopoietic Stem and Progenitor Cell) and lymphoid-primed MPP4 can be distinguished according to the expression of Flt3 in the LSK ($Lin^{-}Sca1^{+}c\text{-}kit^{+}$) cell fraction that does not express lineage markers (i.e., CD4, CD8, NK1.1) expressed by mature blood cells[43,44]. Within the HSPC fraction, LT-HSC, ST-HSC, myeloid-biased MPP3, and MPP2 were identified (Fig. 3A, Table 2).

LT-HSC (long-term HSC), which exists in very small amounts in the hematopoietic lineage and maintains a constant pool from 12.5 dpc, slightly increased in frequency within the bone marrow, but there was no statistically significant difference. There was also no difference in absolute cell number within the bone marrow (Fig. 3B). Both ST-HSC (short-term HSC) and LSK cell populations belonging to multipotent progenitor cells did not show statistically significant differences in frequency and absolute cell number, but both tended to slightly decrease (Fig. 3C). Overall, the frequency and cell number of the multipotent stem cell (LT-HSC) and the multipotent progenitor cell (ST-HSC, LSK) tended to decline when Btg1/2 was knocked out, but there was no significant difference, indicating that hematopoietic homeostasis remained unaffected by Btg1 and Btg2 deletion.

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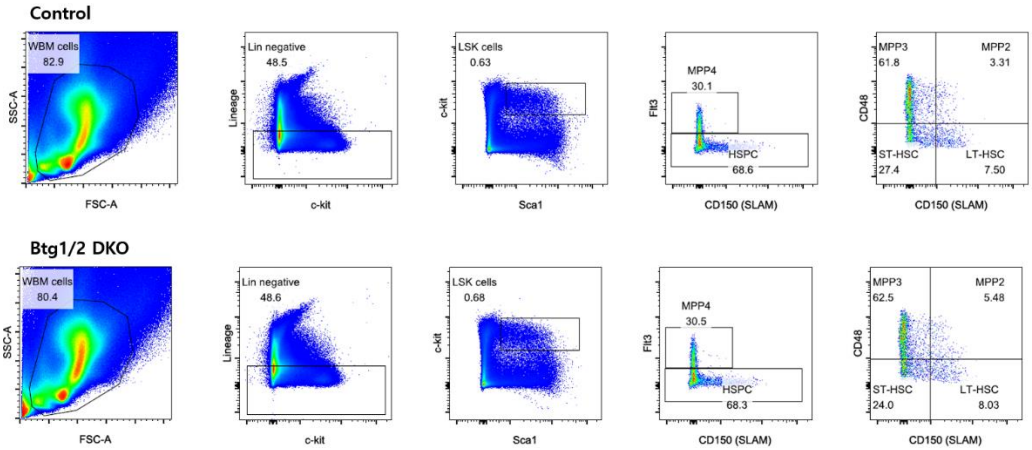

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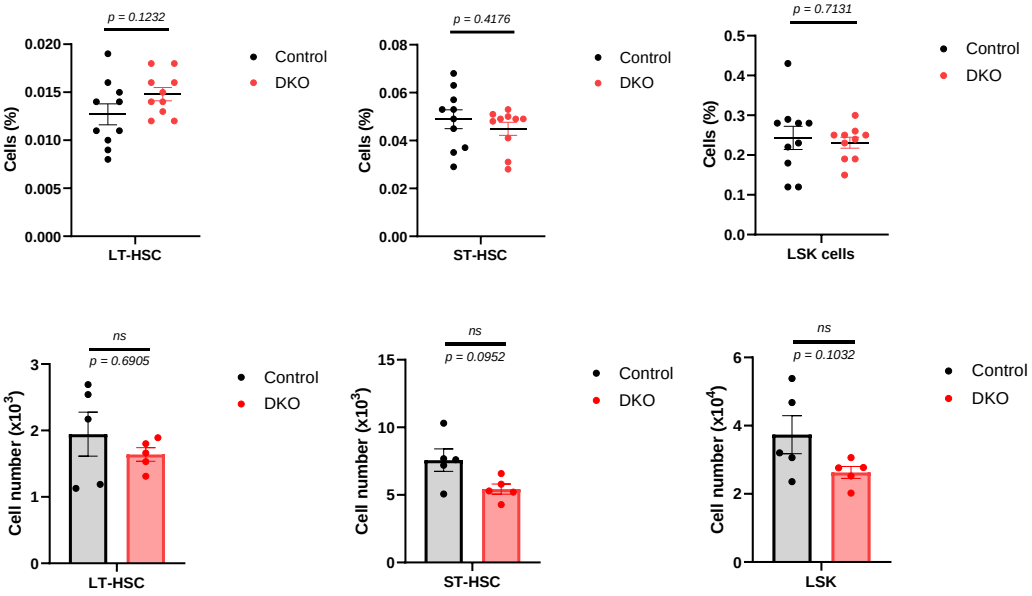

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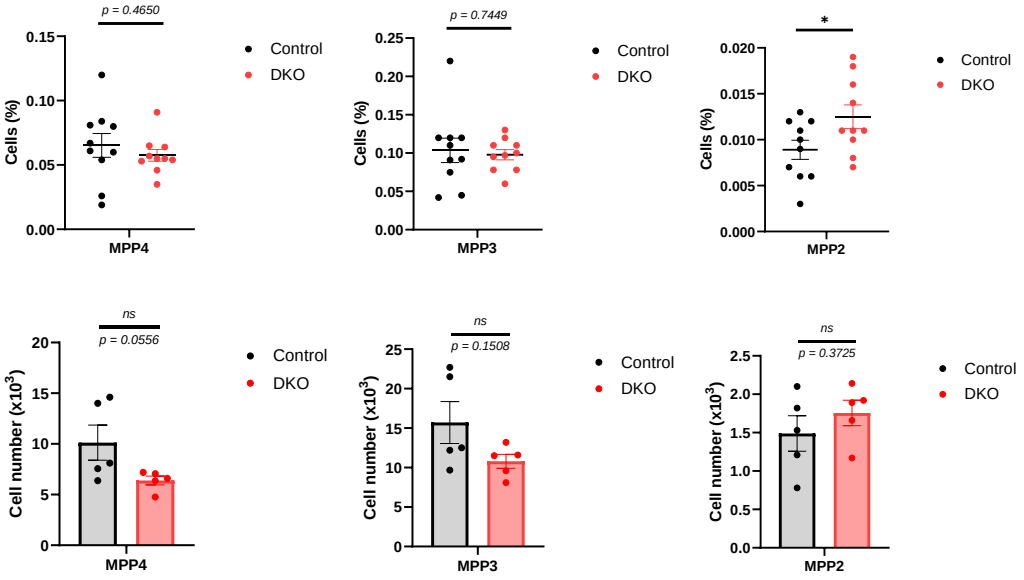

Figure 3. Loss of Btg1 and Btg2 do not affect homeostasis of multipotent stem and progenitor cells except for myeloid biased progenitor cells. **(A)** Gating strategy of Control ($Btg1^{f/f}$ $Btg2^{f/f}$) and Btg1/2 DKO ($Btg1^{f/f}$ $Btg2^{f/f}$ – Flk1-cre) bone marrow for Lineage negative LSKs, LT-HSC, ST-HSC, MPP4, MPP3 and MPP2 cell fraction. **(B)** LT-HSC, ST-HSC and LSK frequency and absolute cell number in bone marrow. **(C)** lineage-primed MPP4, MPP3 and MPP2 frequency and absolute cell number in bone marrow. The cellular frequency panels of (B) and (C) are derived from n=5 mice per group which is combined from two experiments. Absolute cell number panels are derived from n=5 mice per group. Error bars denote the mean ± SEM. (B and C) Statistics were calculated using the unpaired student's *t*-test. P values < 0.05 were considered significant (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001), *ns* = not significant.

4. Btg1 and Btg2 are involved in the homeostasis of myeloid lineage committed progenitor cells

Common Myeloid Progenitor (CMP) differentiates into Megakaryocyte-Erythrocyte Progenitor (MEP) and Granulocyte-Macrophage Progenitor (GMP). All three fractions are progenitor cells in Myelopoiesis hiearchy and are a subgroup of LK ($Lin^{-}Sca1^{-}c\text{-}kit^{+}$) cells[45]. On the other hand, Common Lymphoid Progenitor (CLP) is a progenitor cell in Lymphopoiesis hiearchy and is a subgroup of CD127 (a.k.a. IL-7 receptor alpha), c-kit double positive fraction[46,47]. Both the CMP and CLP lineages have no self-renewal and quiescent activity, unlike multipotent stem and progenitor cells[48]. CMP, MEP, and GMP can be distinguished according to the expression level of CD34 and CD16/32, and CLP can be identified through the expression of Flt3 (Fig. 4A, Table 2). Immnophenyping was carried out to see if Btg1/2 controls homeostasis for the lineage committed progenitor cell fraction.

As a result, there was no significant difference in the absolute cell number of CMP in the bone marrow of Btg1/2 knockout mice compared to littermate controls. However, the frequency was significantly increased (Fig. 4B). This result suggests that Btg1/2 regulates myelopoiesis homeostasis in steady-state condition. Next, both MEP and GMP were analyzed to see if there was a bias toward the erythrocyte or monocyte lineage because both progenitor cells have the capacity to differentiate into erythorcyte and macrophage respectively. As a result, the GMP fraction significantly increased in DKO mice but not in the MEP fraction (Fig. 4B), suggesting that the ablation of Btg1/2 in steady-state conditions disrupts normal homeostasis of the myeloid lineage-committed progenitor population.

Meanwhile, since lymphoid lineage progenitor cells in the bone marrow show high expression of CD127, CLP was defined as CD127, Flt3 double-positive population, which is a differentiation marker for hematopoietic stem cells. But there was no alteration in the CLP population (Fig. 4C). Overall, Btg1/2 regulates myelopoiesis homeostasis in normal hematopoiesis under steady-state conditions.

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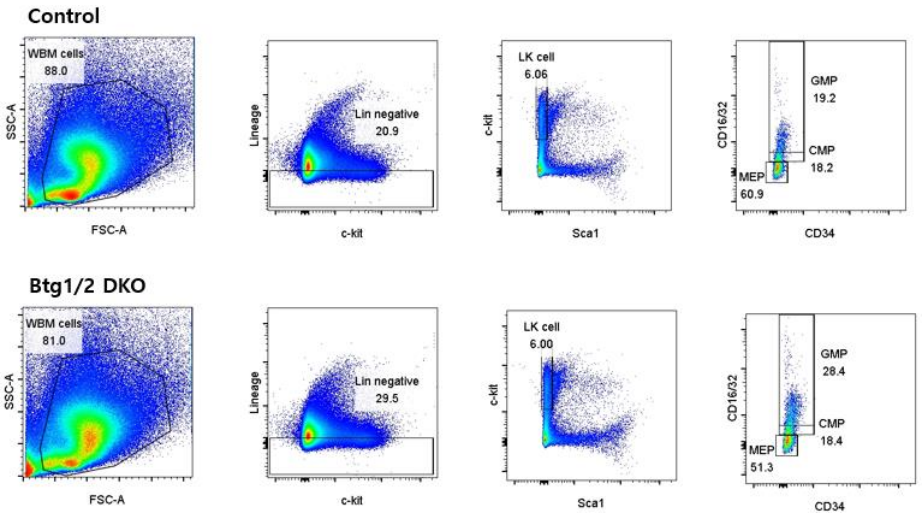

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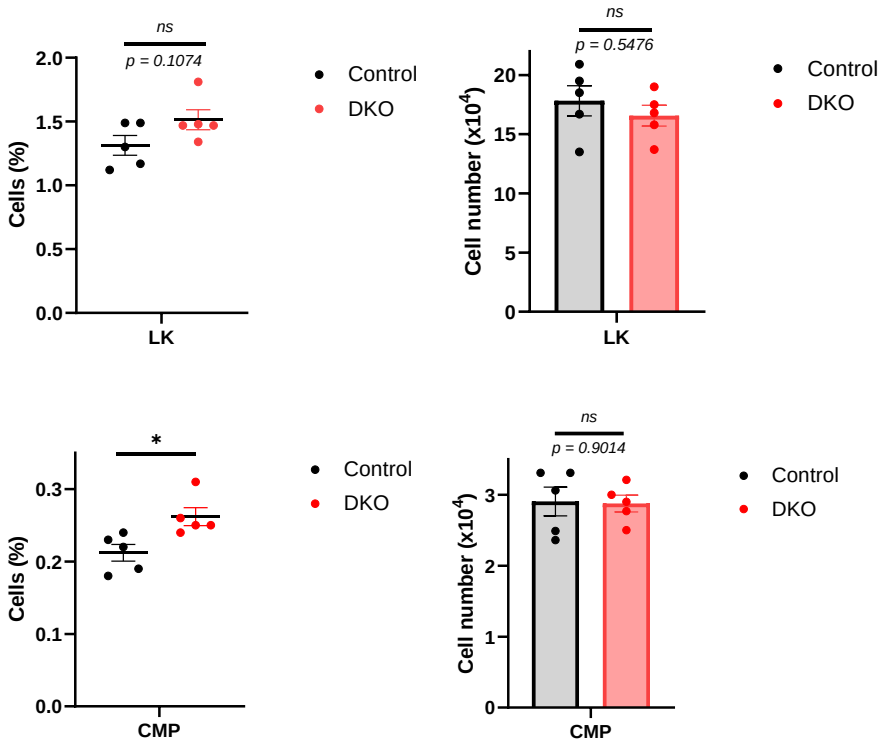

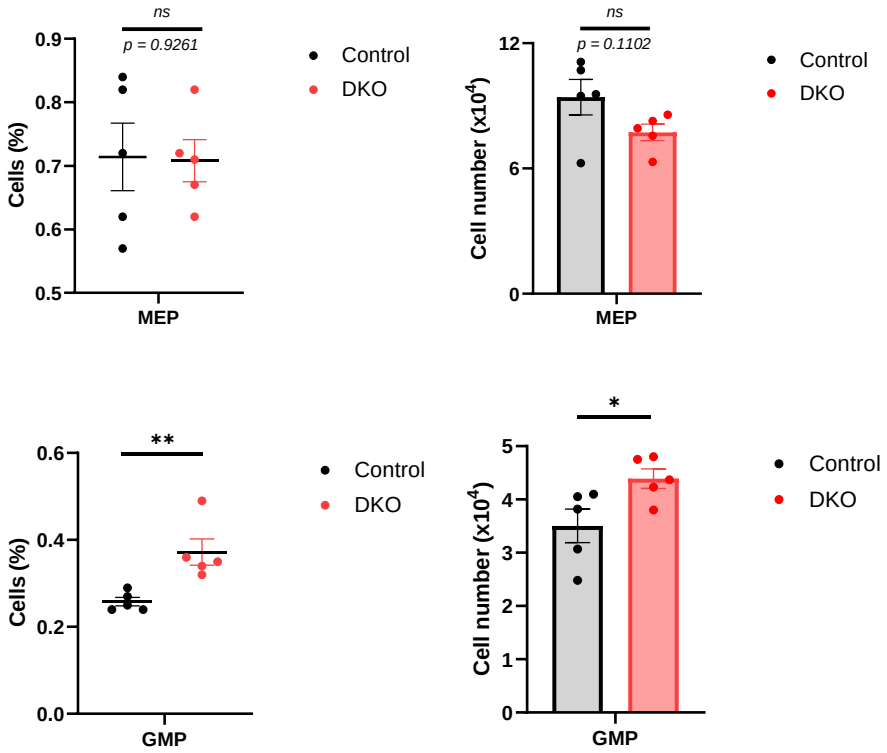

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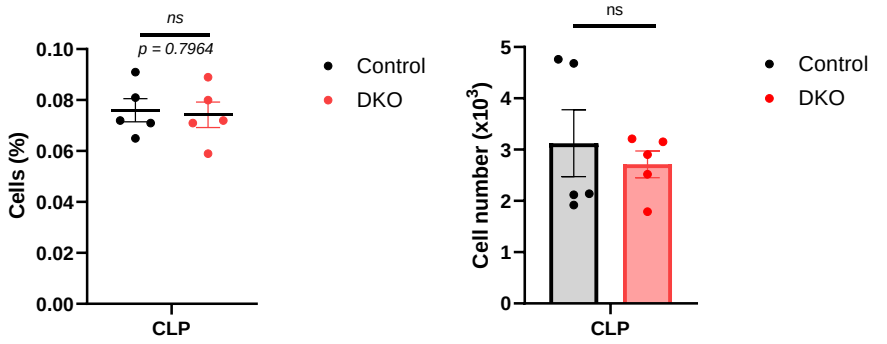

Figure 4. Loss of Btg1 and Btg2 results in myeloid-biased hematopoiesis and disrupts homeostasis. **(A)** Gating strategy of Control ($Btg1^{f/f}$ $Btg2^{f/f}$) and Btg1/2 DKO ($Btg1^{f/f}$ $Btg2^{f/f}$ – Flk1-cre) bone marrow for Lineage negative LKs, CMP, MEP, GMP and CLP fraction. **(B)** LK cell and Myeloid lineage committed cell (CMP, MEP and GMP) frequency and absolute cell number in bone marrow. **(C)** Lymphoid lineage committed cell (CLP) frequency and absolute cell number in bone marrow. The cellular frequency panels of (B) and (C) are derived from n=5 mice per group. Absolute cell number panels are derived from n=5 mice per group. Error bars denote the mean ± SEM. (B and C) Statistics were calculated using the unpaired student's *t*-test. P values < 0.05 were considered significant ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$), *ns* = not significant.

5. Loss of Btg1 and Btg2 in normal hematopoiesis alter the bone marrow resident myeloid and lymphoid compartment

Since myeloid lineage-comiitted progenitor cells, especially CMP and GMP, were increased in Btg1/2 DKO mice, it was hypothesized that there would be changes in terminal differentiation into neutrophils and monocyte-macrophages. Although there was no difference in CLP, it was analyzed together with myeloid cells because Btg1/2 is known to be a key regulator of B cell differentiation. The neutrophil fraction was defined by using CD11b and Ly6g markers, and B cells were distinguished by using B220, which is an early B cell development marker (Fig. 5A).

Consistent with previous studies, the frequency and absolute cell number of B cells were dramatically reduced in the bone marrow of Btg1/2-deficient mice (Fig. 5B). This suggests that although the loss of function of Btg1/2 does not disrupt CLP homeostasis, it can be a possible cell intrinsic regulator of the terminal differentiation of B cells. Interestingly, myeloid cell neutrophil and monocyte-macrophage homeostasis changed significantly. Neutrophil frequency increased by more than 10% compared to littermate control, and monocyte-macrophage frequency and cell number decreased (Fig. 5C). Thus, Btg1/2 can maintain the homeostasis of bone marrow-resident myeloid and lymphoid cells in vivo.

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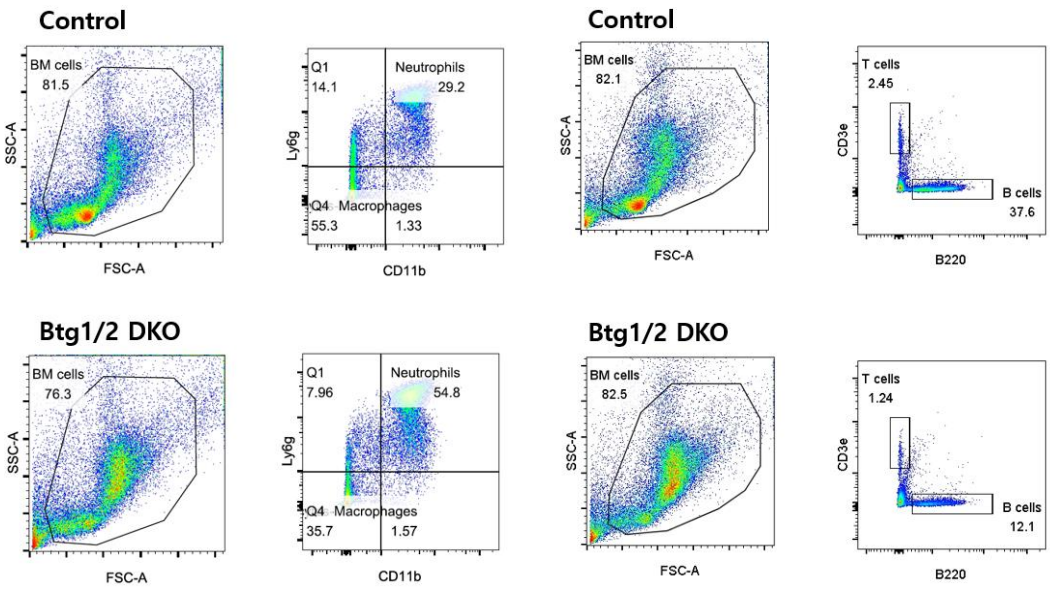

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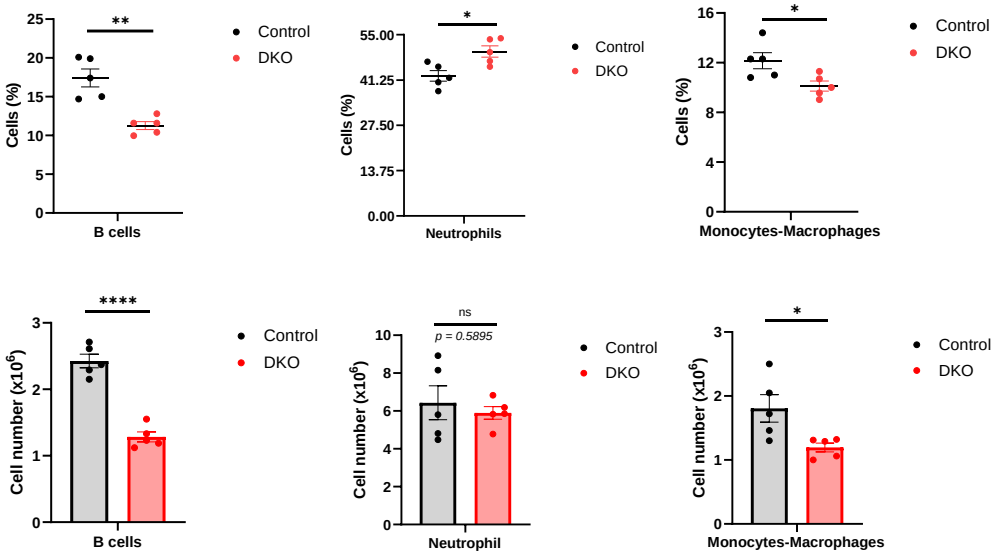

Figure 5. Loss of Btg1 and Btg2 alter the bone marrow resident myeloid and lymphoid compartment. (A) Gating strategy of Control ($Btg1^{f/f}$ $Btg2^{f/f}$) and Btg1/2 DKO ($Btg1^{f/f}$ $Btg2^{f/f}$ – Flk1-cre) for Lymphoid (B cell) and Myeloid (Neutrophil, Monocyte-Macrophage) fraction. **(B)** The frequency and absolute cell number of B cell, Neutrophil and Monocyte-macrophage in bone marrow. The cellular frequency panels of (B) are derived from n=5 mice per group. Absolute cell number panels are derived from n=5 mice per group. Error bars denote the mean ± SEM. (B) Statistics were calculated using the unpaired student's *t*-test. P values < 0.05 were considered significant ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$), *ns* = not significant.

6. Btg1 and Btg2 are not involved in extramedullary hematopoiesis of multipotent stem and progenitor and lineage committed progenitor except for CLP

Embryonic development Hematopoiesis is called primitive hematopoiesis. Red blood cells are mainly produced during this process in the yolk sac, fetal liver, and spleen[49]. The major purpose of primitive heamtopoiesis is the rapid growth of the embryo by activating tissue oxygenation. Definitive hematopoiesis, on the other hand, is a common hematopoiesis that occurs in the bone marrow from 12.5 dpc until adulthood. This refers to the process by which all blood cell lineages are generated from LT-HSC[50]. Extramedullary hematopoiesis (EMH) is the result of a physiological response to inefficient bone marrow production of blood cells and occurs primarily in the red pulp of the spleen[50-52]. In this study, EMH might be affected by Btg1/2 deletion because of the physiological defect of definitive hematopoiesis in bone marrow. However, screening was restricted to lineage-committed progenitor cells and mature blood cells because there are not enough multipotent stem and progenitor cells in the spleen. The markers for identifying lineage-committed progenitor cells and mature blood cells are the same as before (Fig. 4A, 5A).

As a result, the overall frequency and absolute cell number of the myeloid lineage committed cell fraction in spleen did not show statistically significant differences, but showed an increasing tendency consistent with the results of the increase in GMP frequency in bone marrow (Fig. 4B, 6A). Even though definitive hematopoiesis is disrupted by ablating Btg1/2, no alteration in myeloid proenitor cells suggests that there is no indirect effect on EMH.

Interestingly, the CLP fraction, which showed no difference in the bone marrow, increased by more than twofold in frequency due to the EMH of the spleen (Fig. 4C, 6B). This suggests that, even though there is no difference in bone marrow hematopoiesis, EMH toward the lymphoid lineage can at least be stimulated when Btg1/2 loses its function.

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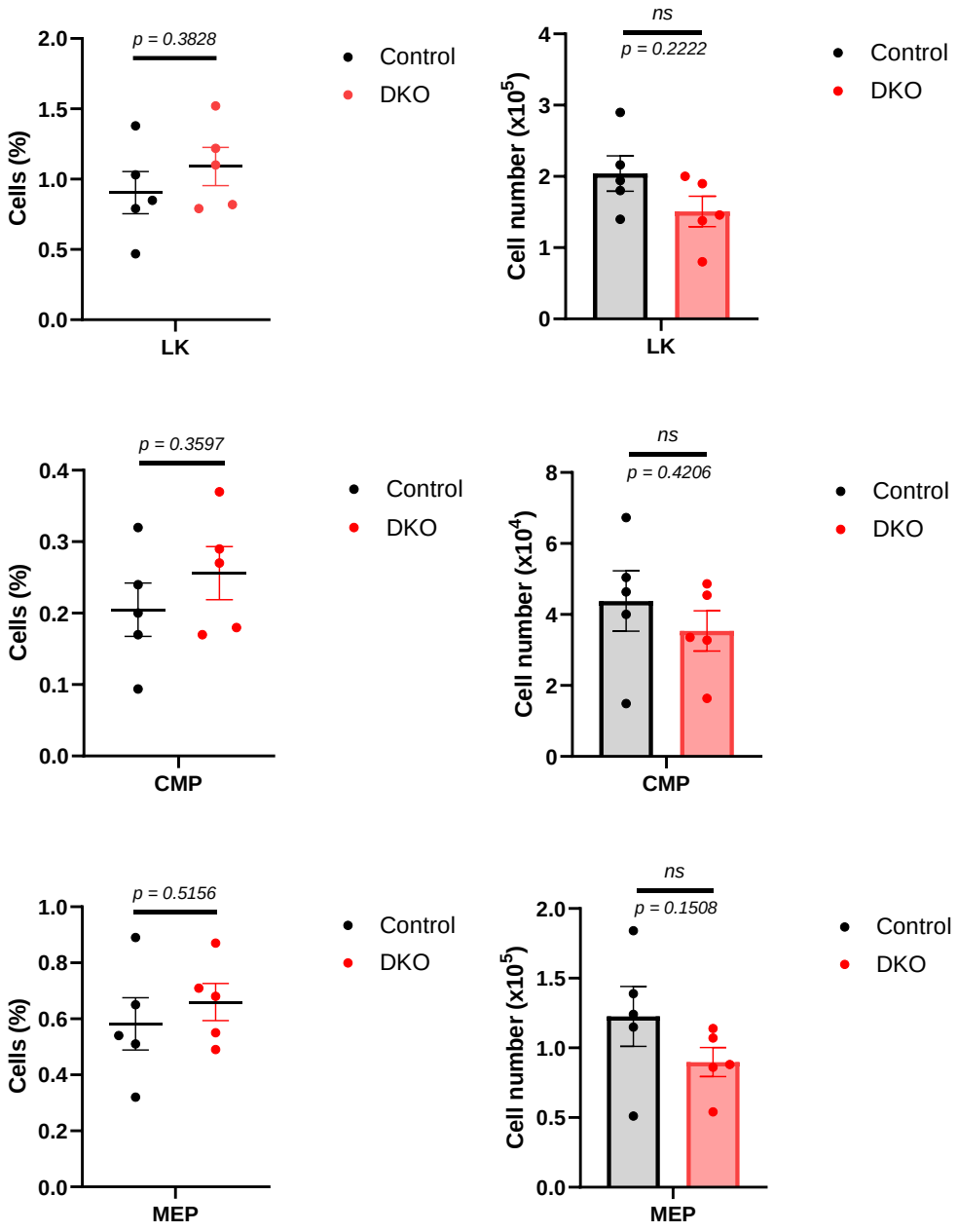

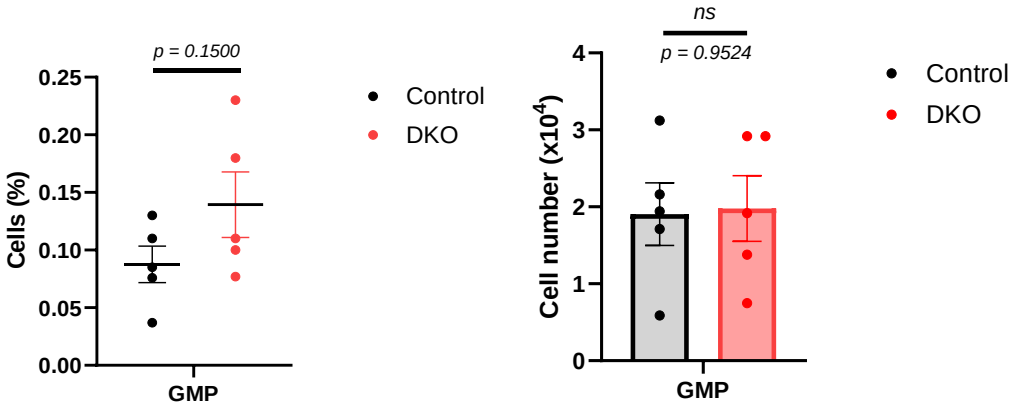

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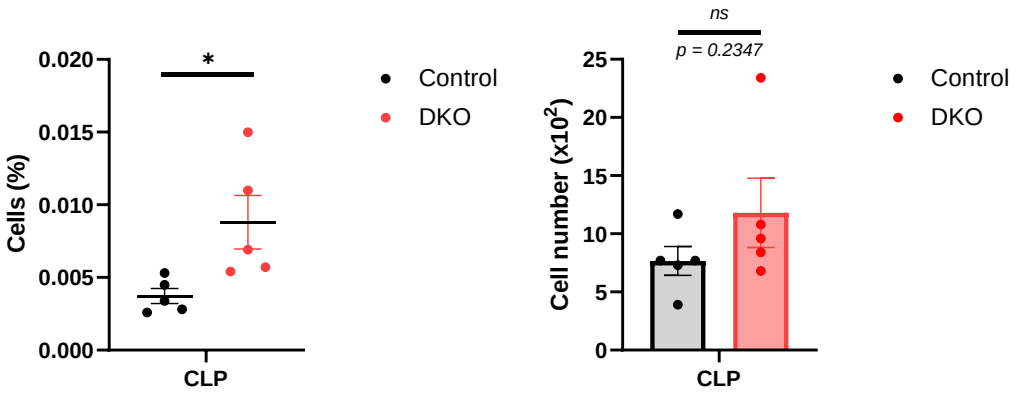

Figure 6. The correlation between Btg1/2 and Extramedullary hematopoiesis of lineage committed progenitor cells. (A) The frequency and absolute cell number of LK cell and myeloid lineage committed progenitor cell (CMP, MEP, GMP) of Control ($Btg1^{f/f}$ $Btg2^{f/f}$) and Btg1/2 DKO ($Btg1^{f/f}$ $Btg2^{f/f}$ – Flk1-cre) Spleen. (B) The frequency and absolute cell number of lymphoid lineage committed progenitor cell (CLP) in Spleen. The cellular frequency panels of (A, B) are derived from n=5 mice per group. Absolute cell number panels are derived from n=5 mice per group. Error bars denote the mean ± SEM. (A, B) Statistics were calculated using the unpaired student's *t*-test. P values < 0.05 were considered significant ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$), *ns* = not significant.

7. The Lack of Btg1 and Btg2 in steady state hematopoiesis results in a disruption of the homeostasis of spleen resident lymphoid and myeloid lineage cells

Because the CLP fraction increased in the spleen, lymphoid lineage of B and T cells was analyzed. Intriguingly, the results showed that both B cells and T cells decreased significantly in frequency and absolute cell number. B cells were greatly decreased, consistent with the results in bone marrow (Fig. 7A). However, this is more likely to be caused by a decrease in peripherial blood (PB) output itself after bone marrow hematopoiesis rather than a result of EMH in the spleen. This is because the number of CLPs are exist in very small amount in the spleen. Furthermore, both T cell frequency and absolute cell number were significantly reduced. In fact, considering that there was no alteration in CLP in bone marrow (Fig. 4C) and that CLP increased in the spleen, the decreased phenotype of T cells is quite contradictory. Therefore, the thymus was examined to verify further changes in CLP's maturation process.

Interestingly, during the maturation process of CLP that migrated to the thymus, there were statistically significant differences in the DN (Double Negative), DP (Double Positive), CD4 SP (Single Positive), and CD8 SP (CD8 Single Positive) fractions (Fig. 7C). Although the frequency of the DP fraction increased, it was confirmed that differentiation into CD4 and CD8 SP cells was strongly suppressed after medullary thymic epithelial cell (mTEC) migration for negative selection. These findings imply that Btg1/2 may function as a cofactor in both B cell terminal differentiation and negative selection, one of the thymus's tolerance mechanisms.

The homeostasis of the lineage-committed progenitor cells CMP and GMP was preserved in the spleen (Fig. 6A), whereas the frequency of the monocyte-macrophage fraction was increased and the absolute cell number remained constant. Neutrophils, however, increased more than twofold (Fig. 7D). This results were consistent with the screening outcomes in bone marrow. The rise in progenitor cell stages, or CMP and GMP fractions, in the bone marrow accounts for the increase in Neutrophil (Fig. 4B). A further

possible cause was required because the CMP and GMP fractions in the spleen did not differ significantly.

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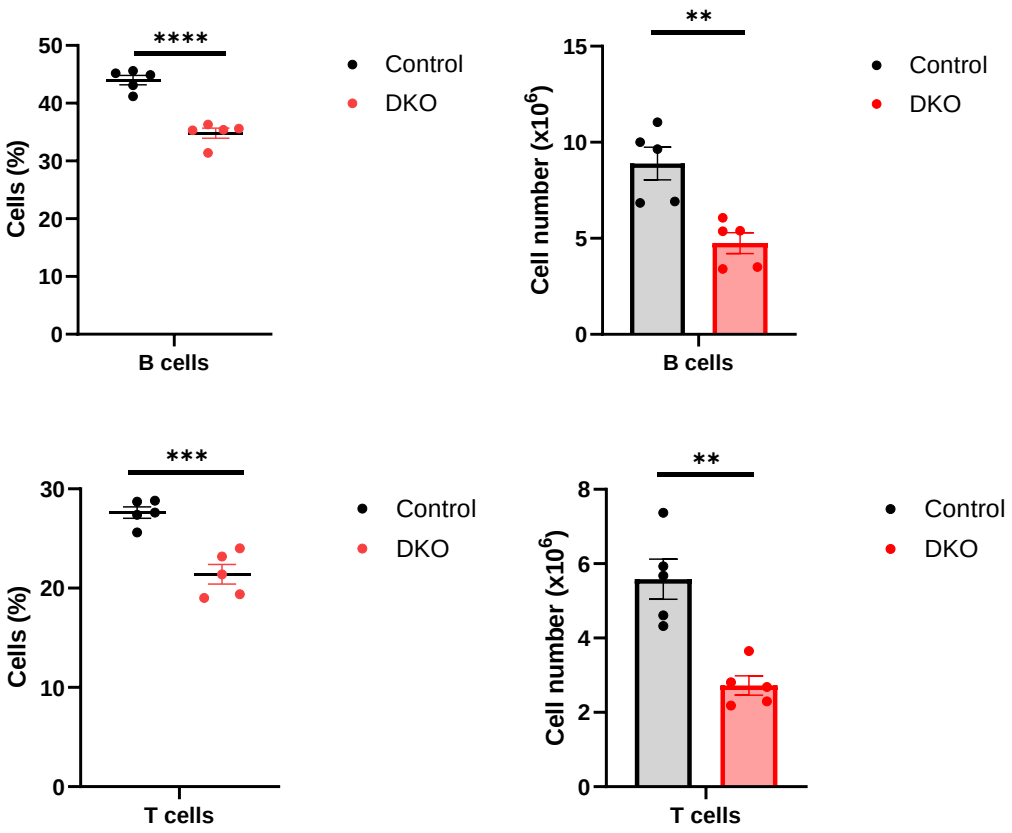

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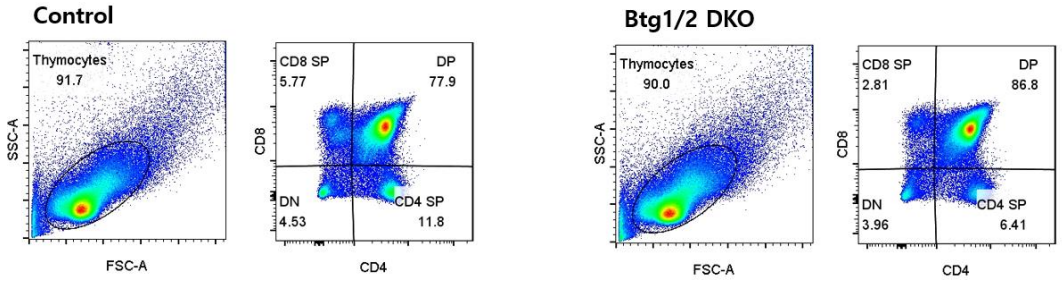

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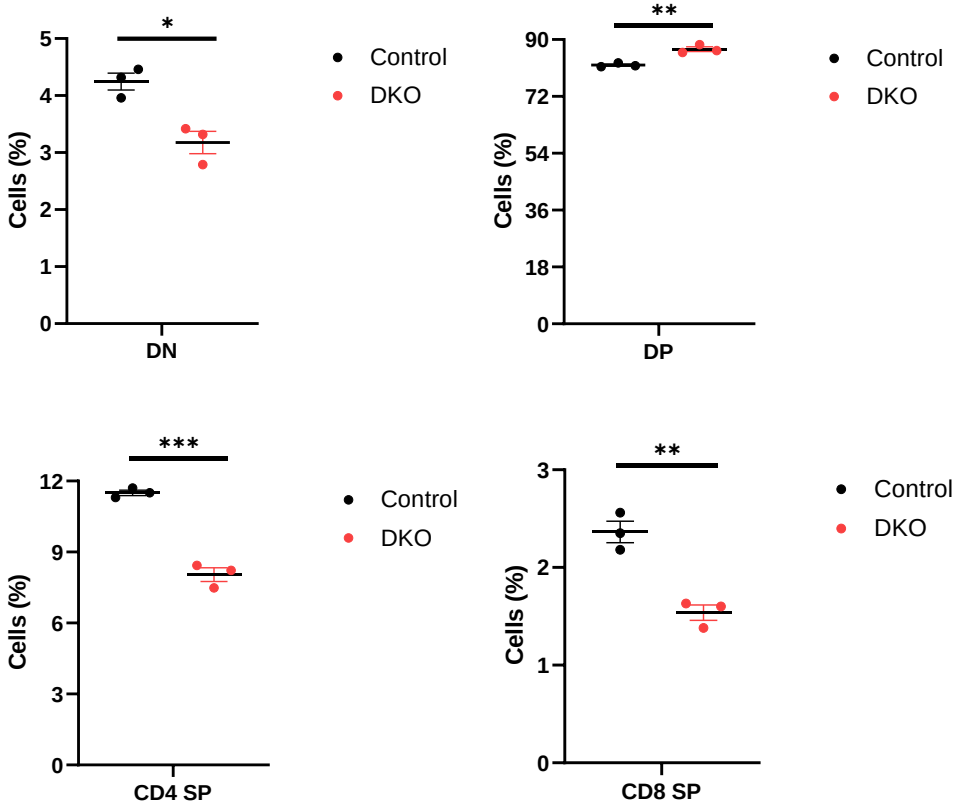

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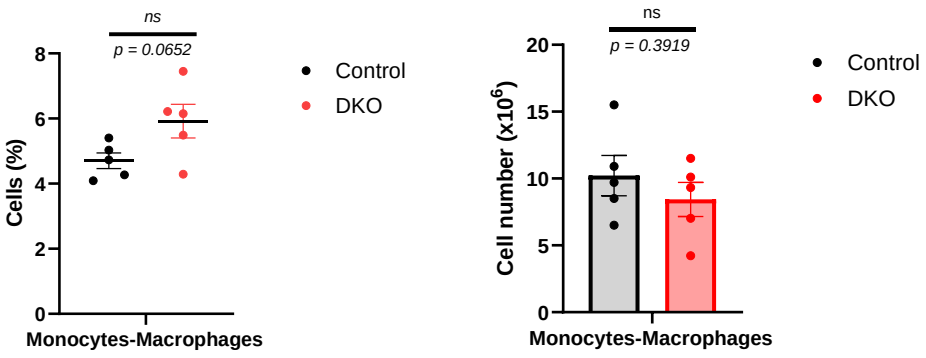

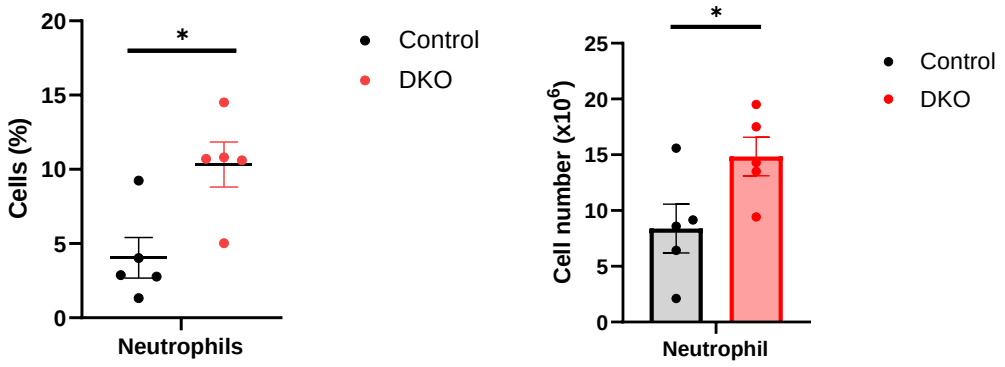

Figure 7. The absence of Btg1 and Btg2 disrupts spleen resident lymphoid and myeloid cell homeostasis. (A) The frequency and absolute cell number of B cell and T cell in spleen. **(B)** Gating strategy of Control ($Btg1^{f/f}$ $Btg2^{f/f}$) and Btg1/2 DKO ($Btg1^{f/f}$ $Btg2^{f/f}$ – Flk1-cre) for DN (Double Negative), DP (Double Positive), CD4 SP (Single Positive) and CD8 SP fraction. **(C)** The frequency of thymocyte population. **(D)** The frequency and absolute cell number of Monocyte-macrophage and Neutrophils in spleen. The cellular frequency panels of (A, D) are derived from n=5 mice per group and (C) is derived from n=3 mice per group. Absolute cell number panels of (A, D) are derived from n=5 mice per group. Error bars denote the mean ± SEM. (A, C-D) Statistics were calculated using the unpaired student's *t*-test. P values < 0.05 were considered significant ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$), *ns* = not significant.

8. Disruption of hematopoietic homeostasis in the bone marrow leads to PB output

As a possible reason for the previous results, firstly, the increase in neutrophils may be due to the EMH of the spleen; secondly, it may be due to the PB output from bone marrow; and thirdly, both the first and second may be the cause. To that end, the Complete Blood Count (CBC) test was performed to examine the peripheral blood composition (Fig. 8A).

The CBC test is recommended for hematology evaluation: total WBC count, red blood cell (RBC) count, hematocrit (HCT), hemoglobin concentration, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and platelet count are provided[53,54]. In this study, the analysis was divided into the WBC (Fig. 8B) and RBC groups (Fig. 8C).

Interestingly, all WBC group parameters were consistent with the bone marrow screening results (Fig. 5B). Total WBC decreased in peripheral blood, and the lymphocyte ratio also decreased significantly in DKO mice. Neutrophils and Monocytes were increased in DKO mice. Eosinophil is the fraction that differentiates from GMP and its frequency to that of littermate control, suggesting that upregulated GMP mostly contributes to Neutrophil and Monocyte differentiation (Fig. 8B). Furthermore, it was confirmed that neutrophils increased in the spleen could be the cause of both the first and second cases.

Next, the results of the RBC group present the MEP-derived PB output. The results of screening were consistent with the outcome of bone marrow screening, which was the same as in the WBC group, but MCV and MCH parameters were significantly increased in DKO mice (Fig. 8C). MCV and MCH are common RBC indices and provide information about the average amount of hemoglobin contained in one RBC[55]. These results imply that the Btg1/2 deletion likely regulates the differentiation process of bone marrow MEPs into erythrocytes. Meanwhile, there are restrictions on the flow cytometry analysis that is utilized for screening when it comes to examining RBCs and other

fractions like progenitor and multipotent stem cells at the same time. Therefore, in this study, the Colony Forming Unit (CFU) assay was performed to analyze the differentiation state between MEPs and RBCs. In addition, because there was a bias toward the myeloid lineage as a result of previous screening in bone marrow, a CFU assay was performed to further confirm this in an in - vitro culture system.

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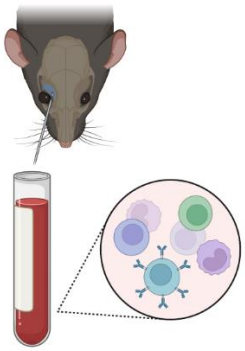

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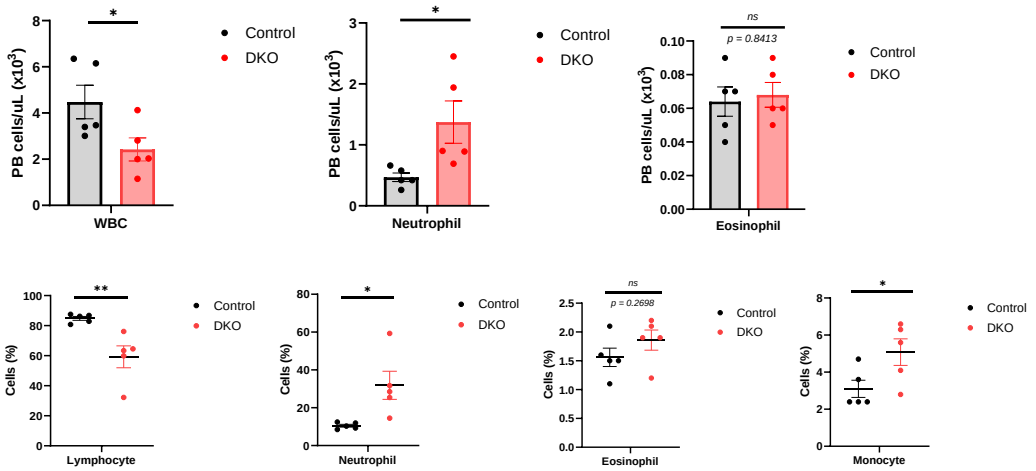

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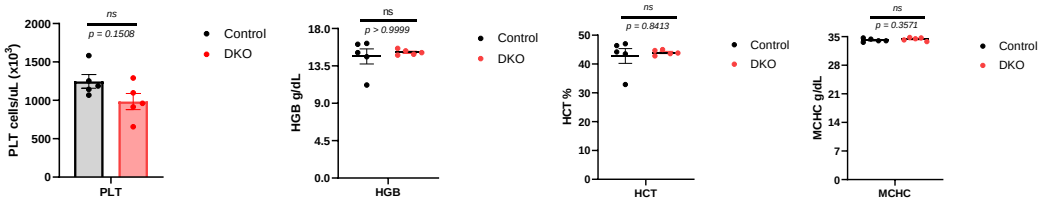

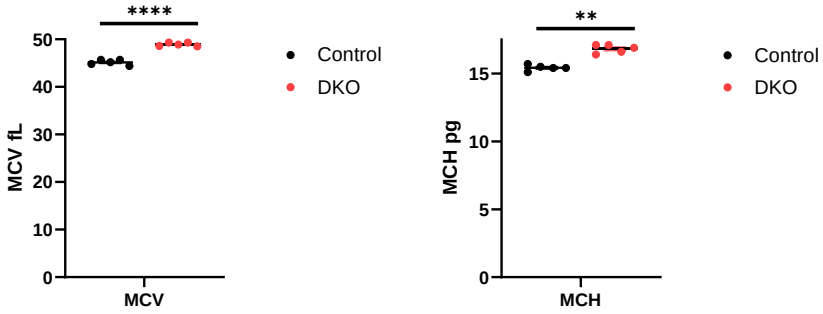

Figure 8. Complete Blood Count (CBC) Test of WBC and RBC group in steady state condition. (A) Schematic illustration for Complete Blood Count (CBC) Test. Peripheral blood were collected by retro-orbital venipuncture of 300 – 500uL per mice **(B)** Number of cells per unit blood volume and the frequency of WBC group. **(C)** Number of cells per unit blood volume and the frequency of RBC group. (B,C) is derived from n=5 mice per group. Error bars denote the mean ± SEM. (B,C) Statistics were calculated using the unpaired student's *t*-test or Mann-Whiteny *U*-test. P values < 0.05 were considered significant (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001), *ns* = not significant.

9. Loss of Btg1 and Btg2 alter the Myeloid-lineage Colony Fomring Unit of CFU-G and CFU-GM

The Colony Forming Unit (CFU) assay is the most commonly used in vitro analysis method to evaluate the ability of multilineage differentiation in multipotent progenitor cells[56]. The CFU assay is also called the colony-forming cell (CFC) assay, and is performed in methylcellulose-based (e.g, MethoCultTM) semi-solid medium under conditions that support the differentiation of individual progenitor cells. It includes cytokines such as IL-3, G-CSF/M-CSF, and EPO that allow the growth of clonal progeny in single progenitor cells[57]. The five major types of CFU (CFU-GEMM, BFU-E, CFU-GM, CFU-G, and CFU-M) can be identified[58-60]. Individual colonies show specific morphology and size (Fig. 9A).

Interestingly, the results of the CFU assay were identical to those in bone marrow (Fig. 4B). The GMP fraction was significantly increased in the form of CFU-GM in the CFU assay, and in CFU-G, which is a later differentiation state, a greater number was formed in DKO mice (Fig. 9B). However, unlike the high RBC indices shown in the CBC Test, there was no significant difference in CFU-GEMM and BFU-E, which can be identified as erythroid progenitor cells in the bone marrow (Fig. 8C, 9B). Thus, this suggests that there are no changes in bone marrow erythropoietic homeostasis due to the Btg1/2 deletion. Overall, the bone marrow flow cytometry results and peripheral blood CBC test results are consistent, showing that Btg1/2 ultimately controls hematopoietic homeostasis, specifically myeloid lineage.

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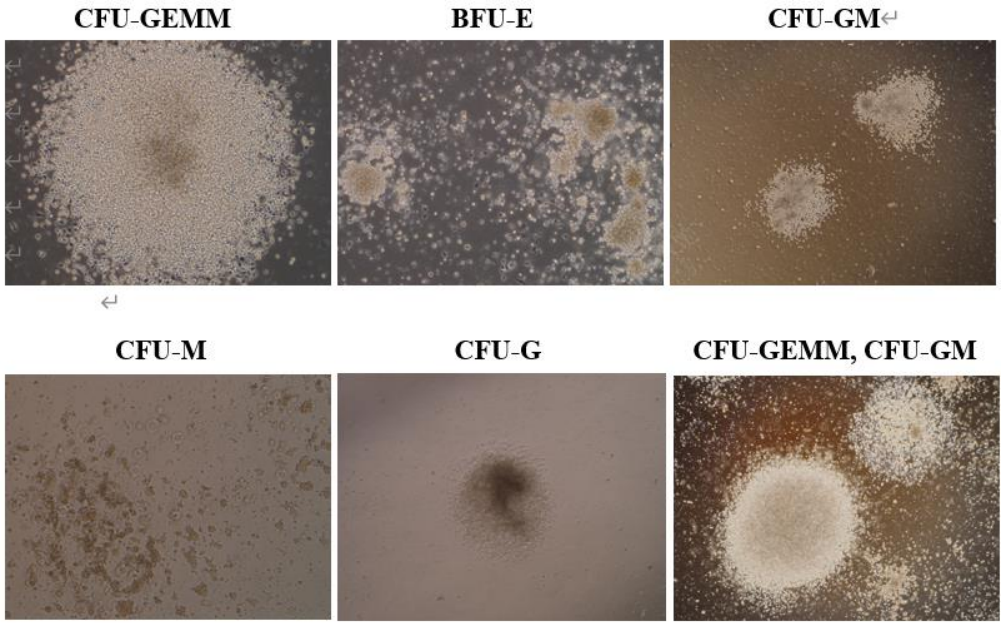

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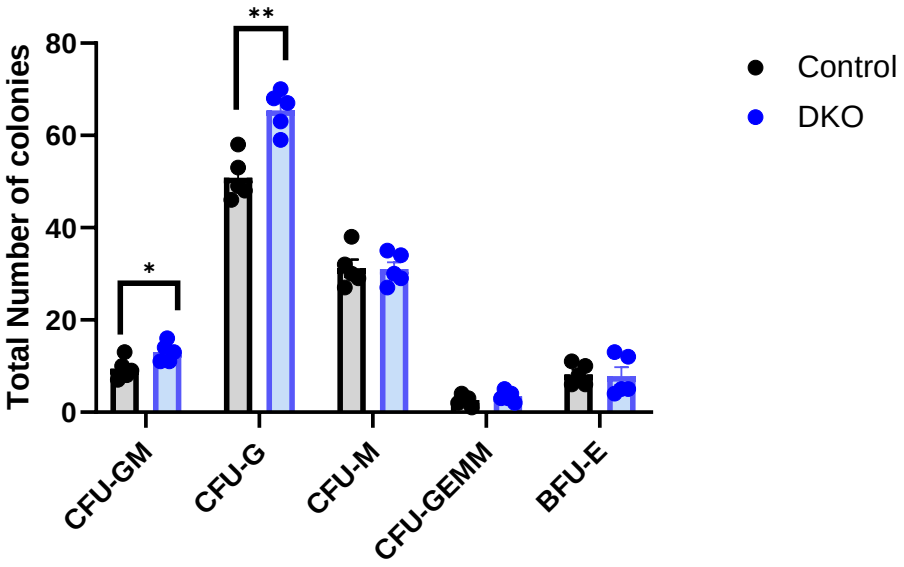

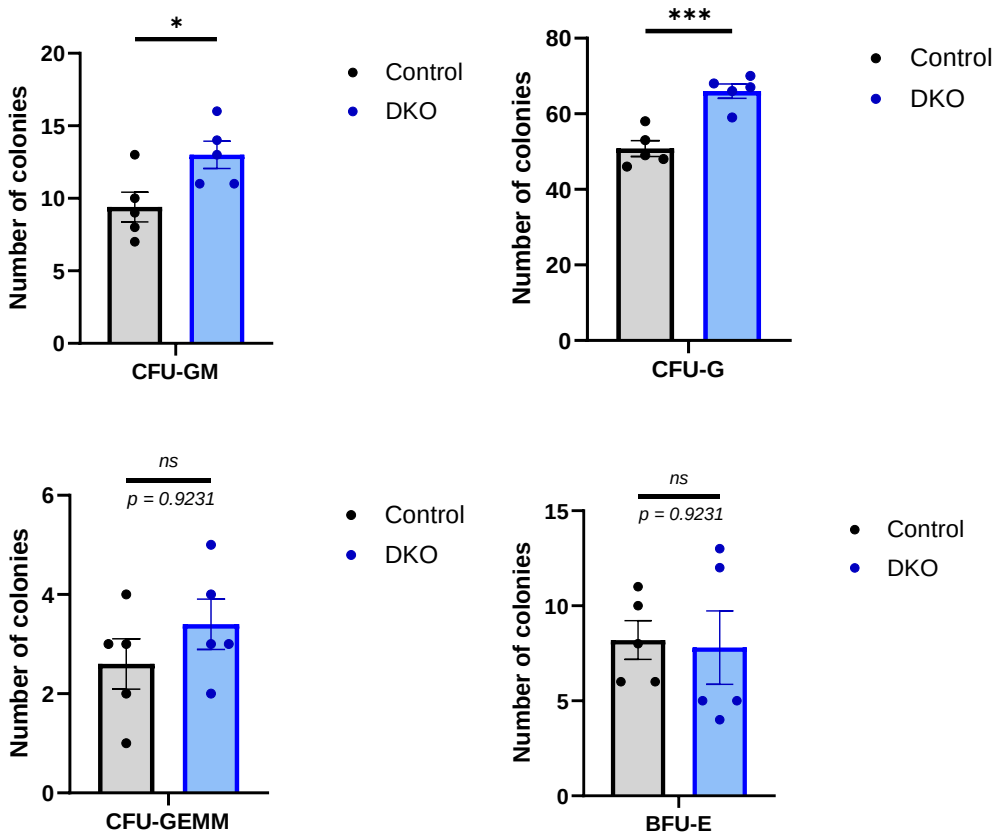

Figure 9. **Colony Forming Unit (CFU) assay of bone marrow in steady state condition.** Colony Forming Unit (CFU) assay from bone marrow of Control ($Btg1^{f/f}$ $Btg2^{f/f}$) and Btg1/2 DKO ($Btg1^{f/f}$ $Btg2^{f/f}$ – Flk1-cre) mice. **(A)** Representative images of different colony morphologies after 10 days culture of bone marrow cell. **(B)** Colony number of five major types of CFU (namely CFU-GEMM, BFU-E, CFU-GM, CFU-G, CFU-M) from control ($Btg1^{f/f}$ $Btg2^{f/f}$) and Btg1/2 DKO ($Btg1^{f/f}$ $Btg2^{f/f}$ – Flk1-cre) mice and is derived from n = 5 per group. Error bars denote the mean ± SEM. (B) Statistics were calculated using the unpaired student's *t*-test. P values < 0.05 were considered significant (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001), *ns* = not significant.

10. Bias toward Myeloid hematopoiesis by Btg1 and Btg2 contributes to Neutrophil and Monocyte

As a result of flow cytometry analysis, the frequency and absolute cell number of the monocyte-macrophage fraction decreased, but CFU-M showed no significant difference (Fig. 9B). This implies that a regulatory effect could exist at a later stage of differentiation than CFU-M. Therefore, the impact of Btg1/2 was examined on BMDM differentiation.

Bone marrow cells differentiate into macrophages by GM-CSF secreted by L929 cells and are called L929-derived macrophages[61,62]. On Day 0, there was no difference in bone marrow resident macrophages between littermate control and DKO, and there was no significant difference in L929-derived macrophages, which were 100% differentiated after 7 days of culture (Fig. 10). This suggests that Btg1/2 does not affect the path to macrophage differentiation and that Btg1/2 has a regulatory effect on monocyte differentiation rather than macrophage differentiation. Overall, when Btg1/2 was deleted under steady-state conditions, lymphoid lineage differentiation was defective and myeloid lineage differentiation was increased. This suggests that Btg1/2 could maintain the balance between lymphopoiesis and myelopoiesis in normal hematopoiesis conditions and can function as a regulator of hematopoietic homeostasis.

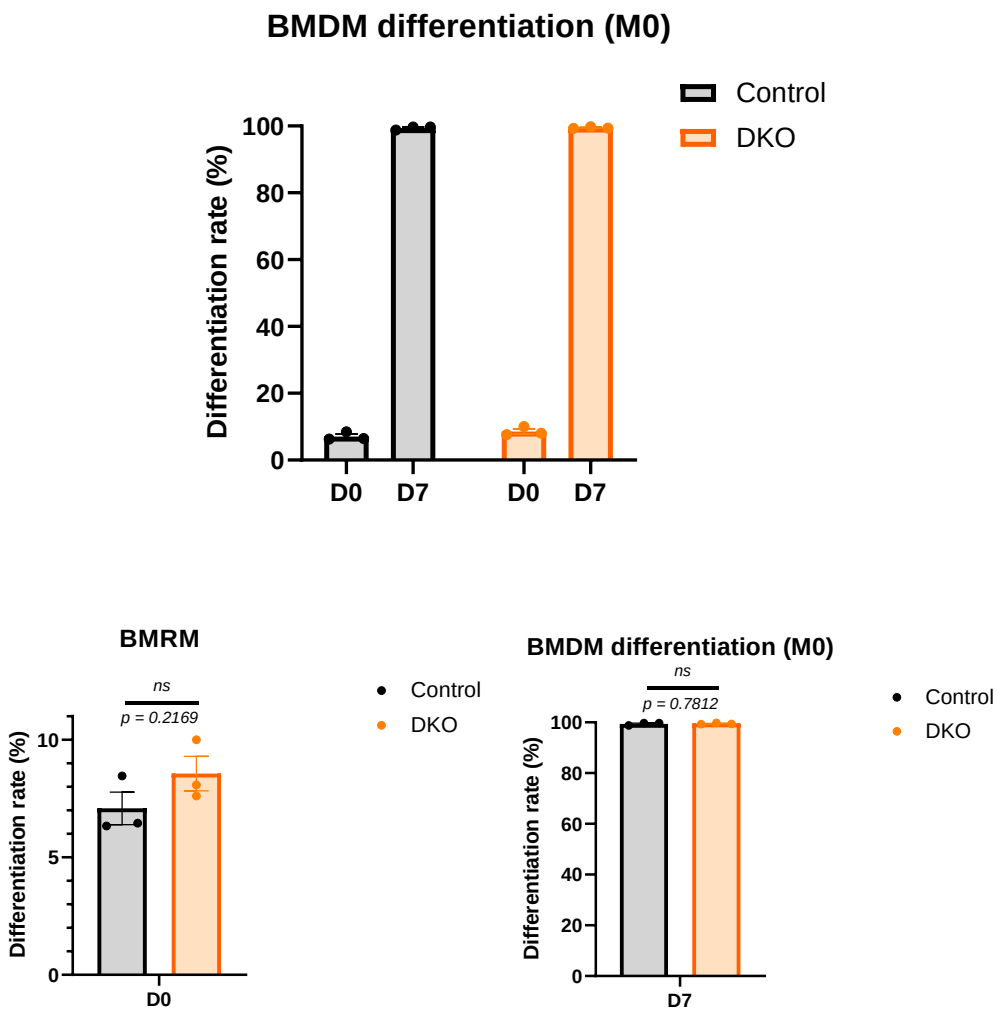

Figure 10. Btg1 and Btg2 cannot affect Differentiation of Bone marrow derived macrophage. Bone marrow derived macrophage diiferentiation rate. BMRM (Bone marrow resident macrophage) at Day 0 and M0 BMDM at Day 7 show no difference between control ($Btg1^{f/f}$ $Btg2^{f/f}$) and Btg1/2 DKO ($Btg1^{f/f}$ $Btg2^{f/f}$ – Flk1-cre) mice and is derived from n = 3 per group. Error bars denote the mean ± SEM. Statistics were calculated using the unpaired student's *t*-test. P values < 0.05 were considered significant (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001), *ns* = not significant.

11. Btg1 and Btg2 do not contribute to Multipotent stem and progenitor cells in stress condition

Next, in order to assess the ability of multipotent stem and progenitor cells to repopulate, hematopoiesis under stress-induced conditions was examined. 5-Fluorouracil (5-FU) can reorganize the bone marrow microenvironment and renew the bone marrow niche surrounding HSCs[63,64]. In other words, the multilineage differentiation capacity of endogenous HSC can be confirmed through 5-FU treatment. In this study, the stress situation through 5-FU was defined as a stress-induced condition, distinct from the steady-state condition.

According to previous studies, when treated with 5-FU, all actively proliferating HSCs disappeared within 2 days[65]. Reconstitution begins on Day 4, and recovery is completed after 10 days (Fig. 11A). Changes in multipotent stem and progenitor cell fraction were evaluated in littermate control and Btg1/2 DKO bone marrow at day 10, when repopulation in bone marrow was completed. 5-FU was treated as a single dose of 150 mg/kg per mouse and injected through the retro-orbital route (Fig. 11A).

The frequency and absolute number of multipotent progenitor cells (MPP4, MPP3, MPP2, ST-HSC and LSK) and multipotent stem cells (LT-HSC) did not differ significantly (Fig. 11B, C). These results suggest that even if Btg1/2 is deleted under stress-induced conditions, the self-renewal activity of LT-HSCs is not impaired, and they can maintain their pool without any depletion. However, in the case of ST-HSC, frequency and absolute cell number tended to slightly decrease, which was the same as normal hematopoiesis in steady-state conditions (Fig. 11B). These results showed possibility that Btg1/2 does not affect repopulation capacity in mulipotent stem and progenitor cells.

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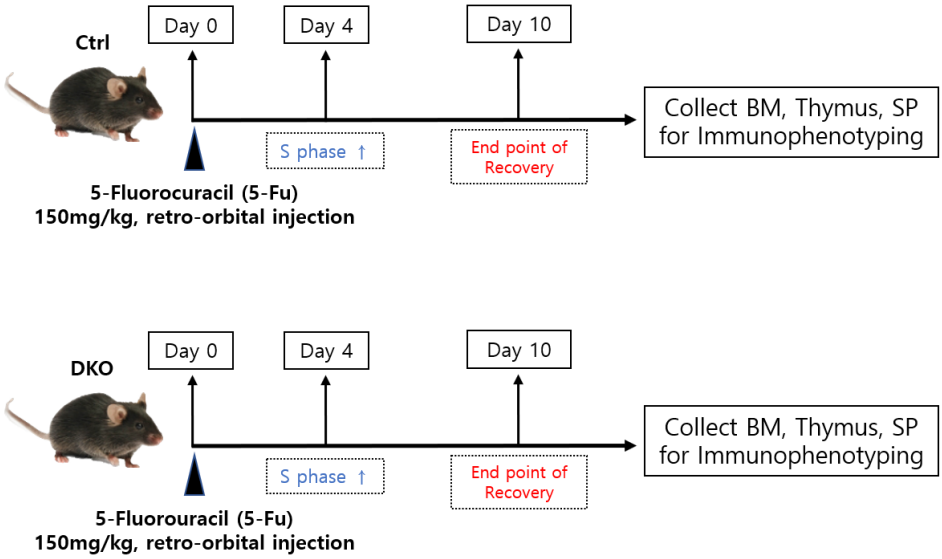

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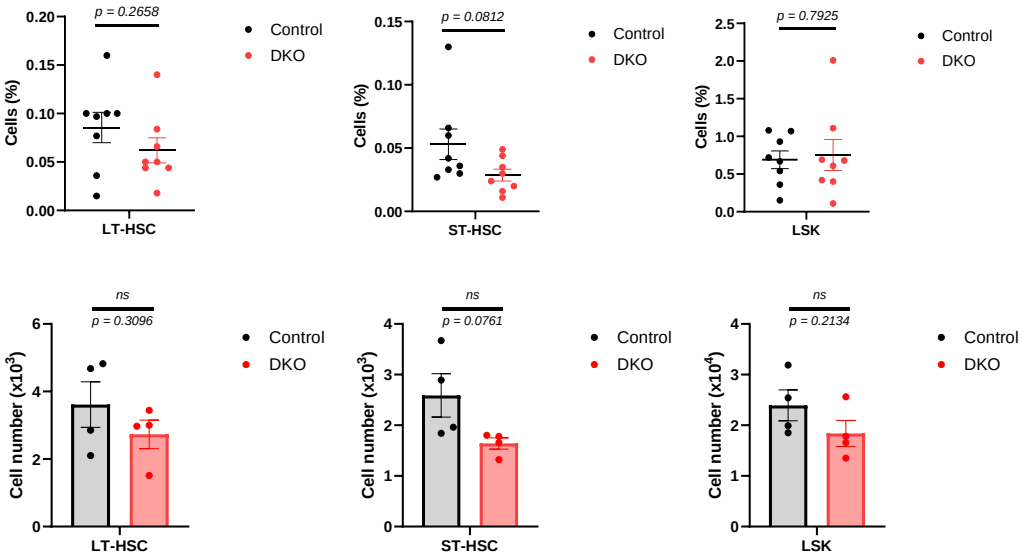

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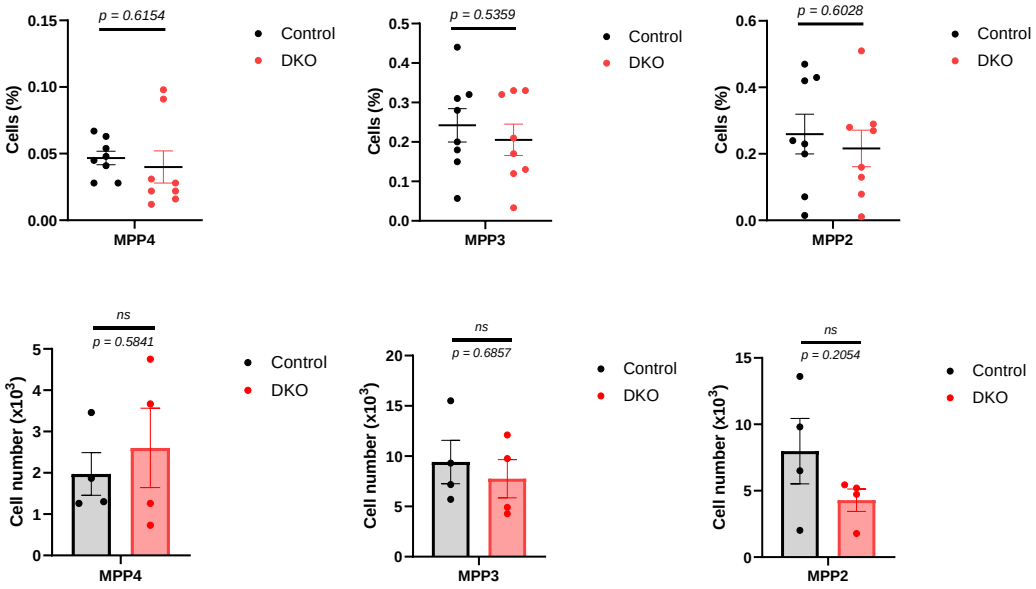

Figure 11. **Btg1 and Btg2 cannot affect stress induced hematopoiesis of multipotent stem and progenitor cells. (A)** Schematic time line of the experiment. Both control ($Btg1^{f/f}$ $Btg2^{f/f}$) and Btg1/2 DKO ($Btg1^{f/f}$ $Btg2^{f/f}$ – Flk1-cre) mice were treated with 5-Fluorouracil (5-FU) by retro-orbital route for single dose of 150mg/kg on Day 0 **(B)** LT-HSC, ST-HSC and LSK frequency and absolute cell number in bone marrow. **(C)** lineage-primed MPP4, MPP3 and MPP2 frequency and absolute cell number in bone marrow. The cellular frequency panels of (B) and (C) are derived from n=8 mice per group which is combined from two experiments. Absolute cell number panels are derived from n=4 mice per group. Error bars denote the mean ± SEM. (B and C) Statistics were calculated using the unpaired student's *t*-test. P values < 0.05 were considered significant (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001), *ns* = not significant.

12. Btg1 and Btg2 regulate homeostasis of lineage committed progenitor cells in stress condition

Next, to evaluate the function of Btg1/2 in stress-induced conditions, the myeloid lineage-comitted progenitor cell fraction was analyzed. As a result, myelopoiesis was still found to be upregulated in Btg1/2-deficient mice. Although the absolute cell number of CMP was not statistically significant, it showed a slight tendency to increase, and GMP increased by more than 1.5-2 times compared to that of littermate control (Fig. 12A). This is consistent with the screening results in steady-state conditions (Fig. 4B), suggesting that even if repopulation of the bone marrow fraction is induced through 5-FU, myelopoiesis still dominates in the absence of Btg1/2, disrupting hematopoietic homeostasis.

It was anticipated that there would be little to no alteration in the lymphoid progenitor cell fraction because the earlier screening results showed that CMP and GMP increased even under stress-induced conditions as well. Interestingly, however, both CLP frequency and absolute cell number in the bone marrow increased more than two-fold after 5-FU treatment (Fig. 12B). Lineage-comitted progenitor cell fraction showed a significant increase in both Myelopoiesis and Lymphpoiesis under 5-FU-induced stress conditions. This shows that not only myelopoiesis but also lymphopoiesis can be enhanced in conditions where there is pressure for repopulation.

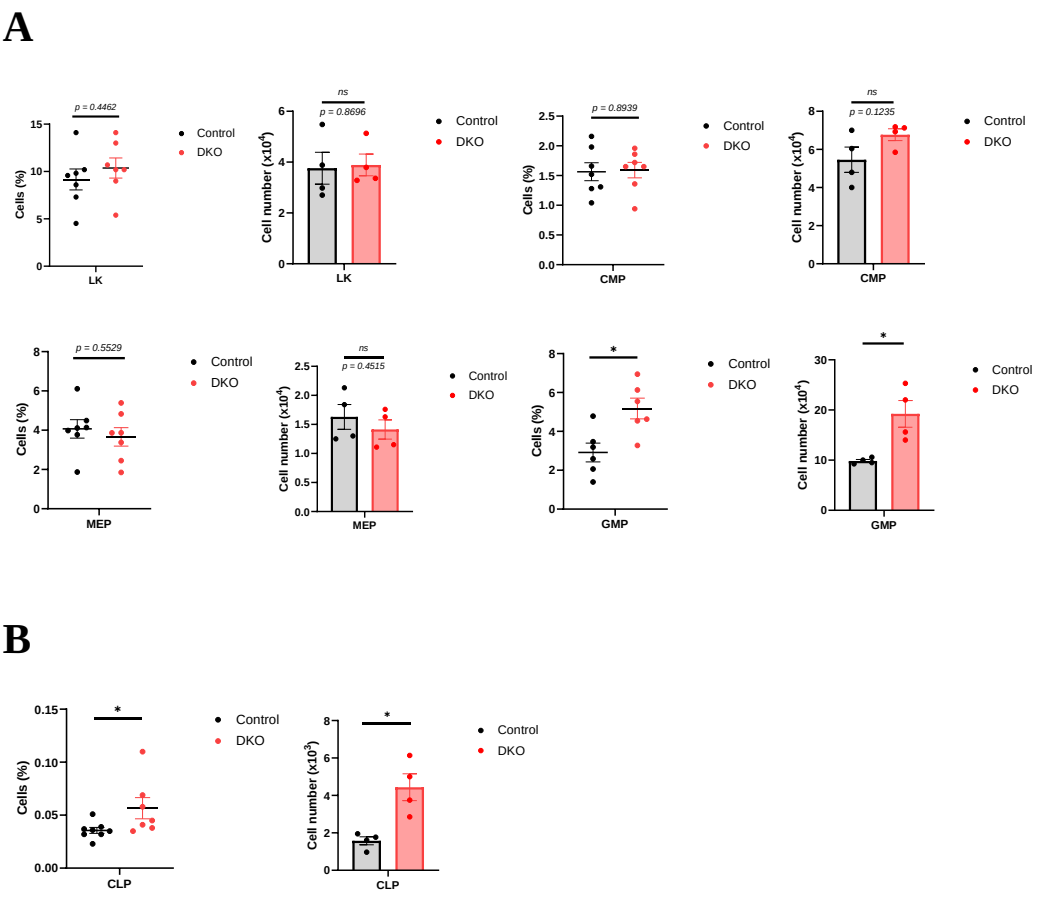

Figure 12. Btg1 and Btg2 regulates lineage committed progenitor cells in stress condition. **(A)** LK cell and Myeloid lineage committed cell (CMP, MEP and GMP) frequency and absolute cell number in control ($Btg1^{f/f}$ $Btg2^{f/f}$) and Btg1/2 DKO ($Btg1^{f/f}$ $Btg2^{f/f}$ – Flk1-cre) mice bone marrow. **(B)** Lymphoid lineage committed cell (CLP) frequency and absolute cell number in bone marrow. The cellular frequency panels of (A,B) are derived from n=8 mice per group. Absolute cell number panels are derived from n=4 mice per group. Error bars denote the mean ± SEM. Statistics were calculated using the unpaired student's *t*-test. P values < 0.05 were considered significant (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001), *ns* = not significant.

13. Btg1 and Btg2 have regulatory potential in both lymphopoiesis and myelopoiesis

Next, bone marrow-resident mature blood cells were investigated. As a result, both B cell frequency and absolute cell number decreased significantly, similar to normal hematopoiesis (Fig. 13). On the other hand, both the frequency and absolute cell number of neutorphils and monocyte-macrophage fractions increased. Interestingly, neutrophils increased consistent with normal hematopoiesis results, but monocyte-macrophage showed a contradictory pattern to the steady-state condition screening outcomes (Fig. 13). It decreased in normal hematopoiesis (Fig. 5B), but increased by more than two times on average in stress-induced conditions. This suggests that Btg1/2 have regulatory potential as one of the mechanisms for maintaining hematopoietic homeostasis. Although detailed follow-up studies are required, the possibility of a regulatory role in both lymphopoiesis and myelopoiesis should be considered.

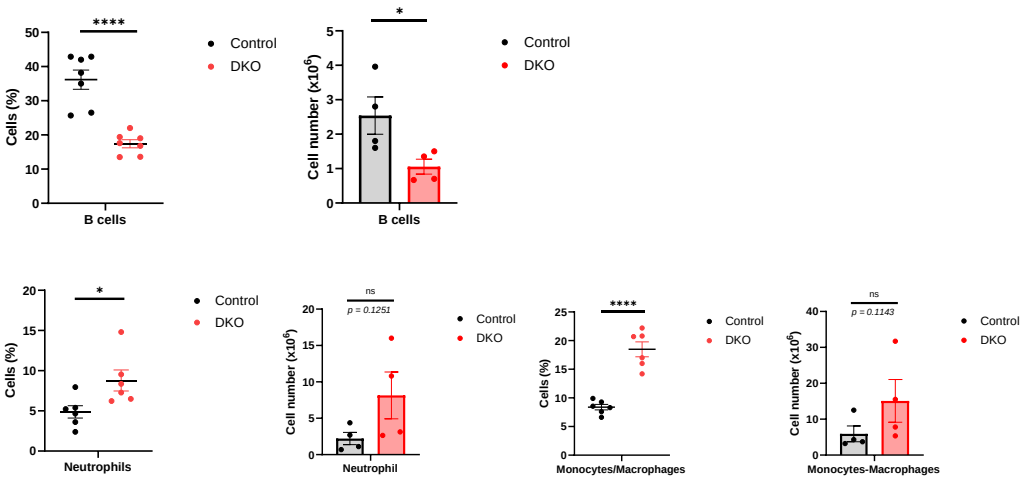

Figure 13. Btg1 and Btg2 increase both lymphopoiesis and myelopoiesis. The frequency and absolute cell number of B cell, Neutrophil and Monocyte-macrophage in control ($Btg1^{f/f}$ $Btg2^{f/f}$) and Btg1/2 DKO ($Btg1^{f/f}$ $Btg2^{f/f}$ – Flk1-cre) mice bone marrow. The cellular frequency panels of are derived from n=6 mice per group. Absolute cell number panels are derived from n=4 mice per group. Error bars denote the mean ± SEM. Statistics were calculated using the unpaired student's *t*-test. P values < 0.05 were considered significant ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$), *ns* = not significant.

14. Possibility that Btg1/2 regulates extramedullary hematopoiesis under stress conditions

Unlike bone marrow, the spleen contains almost no multipotent stem and progenitor cells, so screening was limited to lineage-committed progenitor cells and mature blood cells. Interestingly, under steady-state conditions, the frequency of lineage-committed cells did not differ from control (Fig. 4B, C), but under stress conditions, it tended to significantly increase in all fractions except for mature B cells and T cells (Fig. 14A, B). Although further research is needed, this suggests the possibility that Btg1/2 has homeostatic control over extramedullary hematopoiesis under stress conditions.

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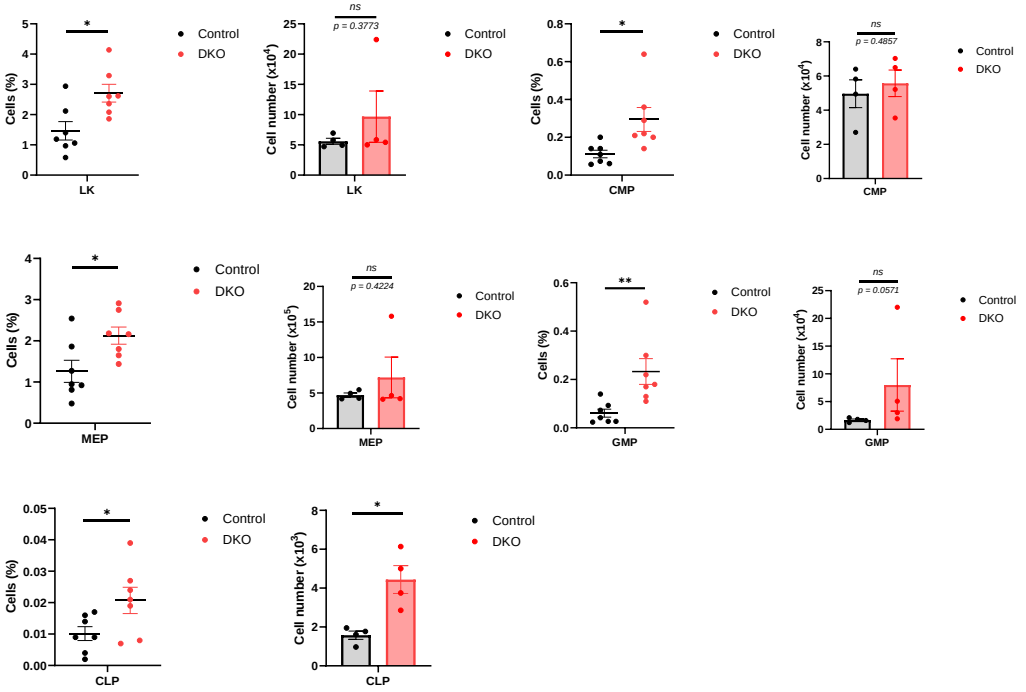

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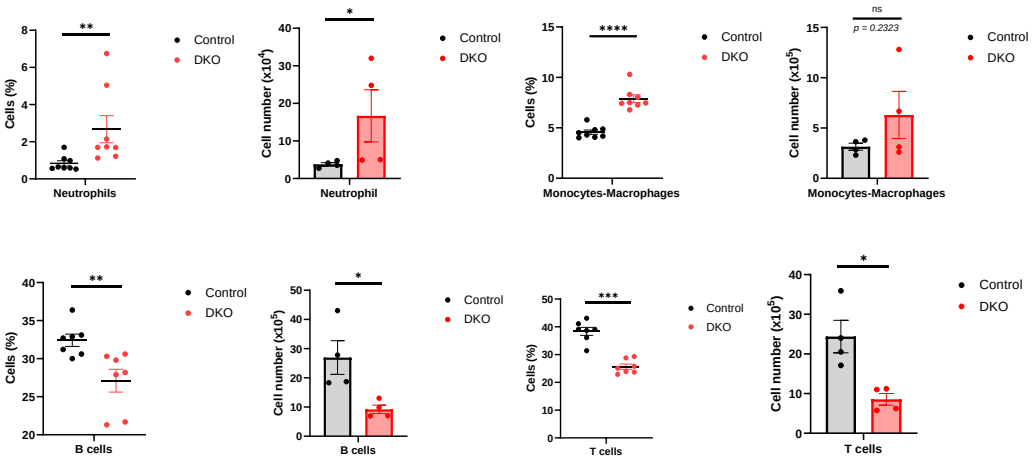

Figure 14. Extramedullary hematopoiesis in 5-Fu induced stress condition. (A) LK cell and lineage committed cell (CMP, MEP, GMP and CLP) frequency and absolute cell number in control ($Btg1^{f/f}$ $Btg2^{f/f}$) and Btg1/2 DKO ($Btg1^{f/f}$ $Btg2^{f/f}$ – Flk1-cre) mice bone marrow. **(B)** The frequency and absolute cell number of B cell, T cell, Neutrophil and Monocyte-macrophage in control ($Btg1^{f/f}$ $Btg2^{f/f}$) and Btg1/2 DKO ($Btg1^{f/f}$ $Btg2^{f/f}$ – Flk1-cre) mice bone marrow. The cellular frequency panels of (A,B) are derived from n=8 mice per group. Absolute cell number panels are derived from n=4 mice per group. Error bars denote the mean ± SEM. Statistics were calculated using the unpaired student's *t*-test. P values < 0.05 were considered significant ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$), *ns* = not significant.

IV. DISCUSSION

In this study, the effect of Btg1/2 loss of function on hematopoietic homeostasis was evaluated under steady state conditions and stress-induced conditions. As a result of investigating Btg1/2-deficient mice, CMP and GMP increased significantly under steady state conditions, and Neutrophil and Monocyte-Macrophage decreased. This suggests that Btg1 and Btg2 can be associated with myelopoiesis. In stress-induced conditions, CLP increased along with CMP and GMP, and both Neutrophil and Monocyte-Macrophage increased. This suggests that Btg1 and Btg2 are involved in both myelopoiesis and lymphopoiesis in proliferative condition. Therefore, this implies that Btg1/2 regulates hematopoietic homeostasis and is particularly involved in maintaining myeloid lineage homeostasis. However, follow-up studies are needed to determine the detailed molecular mechanism mediated by Btg1/2 in abnormally upregulated myelopoiesis.

Meanwhile, in this study, disruption of hematopoietic homeostasis due to loss of Btg1/2 funtion was not observed in the multipotent stem and progenitor cell fraction, including LT-HSC, ST-HSC, LSK, LK, MPP4, MPP3, and MPP2. Primitive LT-HSCs originate from the ebmryo mesoderm-derived aorta-gonad mesoderm (AGM) from the embryo development stage until 10.5 DPC (Day post coitum)[66]. After 10.5 DPC until 12.5 DPC, LT-HSCs migrate to the yolk-sac and fetal liver and colonize, and by this time, primitive hematopoiesis related to red blood cell formation progresses[49]. Hematopoiesis that begins after 12.5 DPC by Flk1-VEGF (Vasculor endothelial growth factor) interaction is called definitive hematopoiesis, and in the Btg1/2-Flk1-cre system, Btg1/2 is deleted from all blood cells from this stage[67,68]. Because multipotent stem and progenitor cells are derived from LT-HSCs at a very early stage, it is expected that there is a physiological mechanism that can compensate for the dysregulation of hematopoietic homeostasis due to loss of Btg1/2 early in fetal development. However, it seems important to identify this loss compensation mechanism through reproducibility experiments and molecular mechanism

studies.

As a result of this study, Myelopoiesis and Lymphopoiesis increased due to loss of function of Btg1/2. This suggests that Btg1/2 is involved in hematopoietic homeostasis. Btg2, which has a high expression level in bone marrow, is involved in the proliferation and differentiation of hematopoietic cells, and has a high expression level in resting thymocytes[69]. The results of this study show that neagtive selection in Btg1/2-deficient CLP is significantly suppressed during development in the thymus. This is consistent with previous findings that the presence of Btg2 maintains the quiescent state of thymocytes[69]. The double positive fraction in the thymus, which maintains the most active proliferative status after positive selection, increased by more than 5% compared to the littermate control. On the other hand, as a result of Spleen screenig, the frequency of mature T cells was significantly reduced. In normal conditions, T cell homeostasis is maintained by suppressing mTOR activity by PTEN (phosphatase and tensin homologue), TSC1 (tuberous sclerosis 1) and LKB1 (liver kinase B1)[70]. Since Btg2 is reported to inhibit mTOR activity[71], it is possible that mTOR activity increases during loss of function and T cells go into apoptotic process before maturation. Therefore, it is important to study the correlation between the mTOR signaling pathway and Btg1/2 in the CLP fraction present in bone marrow and spleen.

In Btg1/2 deficient mice, the greatest difference was observed in B cell fracion. Pax5 (Paired box 5) activates 170 genes involved in differentiation into the B cell lineage, and Btg1 is involved as a downstream effector transcription factor[72]. In addition, Btg2 maintains homeostasis by inducing cell cycle arrest in pre-B cells through PRMT1-mediated methylation of CDK4 (Cyclin dependent kinase 4)[73]. Moreover, the fact that Btg1/2 loss in this study was accompanied by a obvious decrease in B cell frequency in bone marrow and spleen suggests that Btg1/2 plays a unique role in each stage of hematopoietic homeostasis. Although the function of Btg1/2 in B cell devleopmental stage throughout each step of differentiation is well understood, more research is required to fully understand the molecular mechanism of Btg1/2 during myeloid lineage differentiation to

explain outcome of this study.

Normal differentiation of HSCs is tightly regulated by various transcription factors including HLX which is invovled in the formation of functional Hematopoeitic stem cell[74]. Another well-known HSC fate decision transcription factors include Runx1, Gfi1, and Gata2[75,76]. The transcription factor related to myelopoiesis with disrupted homeostasis is Pu.1, which is reported to play a critical role in myeloid-lineage fate decision[77,78]. Meanwhile, Btg1 and Btg2 function as transcriptional coactivators that bind to various intracellular transcription factors. Therefore, it is expected that there are many genes upregulated in myelopoesis and transcription factors that can bind to Btg1/2, and more detailed study on the downstream regulatory pathway is required.

Heterogeneity of hematopoietic stem and progenitor cells is the best known feature of hematopoiesis[79]. Multipotent progentitor cells are a fraction with increased gene expression required for fate decision to each lineage, but are reported to have high plasticity. Even hematopoietic cells with the same surface marker phenotype show different multi-lineage differentiation patterns[80]. This HSC heterogeneity is influenced by extrinsic factors caused by the bone marrow microenvironment and intrinsic factors caused by transcription factors. The fundamental cause of this HSPC heterogeneity, however, is not well characterized to date. Hematopoietic heterogenity was also observed in Btg1/2 knockout mice in this study. Monocyte-Macrophage fraction decreased in steady state condition, but the opposite result was found in stress-induced condition. In addition, CLP, which did not show significant differences in normal hematopoiesis, was highly upregulated in stress-induced conditions. This appears to be a combination of intracellular factors caused by the Btg1/2 and extracellular factors given proliferative pressure to form heterogenity. Indeed, Btg1 and Btg2 are versatile proteins that can perform unique and overlapping roles in growth regulation, differentiation, and apoptosis regulation. This is because PRMT1 (protein arginine N-methyltransferase) binds to the box C domain that Btg1 and Btg2 have in common and promotes methylation of a specific subset[81]. PRMT1 is capable of binding a wide variety of substrates. Given that Btg1/2 loss results in the aforementioned

heterogeneity-related phenotypes, it is important to find the signaling pathways downstream of Btg1/2-PRMT1 interaction in HSPCs, at least in part.

In addition to hematopoietic homeostasis, research on Btg1/2-mediated functions is required in relation to the functional characteristics of hematopoetic stem cells, including quiescent state, repopulation potential, self-renewal potential, and multi-lineage differentiation capacity. If the Myeloid lineage bias identified in this study is proven in in vivo experiments (bone marrow transplantation), it is expected that the function of Btg1/2 in Hmeatopoietic homeostasis will be more closely identified through additional molecular mechanism studies.

V. CONCLUSION

Overall, myelopoiesis caused by Btg1/2 increased in steady state condition and stress-induced condition. In steady state conditions, Btg1/2 regulates differentiation at various stages of progenitor cells. Deletion of Btg1/2 in multipotent stem and progenitor cells did not disrupt hematopoietic homeostasis. Btg1/2-deficient lineage committed progenitor cells showed elevated frequency of CMP and GMP, suggesting that there was a bias toward myelopoiesis. Neutrophil and Monocyte-Macrophage which is Subsequent differentiation cells, showed diminished level in bone marrow, and B cells were also significantly decreased. Lineage committed progenitor cell fraction in stress condition was different from that in steady state condition, suggesting that Btg1/2 plays a mediating role in both myeloid lineage and lymphoid lineage hematopoietic homeostasis. In lymphoid and myeloid cells, neutrophils commonly increased significantly, but Monocyte-Macrophage frequency showed opposite outcome in that of steady state condition. This can be explained by biphasic role of Btg1/2 in hematopoietic homeostasis. This study reveals Btg1/2-mediated hematopoietic homeostasis and suggests that it is an key regulator in maintaining myeloid lineage hematopoietic homeostasis.

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ABSTRACT(IN KOREAN)

조혈 항상성에서 Btg1과 Btg2의 역할 규명

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김상현

조혈 항상성은 조혈 계통에 있는 여러 전구세포들이 일정 비율로 유지되는것을 말하며, 화학요법 개입과 같은 스트레스 자극에 대한 반응으로 활성화 되어 항상성이 파괴된다. 조혈 계통에 존재하는 다양한 형태의 전구세포들은 전사인자에 의해 구동되는 세포 내적 네트워크와 이들이 존재하는 골수 미세환경에 따라 다 계통 분화에 대한 잠재력으로 설명할수 있다. 최근 조혈 항상성에 대한 이해가 크게 발전했음에도 불구하고, 세포 내적 인자에 의한 조절 기전은 여전히 어려운 과제로 남아있다. Btg (B cell translocation gene)1과 Btg2 는 초기 조혈의 중요한 조절자로서, 세포 성장과 휴지상태를 조절하는 세포 내적 자가유지 인자로 기능한다. 본 연구에서, Btg1과 Btg2 매개 기능의 주요 표현형을 조사하기 위해 정상상태 조건과 5-Fluorouracil로 유도한 스트레스 유발 조건에서 면역표현형 검사를 수행하였다. 유세포 분석, in vitro colony forming unit (CFU) assay, 전혈 검사(CBC test)를 통해 Btg1/2 매개 조혈 항상성 조절에 관한 영향이 입증되었다. 정상 상태 조건에서 Btg1/2 결손 마우스 모델은 공통 골수 전구세포(CMP)와 과립구-대식세포 전구세포(GMP)에 대한 상향조절을 보였으며, 이는 골수조혈에 대한 조혈 항상성이 붕괴되었음을 시사한다. 후속 골수 계통 세포인 호중구, 단핵구-대식세포는 각각 큰 폭으로 증가 및 감소하였다. 스트레스 유도 조건에서는 CMP, GMP와 더불어 공통 림프 전구세포 (CLP)가 증가하여 골수구계 조혈

뿐만 아니라 림프구계 조혈에 Btg1/2 매개 효과가 있음이 입증되었다. 호중구 비율 또한 상향 조절 되었으며, 단핵구-대식세포는 스트레스 조건에서 감소하였는데, 이는 Btg1/2 매개 이중역할이 있음을 시사한다. 본 연구는 Btg1/2가 조혈 항상성을 조절하고 있음을 밝히고 골수 계통 항상성 유지에 중요 조절자임을 시사한다.

핵심되는 말 : 조혈 항상성, Btg (B cell translocation gene)1, Btg2, 공통 골수구계 전구세포 (CMP), 과립구-대식세포 전구세포(GMP), 골수조혈