Tumor suppressors BTG1 and BTG2 regulate early mouse B-cell development

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Supplementary Appendix

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Supplementary Methods

Mice

Generation of C57BL/6J *Btg1*$^{-/-}$ mice has previously been reported by Farioli-Vecchiolo et al.[1] The *Btg2*$^{-/-}$ strain was generated at the University of Florida.[2] The *Btg1*$^{-/-}$*;Btg2*$^{-/-}$ mice have been described by Tijchon et al.[3] The animals were housed under specific pathogen-free conditions at the Central Animal Laboratory (Cdl) of the Raboud University Medical Center. All animal experiments were approved by the Animal Experimental Committee of the Radboud University Medical Center and were performed in accordance with institutional and national guidelines. In our experiments we used mice that were between the age of 8-12 weeks old.

Flow cytometric analysis

Bone marrow (BM) cells were harvested by flushing femurs with RPMI medium (Gibco//Life technologies Europe BV, Bleiswijk, The Netherlands) containing 10% FBS (Greiner Bio-One BV, Alphen a/d Rijn, The Netherlands) and 50µM 2-ME (Gibco). Spleen and thymus cells were isolated by gently flushing out the spleen and thymus on a 70µM filter using RPMI medium (Gibco) containing 10% FBS (Gibco) and 50µM 2-ME (Gibco). To isolate peritoneal cavity cells, 5ml ice-cold PBS

(Lonza Benelux BV, Breda, The Netherlands) containing 3% FBS (Gibco) was injected into the peritoneum of euthanized mice followed by gently massage of the abdomen and recovery of the cells with a needle. All cell suspensions were treated with red cell lysis buffer (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands), filtered and counted. Prepared cell suspensions were used for FACS analysis of different hematopoietic lineages (LSRII flow cytometer, BD Biosciences, Breda, The Netherlands). Single cell suspensions were pre-incubated with unlabeled anti–Fcγ III/II receptor (2.4G2) (BD Biosciences) to inhibit unspecific antibody binding. Cells were stained with the following antibodies purchased form BD Biosciences and eBiosciences (Vienna, Austria): anti-B220/CD45R-PE-CY7 (RA3-6B2); BP1-PE (6C3); CD4-FITC (RM4-5); CD8-PE (53-6.7); CD11b/Mac1-FITC (M1/70); CD19-APC-CY7 (1D3); CD24/HSA-FITC (M1/69); CD25-PE-CY7 (PC61.5); CD43-APC (S7); CD44-PERCP-CY5.5 (IM7); CD45.1/Ly-5.1-APC (A20); CD45.2/Ly-5.2-V500 (104); CD117/c-Kit-PERCP-CY5.5 (2B8); CD127/IL-7Ra-FITC (A7R34); CD135/Flt3-PE (A2F10); GR1-PE (RB6-8C5); Lin-APC (145-2C11/M1-70/RA3-6B2/TER119/RB6-8C5); IgM-FITC (II/41); IgD-PE (11-26c); Sca1/Ly6A-PE (D7). Isotype antibodies were used as control. The data were collected and analyzed by FlowJo software.

Competitive repopulation assay

Bone marrow cells were harvested by flushing femurs with RPMI medium (Gibco) containing 10% FBS (Gibco) and 50 uM 2-ME (Gibco), and the cell suspension was treated with red cell lysis buffer (Sigma), filtered and counted. For the competitive *in vivo* repopulation, two experimental groups were created (each 6 mice). In the first group bone marrow cells from wild-type donor mice (C57BL/6; *Ly5.2/CD45.2)* were mixed 1:1 with bone marrow cells from wild-type mice (n=3) of the same strain as the recipient mouse (C57BL/6, *Ly5.1/CD45.1*). In the second group the knockout donor mice (C57BL/6 *Btg1*$^{-/-}$*; Btg2*$^{-/-}$*; Ly5.2/CD45.2*) were mixed 1:1 with bone marrow (BM) cells from wild-type mice (n=3) of the same strain as the recipient mouse (C57BL/6; *Ly5.1/CD45.1*). The BM cells from the CD45.1 were obtained from 3 donor mice and pooled for all the transplantation experiments. The BM cells from the wild-type and *Btg1*$^{-/-}$*;Btg2*$^{-/-}$ CD45.2 mice were each derived from 3 independent animals and also transplanted separately in two independent recipients. The mixture of cells was intravenously injected into lethally irradiated (9 Gy, Cs^{137}) 8–12 week old *Ly5.1/CD45.1* recipient mice, with each recipient receiving 1×10^6 donor (*Ly5.2-CD45.2*) and 1×10^6 competitor (*Ly5.1-CD45.1*) bone marrow cells. The contribution within the CD45.1 and CD45.2 donor fractions for T cells ($CD3^+$), B-cells ($B220^+$), and myeloid cells ($Mac\text{-}1^+$) was assessed 5 and 10 weeks post transplantation by flow cytometry from blood samples and at 11 weeks from spleen. Two animals receiving CD45.2 from wild-type mice died at the age of 3-4 weeks, due to failure of transplantation procedure.

Methylcellulose colony assays

Total bone marrow cells ($1x10^5$ cells/plate) and MACS sorted $CD19^+$ cells ($0.2x10^5$ cells/plate) derived of WT, *Btg1*$^{-/-}$, *Btg2*$^{-/-}$ and *Btg1*$^{-/-}$;*Btg2*$^{-/-}$ mice, were cultured in 1.1ml B-cell specific methylcellulose cultures in 35mm plates (M3630, STEMCELL Technologies, SARL, Grenoble, France), in duplicate, to study the outgrowth of B-cell progenitors.

Microarray gene expression analysis

Hardy fractions ABC and DEF[4] (early and late B-cell stage) isolated from BM of WT and *Btg1*$^{-/-}$;*Btg2*$^{-/-}$ animals were obtained by sorting with FACS AriaII (BD), as follows: $B220^+$ $CD43^{hi}$ (Hardy ABC), $B220^+CD43^-$ (Hardy DEF). Total RNA was extracted of $1\text{-}3x10^6$ cells according to manufacturer protocol (Qiagen RNAeasy kit) (QIAGEN Benelux BV, Venlo, The Netherlands). Biotinylated RNA was prepared using the Illumina TotalPrep RNA Amplification kit (Ambion, Inc., Austin, TX, U.S.A.) according to manufacturer's specifications with an input of 200ng total RNA. Samples were hybridized onto the Illumina MouseRef-8 v2 (Illumina, Inc., San Diego, CA, U.S.A.). Image analysis and extraction of raw expression data was performed with Illumina GenomeStudio v2011.1 Gene. The Quality control, RNA labeling, hybridization and data extraction were performed at ServiceXS B.V (Leiden, The Netherlands). Samples were normalized to Robust Multi-array Average (RMA) and analyzed with ArrayStar software. KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis was performed using the online Gene set enrichment analysis (GSEA) Molecular Signature Database (MSigDB) tool,[5] only including genes with an expression cutoff of ≥ 5 and ≥ 1.6 fold difference. Illumina bead array expression data is submitted to the NCBI Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/), accession code: GSE55997.

Apoptosis and proliferation assays

MACS sorted $CD19^+$ BM cells ($1x10^6$ cells/ml) derived from WT and *Btg1*$^{-/-}$ mice, were cultured in IMEM medium (Gibco) containing 20% FBS for Mouse Pre-B Lymphoid Colony-Forming Cells (STEMCELL Technologies), 1% Penicillin-Streptomycin (Invitrogen/ Life technologies Europe BV, Bleiswijk, The Netherlands), 0,3% w/v Primatone (Sigma) and 55µM 2-ME (Gibco) in the presence of 20ng/ml IL-7 (R&D Systems Europe, Abingdon, United Kingdom) in a 6-well tissue-culture (TC) plate. The cells were cultured for 4 days. Apoptosis was measured using the AnnexinV-PE Apoptosis Detection Kit I (BD Biosciences) and analyzed on a LSRII flow cytometer (BD Biosciences). For measuring cell proliferation, the cell cycle distribution was determined using Ki-67 staining according to the manufacturer's instructions (BD Biosciences).

RNA isolation and quantitative RT-PCR

RNA was isolated from $B220^+$ BM cells derived from WT, *Btg1*$^{-/-}$, *Btg2*$^{-/-}$ and *Btg1*$^{-/-}$;*Btg2*$^{-/-}$ mice according to manufacturer protocol (Qiagen RNeasy kit). For quantitative reverse transcription

polymerase chain reaction (qRT-PCR), cDNA was generated with the iScript cDNA synthesis kit (Bio-Rad Laboratories BV, Veenendaal, The Netherlands) and analyzed by qRT-PCR using SYBR Green method (Bio-Rad). Expression levels were normalized to the housekeeping gene TATA box-binding protein (TBP). Primers sequences used in qRT-PCR experiments are listed in Supplementary Table S2.

Statistical Analysis

Statistical data are expressed as mean ± SEM, as indicated in the text. Statistical differences were determined with 2-sided, unpaired Student's t test when comparing two biological datasets or one-sided ANOVA combined with Dunetts's post-hoc test in case of comparing multiple biological datasets to wild-type controls. P values < 0.05 (*), <0.01 (**) or <0.001 (***) were considered statistically significant.

Supplementary References

1. Farioli-Vecchioli S, Micheli L, Saraulli D, et al. Btg1 is Required to Maintain the Pool of Stem and Progenitor Cells of the Dentate Gyrus and Subventricular Zone. Frontiers in neuroscience. 2012;6(124.
2. Park S, Lee YJ, Lee HJ, et al. B-cell translocation gene 2 (Btg2) regulates vertebral patterning by modulating bone morphogenetic protein/smad signaling. Molecular and cellular biology. 2004;24(23):10256-10262.
3. Tijchon E, van Ingen Schenau D, van Opzeeland F, et al. Targeted Deletion of Btg1 and Btg2 Results in Homeotic Transformation of the Axial Skeleton. PloS one. 2015;10(7):e0131481.
4. Hardy RR, Carmack CE, Shinton SA, Kemp JD, Hayakawa K. Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow. The Journal of experimental medicine. 1991;173(5):1213-1225.
5. Subramanian A, Tamayo P, Mootha VK, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proceedings of the National Academy of Sciences of the United States of America. 2005;102(43):15545-15550.

Supplementary Tables

Table S1. Identification of KEGG pathways in *Btg1*$^{-/-}$*; Btg2*$^{-/-}$ progenitor B-cells by Gene Set Enrichment Analysis

Description pathway	p-Value	Genes up	Genes down
B220*$^{+}$ *CD43*$^{+}$ *cells (Hardy ABC)			
Pathways in cancer	7,15E-10	*Traf2, Stat3, Runx1, Ets1, Tcf7, Pten, Ep300, Cdh1*	*Mmp9, Egf, Csf2ra, Traf5, Csf1r, Hsp90ab1*
Hematopoietic cell lineage	1,37E-09	*Cd38, Cd33, Cd3d, Cd3g, Cd9*	*Gypa, Il1b, Tnf, Csf2ra, Cd19, Ms4a1, Csf1r*
Cytokine-cytokine receptor interaction	6,52E-08	*Cx3cr1, Il6st, Tnfrsf19, Ccr7, Il2rb, Tnfrsf1a, Cxcr4*	*Tnfrsf12a, Egf, Il1b, Tnfrsf4, Tnf, Tnfrsf13b*
Jak-STAT signaling pathway	7,24E-06	*Il6st, Stat3, Il2rb, Cblb, Jak1, Spred1, Socs5, Pik3r1*	*Csf2ra, Osmr, Ccnd3*
T cell receptor signaling pathway	8,67E-06	*Pak1, Cblb, Prkcq, Cd3d, Lcp2, Cd3g, Itk, Pik3r1*	*Tnf, Cd4*
B220*$^{+}$ *CD43*$^{-}$ *cells (Hardy DEF)			
Cell Cycle	8,80E-14	*Ccna2, Mcm2, Rad21, Wee1, Rbl1, Cdc25a, Cdkn1a*	*Gadd45b, Ywhaz, Gadd45a*
Pathways in cancer	4,90E-10	*Itga6, Lamc1, Prkca, Ccne2, Casp3, Runx1, Cdkn1a*	*Lama4, Tcf7l2, Fos, Csf2ra, Traf5, Araf*
p53 signaling pathway	9,27E-07	*Ccnd1, Ccne2, Casp3, Chek1, Cdkn1a, Ccnb1*	*Ddb2, Gadd45b, Pmaip1, Gadd45a*
MAPK signaling pathway	1,63E-06	*Cacna2d1, Ptpn7, Mapk3, Traf2, Mapkapk2, Map4k4*	*Fos, Gadd45b, Mknk1, Hspa8, Mapk11, Gadd45a*
Wnt signaling pathway	1,79E-06	*Tbl1xr1, Fzd6, Dvl2, Axin2, Frat1, Apc, Prkca, Csnk2a1*	*Tcf7l2, Nkd2, Plcb2*

Table S2. Primers for quantitative RT-PCR

Name	**Forward (5' ⟶ 3')**	**Reverse (5' ⟶ 3')**
mBtg1	CCACCATGATAGGCGAGATCG	CTGGGAACCAGTGATGTTTG
mBtg2	GGGTTTCCTCTCCAGTCTCC	GATACGGATACAGCGATAGC
mCd4	TCCCACTCACCCTCAAGATA	CCATCACCTCACAGGTCAAA
mIkzf2	GCCTTTTGAGAGACCTGCTG	TGATGGCTTGGTCCATCATA
mTcf7	CCAGAAGCAAGGAGTTCACA	GCAGGGAAGTGCTGTCTATATC
mPax5	CGCGTGTTTGAGAGACAGCACTACT	GTCTCGGCCTGTGACAATAGGGTAG
mEbf1	CCATCCGAGTTCAGACACCTCCT	ACCTCTGGAAGCCGTAGTCGATG
mTcf3	GGGAGGAGAAAGAGGATGA	GCTCCGCCTTCTGCTCTG
mFoxo1	CATCGAGAGCTCAGCCGAGAA	ATGGACGCAGCTCTTCTCCG
Tbp	GCACAGGAGCCAAGAGTGAA	ACATCACAGCTCCCCACCAT

Supplementary Figures

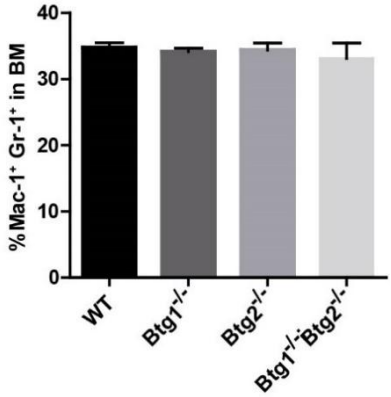
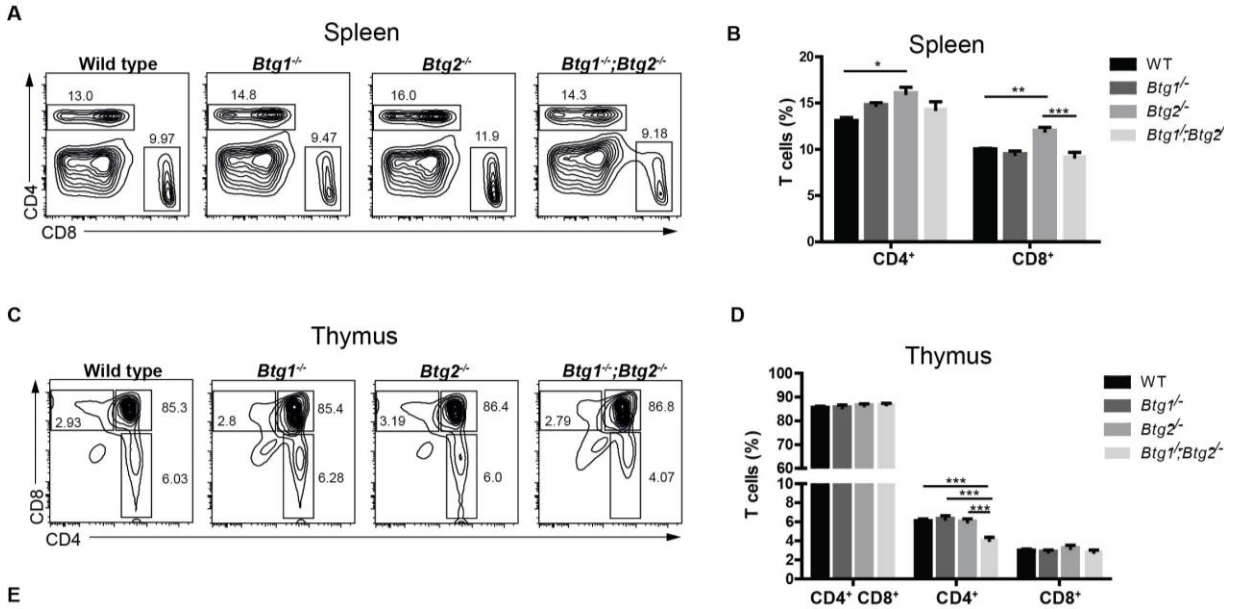


Figure S1. Myeloid development in bone marrow compartment of mice deficient for *Btg1* and *Btg2*. Flow cytometric analysis was performed on mononuclear cells isolated from bone marrow (BM) of wild-type (WT) control (n=6), *Btg1*$^{-/-}$ (n=3), *Btg2*$^{-/-}$ (n=4) and *Btg1*$^{-/-}$*;Btg2*$^{-/-}$ (n=4) mice. The percentage of Mac-1^{+}Gr-1^{+} myeloid progenitor cells in BM is indicated.



Genotype	DN	DN1	DN2	DN3	DN4
WT	1,98	0,044	0,171	0,941	0,568
Btg1$^{-/-}$	1,78	0,033	0,189	0,866	0,464
Btg2$^{-/-}$	1,37	0,029	0,120	0,670	0,393
Btg1$^{-/-}$*;Btg2*$^{-/-}$	1,83	0,039	0,082	0,793	0,555

Figure S2. T-lymphopoiesis in thymus and spleen of *Btg1* and *Btg2* knockout animals. (A-E) Flow cytometric analysis was performed on mononuclear cells isolated from spleen and thymus of wild-type control (WT) (n=10-16), *Btg1*$^{-/-}$ (n=6-7) *Btg2*$^{-/-}$ (n=5-8) and *Btg1*$^{-/-}$*;Btg2*$^{-/-}$ mice (n=9-10). (A-B) The

percentage of $CD4^+$ and $CD8^+$ T cells in spleen (C-D) and thymus are indicated. (E) Early T-cell development can be defined as lineage negative (Lin^-) $CD4^-CD8^-$, double negative T-cells. To distinguish the double negative (DN) T-cell fractions, DN1-DN4, cells were gated for CD25 and CD44. Each bar represents the mean of each group of mice ± the standard error of the mean (SEM). *, $P< 0.05$, **, $P< 0.01$, ***, $P< 0.001$.

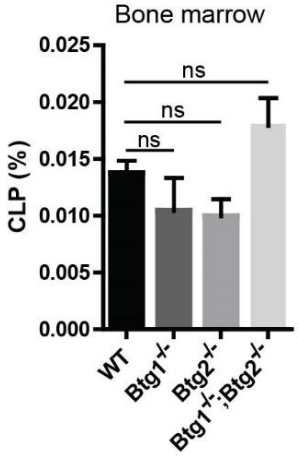


Figure S3. Analysis of common lymphoid progenitor population of *Btg1* and *Btg2* knockout mice. Flow cytometric analysis was performed on mononuclear cells isolated from bone marrow (BM) of wild-type control (WT) (n=15), $Btg1^{-/-}$ (n=7) $Btg2^{-/-}$ (n=8) and $Btg1^{-/-};Btg2^{-/-}$ mice (n=11). BM cells were stained with lineage cocktail, SCA-1/Ly6A/E, c-KIT/CD117, FLT-3L/CD135 and IL-7Rα/CD127 antibodies to determine the percentage of common lymphoid progenitor (CLP) cells.

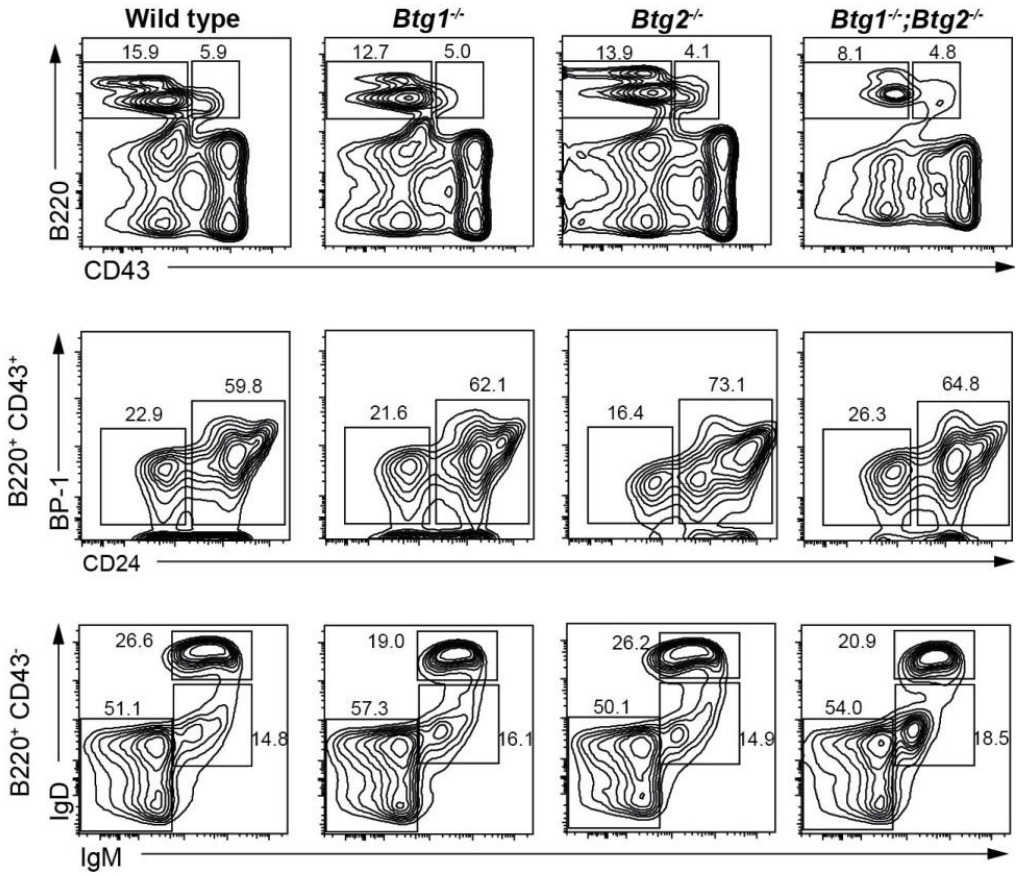


Figure S4. Flow cytometric analysis of the early B-cell developmental stages of mice deficient for *Btg1* and *Btg2***.** Bone marrow (BM) cells from wild-type (WT) control (n=16), *Btg1*$^{-/-}$ (n=7), *Btg2*$^{-/-}$ (n=8), and *Btg1*$^{-/-}$*;Btg2*$^{-/-}$ (n=11) mice were stained with a cocktail of antibodies directed against B220, CD43, BP-1, CD24, IgD and IgM. Cells were first gated according to the expression levels of B220 and CD43, followed by quantitative analyses of BP-1 and CD24 on the B220^{+}CD43^{+} cells to identify Hardy fractions A (B220^{+}CD43^{+}CD24^{-}) and BC (B220^{+}CD43^{+}CD24^{+}), or IgD and IgM expression on B220^{+}CD43^{-} cells to distinguish Hardy fractions D (B220^{+}CD43^{-}IgM^{-}IgD^{-}), E (B220^{+}CD43^{-}IgM^{+}IgD^{lo}) and F (B220^{+}CD43^{-}IgM^{+}IgD^{+}). The mean percentage for each population of cells is indicated for the different genotypes.

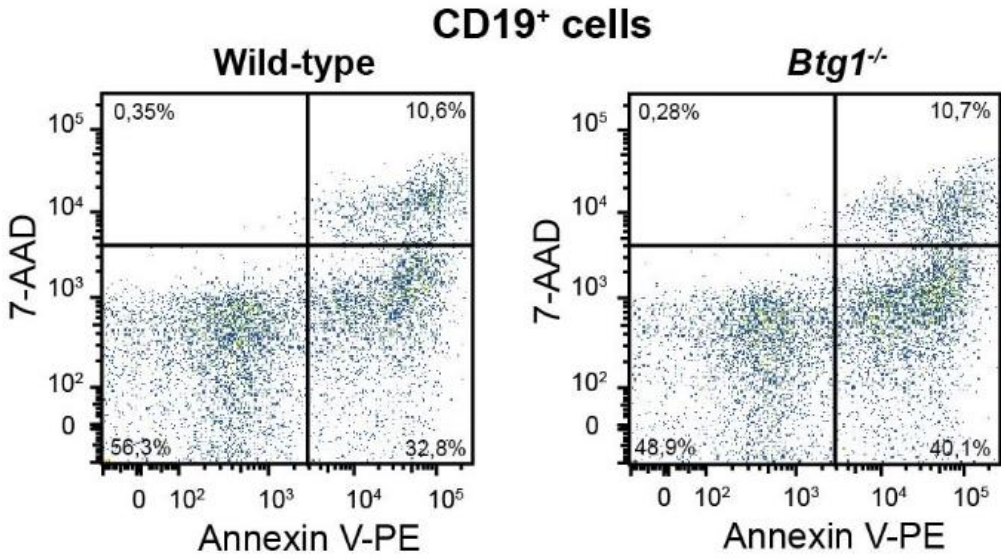


Figure S5. Analysis of apoptosis in CD19^{+} cells of *Btg1*-deficient mice by AnnexinV staining**.** Apoptosis assay was performed on CD19^{+} cells isolated from bone marrow of wild-type (WT) (n= 4) and *Btg1*$^{-/-}$ (n=7) mice that were cultured for 4 days in B-cell specific culture medium by measuring AnnexinV and 7-AAD with flow cytometry.

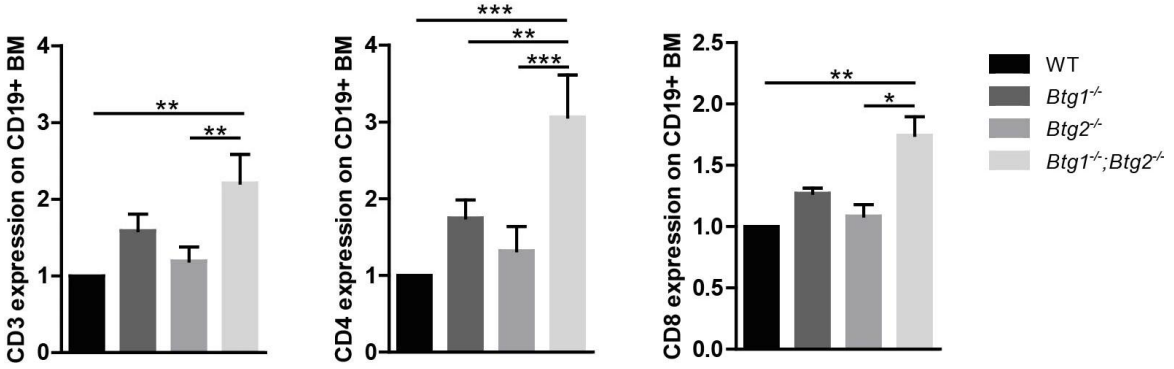


Figure S6. Btg1 and Btg2 suppress expression of inappropriate T-lineage markers on committed B-cells. Bone marrow (BM) cells derived from wild-type (WT) (n=6), *Btg1*(n=9), *Btg2* (n=6) and *Btg1;Btg2* (n=6) knockout mice were stained for CD19 in combination with T-cell surface markers (CD3, CD4, CD8) and analyzed by flow cytometry. Cells were first gated for CD19^{+} B-cells and within that gate we analyzed the fraction of CD3, CD4 and CD8 T-cell marker expression. Data shown are representative of three separate experiments and indicate the mean value (and SEM) of at least two independent biological samples, and compared relative to WT levels. *, $P< 0.05$, **, $P< 0.01$, ***, $P< 0.001$.