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

Transcription factors that regulate B-cell differentiation are frequently targeted by genetic aberrations in B-cell malignancies. The B-cell translocation gene 1 (BTG1), which encodes a transcription coregulator, is recurrently affected by gene deletions in B-cell precursor acute lymphoblastic leukemia (BCP-ALL) and targeted by missense mutations in diffuse large B-cell lymphomas (DLBCL). Similarly, the closely related BTG2 gene is subject to mutations in DLBCL, Burkitt lymphoma and follicular lymphoma. To establish the role of BTG1 and BTG2 in B lymphopoiesis, we studied the composition of the hematopoietic compartment in mice deficient for either Btg1 or Btg2, as well as in Btg1;Btg2-double knockout animals. We found that Btg1 and Btg2 are both required at multiple stages of early B-cell development, but also display specific functions in progenitor B-cells. Our data demonstrate that transcriptional cofactors BTG1 and BTG2 act as important regulators of B-cell differentiation, providing an important link to their role as tumor suppressors in B-cell malignancies.

To determine the function of Btg1 and Btg2 during normal hematopoiesis we performed flow cytometric analysis of the bone marrow (BM) compartment and spleen. The fraction of B220+ progenitor B-cells was significantly reduced in Btg1- and Btg2-deficient mice, ranging from a 10% decrease in the Btg2–/– mice, 20% in Btg1–/– mice, to 40% in the Btg1–/–;Btg2–/– double knockout animals relative to control mice (Figure 1A). Similarly, B-cell counts in the...

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**Figure 1. Decreased B-cell numbers in mice deficient for Btg1 and Btg2.** (A-B) Flow cytometric analysis was performed on mononuclear cells isolated from bone marrow (BM) and spleen of wild-type (WT) control (n=16), Btg1–/– (n=7), Btg2–/– (n=8) and Btg1–/–;Btg2–/– mice (n=11). The fractions of B220+ BM cells (A) and B220+IgM+ splenocytes (B) are indicated. Each dot represents an individual sample and the horizontal line indicates the mean. (C) Competitive repopulation assay of wild-type and Btg1–/–;Btg2–/– donor cells (CD45.2) derived of BM mixed 1:1 with CD45.1 wild-type cells which were transplanted into 9Gy (Gray) irradiated C57BL6/J mice (CD45.1). Flow cytometric analysis was performed on blood and spleen. The fraction of B220, CD3 and Mac-1 of WT:WT (CD45.1:CD45.1) (n=4) and WT:Btg1–/–;Btg2–/– (CD45.1:CD45.2) (n=6) cells are indicated. (D) Schematic representation of the different B-cell developmental stages according to Hardy nomenclature. Cell surface markers that distinguish specific Hardy fractions are indicated. (E) The percentage for the different Hardy B-cell fractions within BM is indicated as determined in WT control (n=16), Btg1–/– (n=7), Btg2–/– (n=8), and Btg1–/–;Btg2–/– (n=11) mice. 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.
spleen were most significantly reduced in $Btg1^{-/-}$; $Btg2^{-/-}$ mice, with about 30% fewer B220$^+$IgM$^+$ B-cells (Figure 1B). In contrast, the fraction of myeloid progenitor cells in BM was not altered in the $Btg1^{-/-}$ and $Btg2^{-/-}$-deficient animals (Online Supplementary Figure S1). In addition, we did not observe any specific defects in T-cell development in mice lacking $Btg1$ and $Btg2$ expression (Online Supplementary Figure S2).

To assess whether the defect in B lymphopoiesis was not related to defective support from the BM microenvironment in the $Btg1^{-/-}$ and $Btg2^{-/-}$ knockout animals, competitive reconstitution assays were performed. BM cells derived from either wild-type (CD45.2) or $Btg1^{-/-}$; $Btg2^{-/-}$ mice (CD45.2) were mixed with competitor wild-type (CD45.1) mice (CD45.1). FACS analysis on peripheral blood and spleen demonstrated that contribution to the B-cell lineage was significantly reduced using $Btg1^{-/-}$; $Btg2^{-/-}$ donor cells when compared to wild-type donor cells (Figure 1C), arguing that the observed defect in B-cell development is cell autonomous.

To establish at what stage of early B-cell development $Btg1$- and $Btg2$-deficiency affect B-cell differentiation, we investigated the relative frequencies of the different B-cell subsets within the BM compartment. Analysis of the common lymphoid progenitor (CLP) fraction, defined as lineage negative (Lin$^-$) Sca-1$^+$c-Kit$^+$Flt3$^+$IL-7R$^+$ cells, revealed no significant differences in $Btg1^{-/-}$, $Btg2^{-/-}$, and $Btg1^{-/-}$; $Btg2^{-/-}$ mice compared to wild-type controls (Online Supplementary Figure S3). Next, we employed the cell surface markers B220, CD43, CD24, IgD and IgM, to distinguish different B-cell precursor populations, generally known as Hardy fractions (Figure 1D). When we examined the single knockout animals, the $Btg2^{-/-}$ mice displayed decreased B-cell fractions at both the early and later stages of B-cell differentiation, while $Btg1$-deficiency resulted only in reduced B-cell fractions beyond the pre-B cell stage, including the immature B-cells (Hardy fractions E and F) (Figure 1E). In the $Btg1^{-/-}$; $Btg2^{-/-}$ mice, we observed a similar phenotype as the $Btg2^{-/-}$ mice at the early stages of B-lineage differentiation, with a moderate decrease in the pool of pro-B cells (Hardy BC) compared to control mice. However, from the pre-B cell stage onwards, the $Btg1^{-/-}$; $Btg2^{-/-}$ donor cells displayed a more pronounced phenotype in comparison to the $Btg1^{-/-}$ and $Btg2^{-/-}$ single knockout animals, showing a 2-fold reduction in the B220$^+$CD43$^-$ cell fraction relative to control mice (Hardy D, E and F) (Online Supplementary Figure S4).

**Figure 2.** $Btg1$ stimulates B-cell progenitor outgrowth in response to IL-7. (A) Bone marrow (BM) mononuclear cells (1 x $10^5$ cells) were isolated from wild-type (WT) control (n=18), $Btg1^{-/-}$ (n=7), $Btg2^{-/-}$ (n=8) and $Btg1^{-/-}$; $Btg2^{-/-}$ mice (n=11) and added in methylcellulose containing 10 ng/mL IL-7. (B) BM mononuclear cells were isolated from WT control (n=10), $Btg1^{-/-}$ (n=7), $Btg2^{-/-}$ (n=4) and $Btg1^{-/-}$; $Btg2^{-/-}$ mice (n=4) and added after CD19 isolation (2 x $10^5$ CD19$^+$ cells) in methylcellulose containing 10 ng/mL IL-7. (A, B) Mean colony counts (and SEM) were determined (>30 cells/colony) after seven days of culture. Data are representative of at least two independent experiments. ***P<0.001, **P<0.01. (C) BM cells from WT, $Btg1^{-/-}$, $Btg2^{-/-}$, and $Btg1^{-/-}$; $Btg2^{-/-}$ mice (all n=2) were stained with B220 and IL-7Rα/CD127 antibodies and the percentage of IL-7Rα-positive cells within the B220$^+$ fraction was determined. The black line indicates IL-7Rα expression in the WT cells, while the blue peak shows the IL-7Rα expression in the $Btg1^{-/-}$, $Btg2^{-/-}$ and $Btg1^{-/-}$; $Btg2^{-/-}$ double knockout cells. (D) Apoptosis assay of CD19$^+$ BM cells isolated from WT (n=4) and $Btg1^{-/-}$ (n=7) cultured for 4 days in B-cell specific medium, by measuring Annexin V using flow cytometry. The percentage of Annexin V-positive cells in CD19$^+$ BM cells is indicated. (E) Proliferation assay of CD19$^+$ BM cells isolated from WT (n=3) and $Btg1^{-/-}$ (n=3) cultured for 4 days in B-cell specific medium, measuring Ki-67 by flow cytometry. The cell cycle distribution according to Ki-67 staining in CD19$^+$ BM cells is indicated. Each bar represents the mean of each group of mice ± the standard error of the mean (SEM). ***P<0.001, **P<0.01.
We conclude from these results that Btg1 and Btg2 are both required for mouse B-cell development, while deficiency for each of the genes shows a different outcome on the relative abundance of the specific Hardy fractions.

To more specifically assess the function of Btg1 and Btg2 in early B lymphopoiesis, we investigated whether interleukin-7 (IL-7) dependent outgrowth of B-lymphoid progenitor cells was affected in the absence of Btg1 and Btg2 expression. BM cells derived from Btg1−/−, Btg2−/− single knockout and Btg1−/−;Btg2−/− double knockout mice were plated in methylcellulose and cultured for one week in the presence of cytokine IL-7. The number of colonies in methylcellulose was about 4-fold lower in cultures derived from Btg1−/− and Btg1−/−;Btg2−/− BM cells relative to those derived from control BM cells (Figure 2A). Moreover, colony size was also reduced in these two knockout genotypes (data not shown). In contrast, Btg2-deficiency had no significant impact on the outgrowth of lymphoid progenitor cells in the IL-7-dependent methylcellulose colony formation assay. Given the fact that the number of B220+ B-cell progenitors was decreased in the BM of the different Btg1 and Btg2 knockout lines, we also analyzed the colony outgrowth of similar amounts of purified CD19+ progenitor B-cells under the same conditions. Again, colony formation was significantly impaired using CD19+ cells derived from Btg1−/− and Btg1−/−;Btg2−/− mice, but not with Btg2−/− progenitor B-cells, which indicates that the loss of Btg1 function is
largely responsible for the defective outgrowth of B-lymphoid progenitors (Figure 2B).

To assess whether differences in colony formation could be attributed to reduced expression levels of the IL-7 receptor alpha (IL-7Rα/CD127), CD127 expression was measured on B220+ BM cells by flow cytometry. We observed that IL-7Rα expression was not decreased in Btg1−/− progenitor B-cells, but was even upregulated in Btg1−/−Btg2−/− cells (Figure 2C), arguing that the low number of colonies due to Btg1-deficiency was not related to reduced IL-7Rα expression levels. Next, we examined whether Btg1-deficient CD19+ cells displayed intrinsic differences in the level of apoptosis and proliferation. We observed moderate but significant increase in the fraction of Annexin V-positive cells derived from Btg1−/− mice (Figure 2D; Online Supplementary Figure S5), while the cell cycle distribution, as determined by Ki-67 staining, was not affected (Figure 2E). Thus, Btg1 appears to be required for optimal IL-7 dependent B-lymphoid colony formation, in part by promoting cell survival in early progenitor B-cells.

To elucidate how Btg1 and Btg2 regulate B-cell development in vivo, we examined the expression of key transcription factors involved in early B-cell differentiation, including E2a, Tcf3, Foxo1, Ebf1, and Pax5.11,12 Quantitative reverse transcription polymerase reaction (qRT-PCR) on RNA isolated from B220+ sorted BM cells of Btg1−/−, Btg2−/−, and Btg1−/−Btg2−/− mice demonstrated that the expression of these master regulators of B-cell differentiation was not decreased (Figure 3A). On the contrary, mRNA expression levels of these transcription factors were even somewhat elevated in the absence of Btg1 and Btg2. Hence, the partial block in B-cell differentiation observed in the Btg1−/−Btg2−/− animals cannot be explained by diminished expression levels of these B-lineage transcription factors.

Next, we performed microarray based gene expression profiling using early (B220+CD43+) and late progenitor B-cells (B220+CD43−) isolated from control and Btg1−/−; Btg2−/− mice and performed Gene Set Enrichment Analyses (GSEA) using the Molecular Signatures Database (MSigDB) to define KEGG pathways that were specifically affected (Figure 3B). Interestingly, several signaling pathways were identified, including the presence of a T-cell receptor signaling signature in early B-cells of Btg1−/−;Btg2−/− mice showing upregulation of Cd3d (Cd3delta) and Cd3g (Cd3gamma) (Online Supplementary Table S4). Therefore, we aimed to assess whether Btg1/Btg2-deficient progenitor B-cells displayed aberrant expression of other T-lineage specific genes, including Cd4, Ileif2, Tcfl7, Gata3 and Notch1. Indeed, qRT-PCR revealed that Btg1−/−;Btg2−/− B220+ BM cells showed significantly increased expression levels of these T-lineage marker genes (Figure 3C), while we also observed higher expression of the CD3, CD4 and CD8 cell surface markers on Btg1−/−;Btg2−/− committed B-cells (Online Supplementary Figure S6). These findings suggest that Btg1 and Btg2 are implicated in regulating B-lineage commitment by suppressing the expression of lineage-inappropriate genes.

In summary, using genetic mouse models, we demonstrate that both Btg1 and Btg2 promote mouse B-cell differentiation at multiple stages of development, and mostly display overlapping functions during B lymphopoiesis, which correlates with an additive phenotype in the Btg1−/−Btg2−/− mice. On the other hand, Btg1 fulfills a more prominent role in promoting the proliferative expansion of lymphoid progenitors by inhibiting apoptosis. This is in agreement with the finding that Btg1 ablation in the mouse brain decreases the pool of proliferating stem and progenitor cells, which is accompanied by increased apoptosis.13 Importantly, Btg1 and Btg2 are both required to repress T-lineage gene expression in progenitor B-cells. These findings are partially overlapping with the phenotype observed in Ebf1 and Pax5 knockout mice, where it has been shown that EBF1 and PAX5 are critical regulators of B-lineage specification and commitment by repressing alternative non-B-cell fates.14,15 As the expression of Ebf1 and Pax5 itself is not inhibited in Btg1−/−Btg2−/− progenitor B-cells, the BTG1 and BTG2 transcriptional coregulators may affect B-lineage commitment by modifying the function of these B-cell specific transcription factors or by epigenetic regulation of their target genes. Notably, PAX5 and EBF1, similar to BTG1, are also subject to gene deletions in BCP-ALL, and combinations of these deletions often co-occur in the same leukemia.4 Together our findings suggest that the block in differentiation observed in BCP-ALL may result from gene alterations affecting multiple master regulators of B-cell differentiation.

References