SUPPLEMENTARY APPENDIX

Tumor suppressors BTG1 and IKZF1 cooperate during mouse leukemia development and increase relapse risk in B-cell precursor acute lymphoblastic leukemia patients

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Supplementary Material

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Supplementary methods

Cytogenetic and molecular analysis of patient samples

Cases were screened for high hyperdiploidy (51-65 chromosomes), *ETV6-RUNX1* and *BCR-ABL1* fusion products and rearrangements of *TCF3* and *MLL* by routine diagnostic procedures. Cases in the discovery cohort were identified as *BCR-ABL1*-like, based on a previously described expression signature of 110 probe sets.[1, 2] The multiplex ligation-dependent probe amplification assay SALSA MLPA P335 ALL-IKZF1 (MRC-Holland, Amsterdam, the Netherlands) was performed according to the manufacturer's protocol and analyzed as previously described.[1] A ratio lower than 0.75 for the exon intensity in patients versus the healthy control reference was considered to represent a deletion. For scoring *CDKN2A/B* deletion, it was sufficient to have either *CDKN2A* or *CDKN2B* deleted. Mutation status for *IKZF1* was neither assessed in discovery (n=533) nor validation (n=515) cohort.

Flow cytometric analysis

Murine 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 β-Mercaptoethanol (Gibco). Cells from spleen and thymus were isolated through a 70µM filter using RPMI medium containing 10% FBS and 50µM β-Mercaptoethanol. Leukemic blasts isolated from either lymph nodes, spleen or thymus were analyzed by FACS using the LSRII flow cytometer (BD Biosciences, Breda, The Netherlands). Single cell suspensions were treated with red blood cell lysis buffer (Sigma-Aldrich, Zwijndrecht, The Netherlands) and 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 from BD Biosciences (Franklin Lakes, New Jersey) and eBiosciences (Vienna, Austria): anti-B220/CD45R (RA3-6B2); BP1 (6C3); CD4 (RM4-5); CD8 (53-6.7); CD11b/Mac1 (M1/70); CD24/HSA (M1/69); CD25 (PC61.5); CD43 (S7); CD44 (IM7); IgM (II/41); IgD (11-26c). The data were collected and analyzed by FlowJo V10 software (FlowJo, Ashland, Oregon).

Immunohistochemistry

Tissues isolated from diseased mice were fixed in 4% formalin for 24 hours. Specimens were embedded in paraffin, 4 µM serial sections were prepared and stained with hematoxylin and eosin (HE). For immunohistochemistry, slides were dewaxed and rehydrated prior to heat-induced antigen retrieval using sodium citrate buffer. After blocking with 2% goat serum and 2% BSA in 0.05% Tween-PBS, the slides were incubated overnight with B220/CD45R (RM2600) (Invitrogen, Amsterdam, The Netherlands) and CD3 (A0452) (DAKO Netherlands

BV, Heverlee, Belgium) antibodies. Biotinylated anti-rat IgG (BA9401) (Vector Laboratories, Peterborough, United Kingdom) and anti-rabbit IgG (BA1000) (Vector Laboratories) were used as secondary antibody and subsequently the slides were incubated with metal-enhanced diaminobenzidine in stable peroxide substrate buffer (Thermo Fisher Scientific Inc., Amsterdam, The Netherlands), counterstained with hematoxylin, and after dehydration coverslipped with Entellan. Four selected regions per murine liver or lung histological slide were used for the quantification of $CD3^+$ positively stained areas. The analysis was performed with FIJI software.[3] For the quantification of the blood smears, 10 visual fields with a 40x objective were scored for the number of blasts. In total, 10 visual fields per blood smear were averaged and for each genotype the percentage of blasts was determined for three independent mice.

VDJ rearrangement analysis

DNA was extracted from enlarged spleen or lymph nodes using the Gentra Puregene Mouse Tail kit (Qiagen, Venlo, The Netherlands). PCR analysis was performed with primers specific for the DB1-JB region of Trb1, Dβ2.1-Jβ2.7 region of Trb2, and 5'VH2-3'Cμ and 5'VH2-3'Cκ primers for the Igh and Igk genes, respectively (*Supplementary Table S1*).

Viability assays on murine splenic B-cells

Spleens from healthy young (6-12 weeks of age) wild-type, *Ikzf1*$^{+/-}$, *Btg1*$^{-/-}$ and *Btg1*$^{-/-}$;*Ikzf1*$^{+/-}$ mice were isolated and single mononuclear cells were harvested through a 70 μM cell strainer. Erythrocytes within the cell suspensions were removed by Red blood cell lysis buffer (Sigma-Aldrich, Zwijndrecht, The Netherlands). Next, splenocytes were cultured for 48 hours in RPMI 1640 medium (Life technologies, Carlsbad, CA) supplemented with 10% heat-inactivated fetal calf serum (FCS), 1% penicillin/streptomycin (Invitrogen), and 50 μM β-Mercaptoethanol in the presence of 5 μg/mL lipopolysaccharide (LPS). Subsequently, splenic B-lymphocytes obtained after ficoll gradient were cultured in a 96-well plate at a density of 1x 10^5 cells/well in the presence of 5 μg/mL LPS. Splenic B-cells were treated with increasing concentrations of prednisolone or dexamethasone (both Centrafarm, Etten-Leur, the Netherlands). After 48 hours, relative cell viability was assessed using the CellTiter 2 96® AQueous One Solution Cell Proliferation (MTS) Assay (Promega, Madison, WI). Absorbance was acquired using a plate reader (Infinite F50; TECAN, Männedorf, Switzerland). For AnnexinV analyses, splenic B-lymphocytes obtained from young (6-12 weeks of age) wild-type, *Ikzf1*$^{+/-}$, *Btg1*$^{-/-}$ and *Btg1*$^{-/-}$;*Ikzf1*$^{+/-}$ mice were treated for 48 hours with prednisolone or dexamethasone and stained with the AnnexinV-PE/7-AAD Viability Kit (BD Biosciences) according to manufacturer's instructions and analyzed by FACS. AnnexinV-positive cell fractions were determined by FlowJo software Version 10 (Treestar, Ashland, OR).

References

1. van der Veer A, Waanders E, Pieters R, et al. Independent prognostic value of BCR-ABL1-like signature and IKZF1 deletion, but not high CRLF2 expression, in children with B-cell precursor ALL. *Blood*. 2013;**122**(15):2622-9.

2. Den Boer ML, van Slegtenhorst M, De Menezes RX, et al. A subtype of childhood acute lymphoblastic leukaemia with poor treatment outcome: a genome-wide classification study. *Lancet Oncol.* 2009;**10**(2):125-34.

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Supplementary Tables

Supplementary Table S1. Oligonucleotide primers used for genotyping and IG/TR clonality analysis.

Gene locus	Forward primer (5'-3')	Reverse primer (5'-3')
Wild-type *Btg1*	CCATGCATCCCTTCTACACCC	TGCAGGCTCTGGCTGAAAGT
Knockout *Btg1*	CCATGCATCCCTTCTACACCC	CGGAGAACCTGCGTGCAATC
Wild-type *Ikzf1*	ATGGATGTCGATGAGGGTCAAGAC	AGTTCTCTGAACACAGACACACGT
Knockout *Ikzf1*	GCGGAGCTCCTCAGGTGCAGGCTGCCTATC	GACGCGTCGACGCTAACATCCTGAGGGACTGT
Trb1 (DJ)	CAGCCCCTTCAGCAAAGAT	CCTAAGTTCCTTTCCAAGACCAT
Trb2 (DJ)	GTAGGCACCTGTGGGGAAGAAACT	TGAGAGCTGTCTCCTACTATCCATT
Igh	CCCGAATTCCAGGTCCAGTTGCAGCAGWCWGG	CCCGAATTCGCTCTCGCAGGAGAC
Igk	CCCGAATTCCAGGTCCAGTTGCAGCAGWCWGG	CCCGAATTCTAACTGCTCACTGGA

Supplementary Table S2. Representation of pediatric ALL subtypes in DCOG and COALL cohorts

ALL subtypes	DCOG (N=470)		COALL (N=128)		Total (N=598)		Literature*
	N	Perc	N	Perc	N	Perc	Perc
ETV6-RUNX1	120	26%	34	27%	154	26%	25%
High hyperdiploid (51-65 chr)	95	20%	31	24%	126	21%	25%
Non-*BCR-ABL1*-like B-other	80	17%	27	21%	107	18%	10-15%
BCR-ABL1-like B-other	69	15%	23	18%	92	15%	10-15%
TCF3-rearranged	17	4%	2	2%	19	3%	5%
BCR-ABL1	22	5%	2	2%	24	4%	3%
MLL-rearranged	10	2%	1	1%	11	2%	2%
T-ALL	57	12%	8	6%	65	11%	15%

* Pui et al, Journal of Clinical Oncology 2011

Supplementary Table S3. Co-occurrence of *BTG1* deletion with other common gene deletions in BCP-ALL

	BTG1 no deletion		*BTG1* deletion		Fisher Test	
	N	Perc	N	Perc	*P*-value	Odds ratio
IKZF1 no deletion	397	0.82	31	0.65	0.0071	2.5
IKZF1 deletion	88	0.18	17	0.35		
PAX5 no deletion	352	0.73	33	0.69	0.61	1.2
PAX5 deletion	133	0.27	15	0.31		
CDKN2A/B no deletion	323	0.67	28	0.58	0.27	1.4
CDKN2A/B deletion	162	0.33	20	0.42		
EBF1 no deletion	462	0.95	39	0.81	0.0011	4.6
EBF1 deletion	23	0.05	9	0.19		
RB1 no deletion	451	0.93	40	0.83	0.042	2.6
RB1 deletion	34	0.07	8	0.17		
ETV6 no deletion	351	0.72	25	0.52	0.0046	2.4
ETV6 deletion	134	0.28	23	0.48		

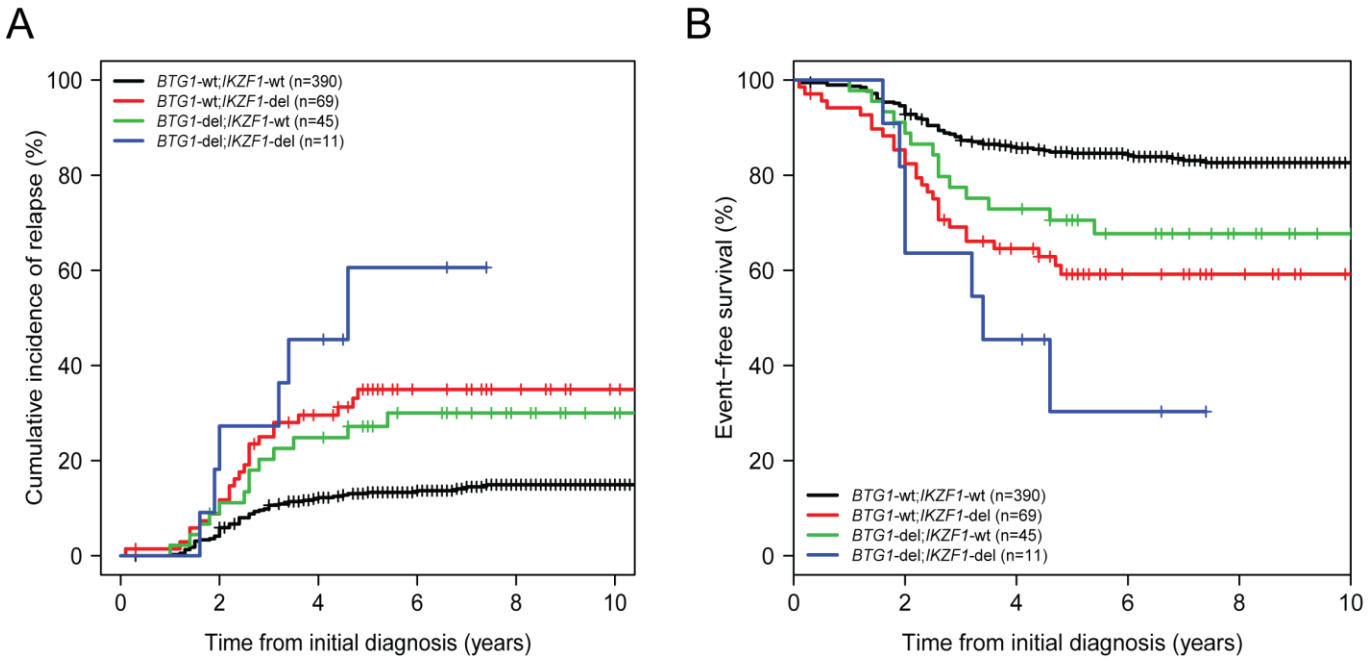
Supplementary Table S4. Outcome analysis in BCP-ALL cases double-deleted for common gene deletions and *IKZF1* versus *IKZF1*-only deleted cases

PAX5				**Univariate**				**multivariate**	
Deletion	**Total**	**Relapse**	**Death**	**5-yr CIR (SE)**	**Gray P**	**EFS HR (95% CI)**	**Cox P**	**EFS HR (95% CI)**	**Cox P**
PAX5;IKZF1	50	17	3	32% (7%)	0.8	1.0 (0.6-1.9)	0.9	0.7 (0.3-1.3)	0.2
IKZF1	55	18	4	33% (7%)					
PAX5	98	19	1	19% (4%)					
none	330	36	11	11% (2%)					
CDKN2A/B				**Univariate**				**multivariate**	
Deletion	**Total**	**Relapse**	**Death**	**5-yr CIR (SE)**	**Gray P**	**EFS HR (95% CI)**	**Cox P**	**EFS HR (95% CI)**	**Cox P**
CDKN2A/B; IKZF1	52	20	3	39% (7%)	0.2	1.4 (0.8-2.5)	0.3	0.9 (0.4-1.8)	0.7
IKZF1	53	15	4	26% (6%)					
CDKN2A/B	130	23	5	18% (4%)					
none	298	32	7	10% (2%)					
EBF1				**Univariate**				**multivariate**	
Deletion	**Total**	**Relapse**	**Death**	**5-yr CIR (SE)**	**Gray P**	**EFS HR (95% CI)**	**Cox P**	**EFS HR (95% CI)**	**Cox P**
EBF1;IKZF1	9	3	1	22% (15%)	0.9	1.2 (0.4-3.3)	0.7	2.1 (0.7-6.9)	0.2
IKZF1	96	32	6	33% (5%)					
EBF1	23	3	0	15% (8%)					
none	405	52	12	13% (2%)					
RB1				**Univariate**				**multivariate**	
Deletion	**Total**	**Relapse**	**Death**	**5-yr CIR (SE)**	**Gray P**	**EFS HR (95% CI)**	**Cox P**	**EFS HR (95% CI)**	**Cox P**
RB1;IKZF1	14	5	1	37% (14%)	0.7	1.2 (0.5-2.9)	0.6	0.6 (0.2-1.6)	0.3
IKZF1	91	30	6	31% (5%)					
RB1	28	5	1	18% (8%)					
none	400	50	11	12% (2%)					
ETV6				**Univariate**				**multivariate**	
Deletion	**Total**	**Relapse**	**Death**	**5-yr CIR (SE)**	**Gray P**	**EFS HR (95% CI)**	**Cox P**	**EFS HR (95% CI)**	**Cox P**
ETV6;IKZF1	25	10	0	40% (10%)	0.4	1.0 (0.5-2.0)	0.9	1.5 (0.7-3.4)	0.3
IKZF1	80	25	7	30% (5%)					
ETV6	132	11	1	8% (3%)					
none	296	44	11	15% (2%)					

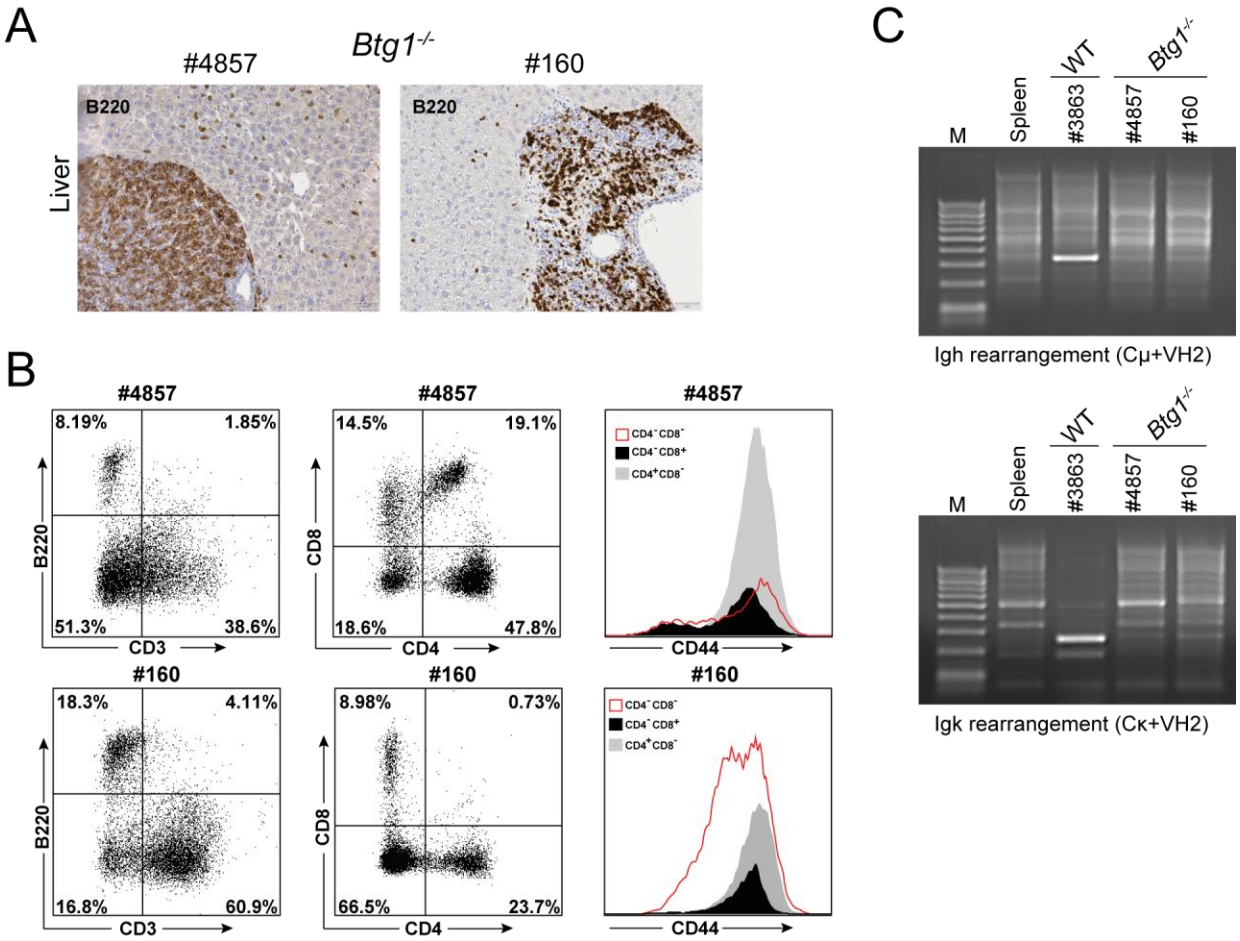
CIR, cumulative incidence of relapse; SE, standard error; EFS, event-free survival; HR, hazard ratio; CI, confidence interval; multivariate, corrected for BCP-ALL subtype and stratified for study cohort (DCOG, COALL)

Supplementary Table S5. Excel data sheet with leukemia phenotype mouse tumors

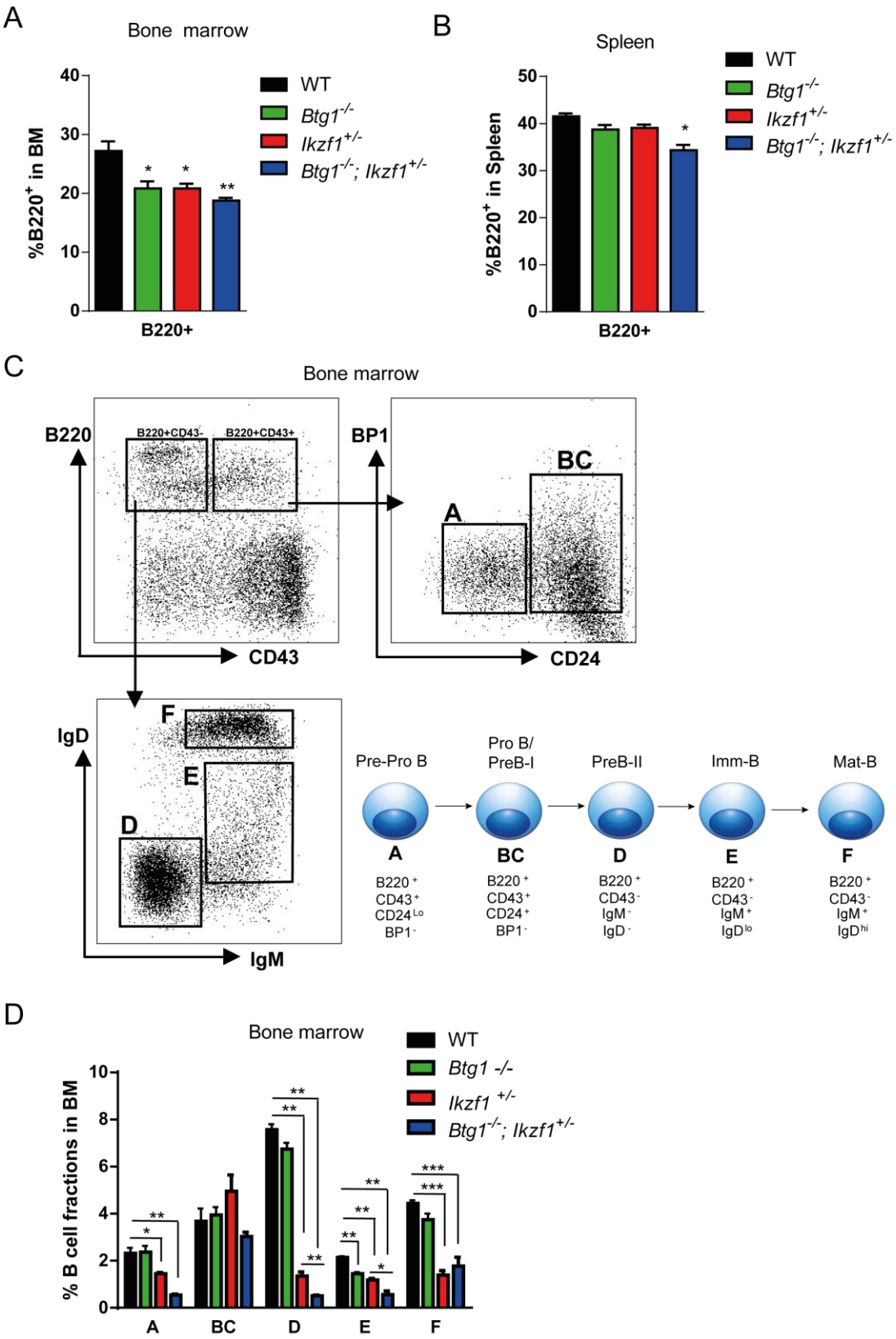
Supplementary Figures



Supplementary Figure S1. Cumulative incidence of relapse (CIR) and event-free survival (EFS) curves for pediatric BCP-ALL cases from the Australian and New Zealand Children's Oncology Group (ANZCHOG) ALL8 trial with or without *IKZF1* and *BTG1* deletions. (**A**) CIR and (**B**) EFS curves for total BCP-ALL cohort (n=515). Colors: black, *IKZF1* and *BTG1*-wildtype; green, *IKZF1*-wildtype, *BTG1*-deleted; red, *IKZF1*-deleted, *BTG1*-wildtype; blue, both *BTG1* and *IKZF1*-deleted. Abbreviations: wt, wildtype; del, deletion; n, total number.

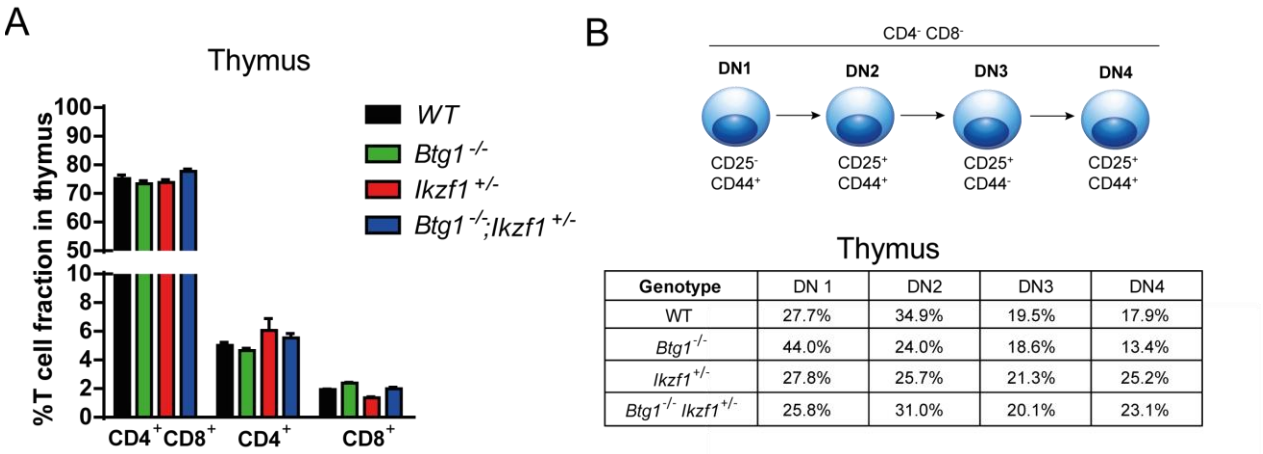


Supplementary Figure S2. Characteristics of *Btg1*-deficient T-cell leukemias. (**A-B**) T-cell leukemias in *Btg1*-deficient mice are characterized by a significant proportion of B220^{+} B-lymphocytes within the tumor area, which can be demonstrated by B220-positive staining as determined by immunohistochemistry of infiltrated liver (A) and flow cytometry of the primary leukemia (B). (**B**) T-cell leukemias in *Btg1* knockout mice are characterized by relative high expression of CD44, which is evident in both CD3^{+}CD4^{-}CD8^{-} fraction (red line), CD3^{+}CD4^{-} CD8^{+} fraction (black curve) and CD3^{+}CD4^{+}CD8^{-} fraction (gray curve). (**C**) Immunoglobulin gene rearrangements for heavy chain (Igh) and kappa light chain (Igk) show clonal rearrangement in B-cell malignancies derived from wild-type C57Bl/6J mice, but not in leukemias derived from *Btg1*$^{-/-}$ mice, indicating that B220^{+} cells in leukemic infiltrates of *Btg1*$^{-/-}$ mice represent non-leukemic B-cells.



Supplementary Figure S3. Analysis of B-cell development in *Ikzf1*$^{+/-}$, *Btg1*$^{-/-}$ and *Btg1*$^{-/-}$;*Ikzf1*$^{+/-}$ mice in bone marrow and spleen. Bone marrow (BM) and splenic cells from WT, *Ikzf1*$^{+/-}$, *Btg1*$^{-/-}$ and *Btg1*$^{-/-}$;*Ikzf1*$^{+/-}$ mice were stained with an antibody cocktail against B220, CD43, BP-1, IgD and IgM. (**A-B**) Flow cytometric analysis on the fraction of B220^{+}-cells in bone marrow (A) and spleen (B) of the

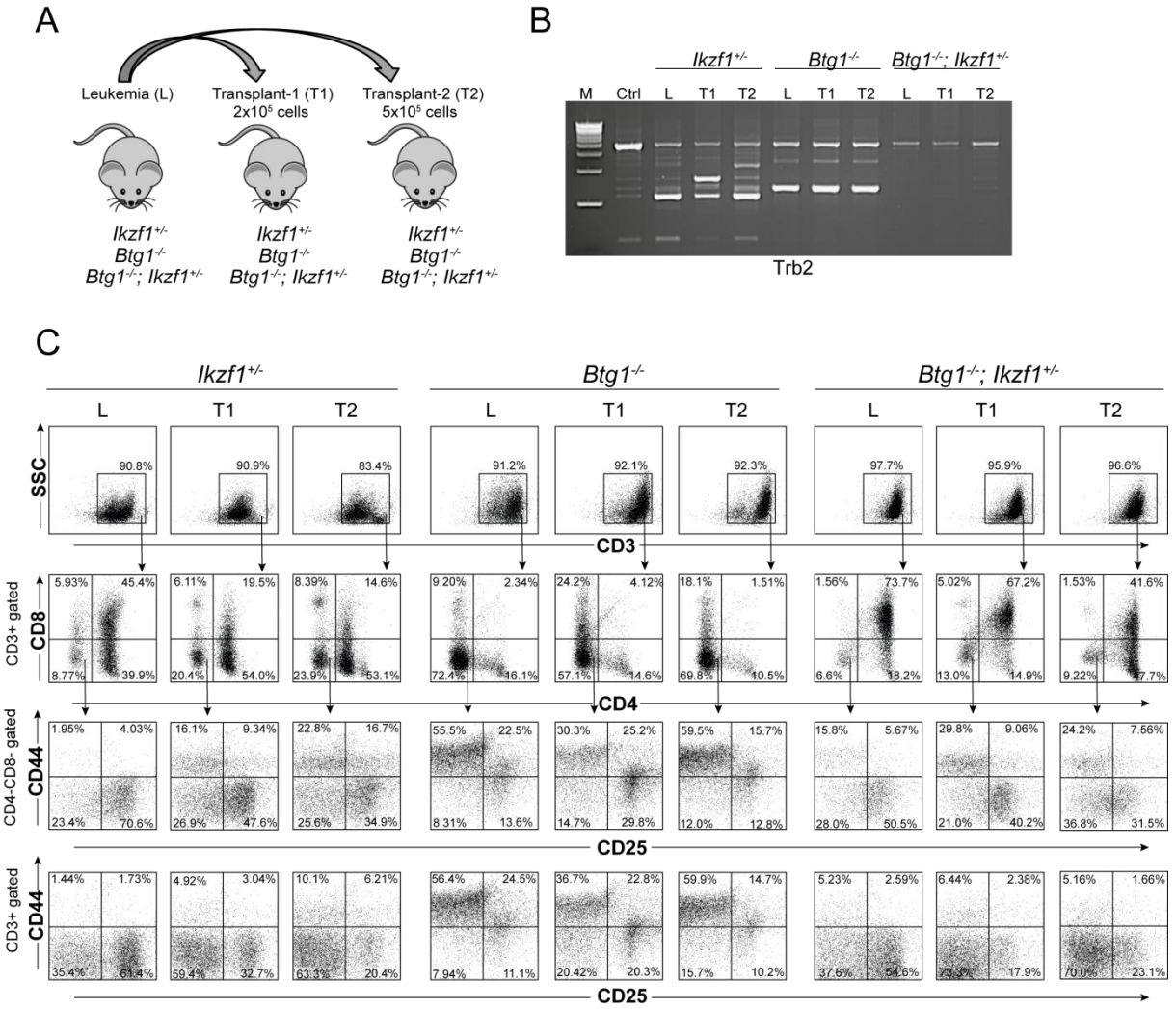
different genotypes (n=4). (**C**) Representative gating strategy of the early B-lymphoid populations in the bone marrow compartment. After gating on the viable cell population and according to B220 and CD43 expression levels, the $B220^{+}CD43^{+}$ population was further analyzed for their BP-1 and CD24 expression to identify Hardy Fraction A ($B220^{+}CD43^{+}BP\text{-}1^{-}CD24^{-}$) and BC ($B220^{+}CD43^{+}BP\text{-}1^{-}CD24^{+}$). Hardy fraction D, E and F were identified by gating for the $B220^{+}CD43^{-}$ population. Subsequently IgD and IgM expression levels in the $B220^{+}CD43^{-}$ gate were used to distinguish Hardy fraction D ($B220^{+}CD43^{-}IgM^{-}IgD^{-}$) Hardy fraction E ($B220^{+}CD43^{-}IgM^{+}IgD^{low}$) and F ($B220^{+}CD43^{-}IgM^{+}IgD^{high}$).(**D**) The relative percentages of different Hardy fractions within the bone marrow compartment is indicated for BM of WT, *Ikzf1*$^{+/-}$, *Btg1*$^{-/-}$ and *Btg1*$^{-/-}$;*Ikzf1*$^{+/-}$ mice (n=4). Data for all experiments are means, and error bars represent SEM. *P* values (two-sided *t* test) are indicated. **P*<0.05, ***P*<0.01, and ****P*<0.001.



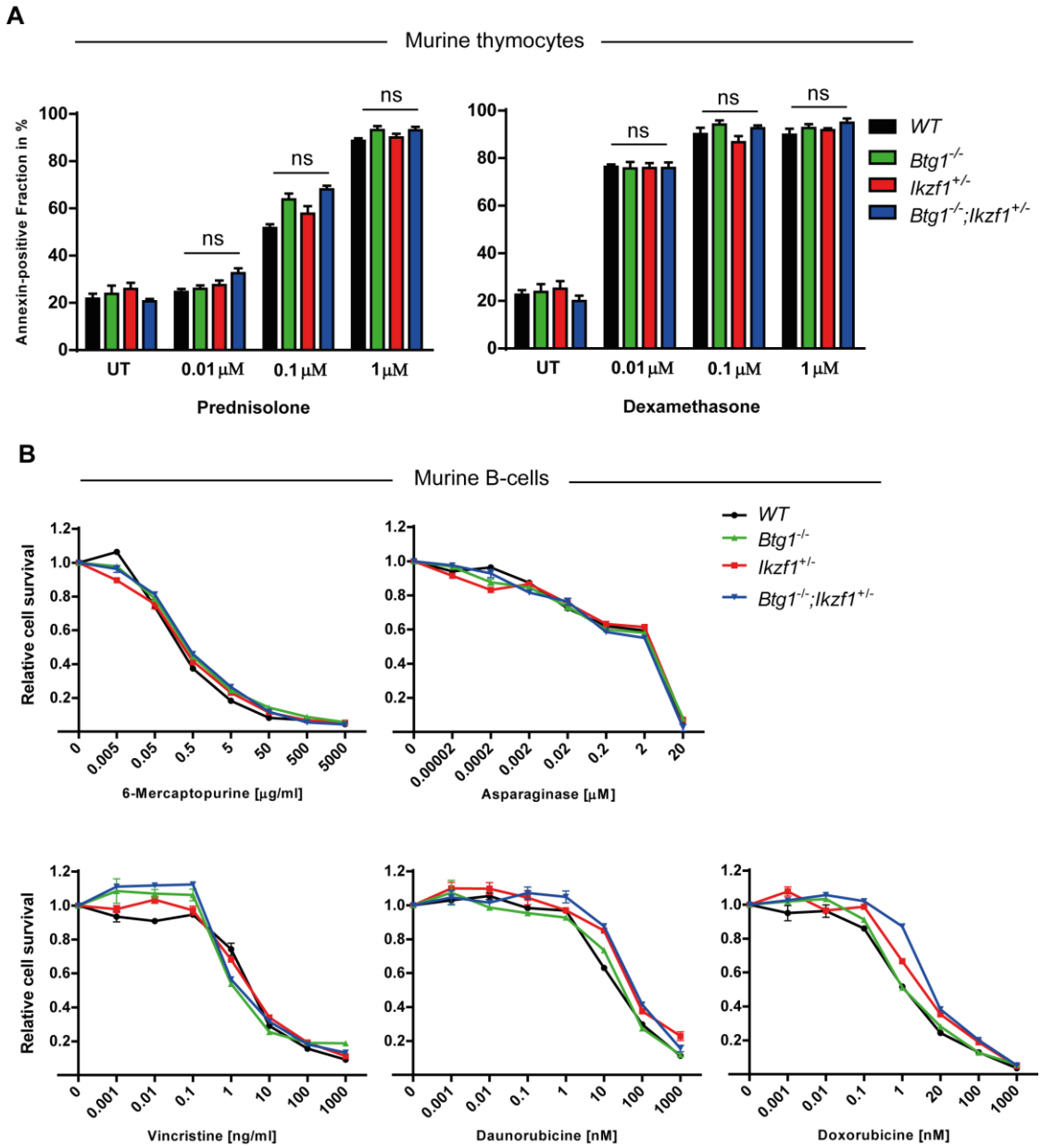
Thymus

Genotype	DN 1	DN2	DN3	DN4
WT	27.7%	34.9%	19.5%	17.9%
Btg1$^{-/-}$	44.0%	24.0%	18.6%	13.4%
Ikzf1$^{+/-}$	27.8%	25.7%	21.3%	25.2%
Btg1$^{-/-}$ *Ikzf1*$^{+/-}$	25.8%	31.0%	20.1%	23.1%

Supplementary Figure S4. Analysis of T-lymphopoiesis in *Ikzf1*$^{+/-}$, *Btg1*$^{-/-}$ and *Btg1*$^{-/-}$;*Ikzf1*$^{+/-}$ mice. (**A-B**) Flow cytometric analysis was performed on the lymphoid cells isolated from thymus of wild-type (WT), *Ikzf1*$^{+/-}$, *Btg1*$^{-/-}$ and *Btg1*$^{-/-}$;*Ikzf1*$^{+/-}$ mice (n=3). Cells were stained with an antibody cocktail against CD3, CD4, CD8, CD44 and CD25 to identify the different stages of T-cell development. (A) Thymocytes were analyzed for expression of CD4 and CD8. (B) Early T-cell development can be identified in the $CD4^{-}CD8^{-}$ double negative (DN) T-cell fraction. To further distinguish the DN stages of T cell development, the $CD4^{-}CD8^{-}$ cells were gated for CD25 and CD44 expression. DN1 is defined as $CD44^{+}CD25^{-}$ fraction, DN2 as $CD44^{+}CD25^{+}$ fraction, DN3 as $CD44^{-}CD25^{+}$ fraction and DN4 as $CD44^{-}CD25^{-}$ fraction in the thymus. Each value represents the mean of three mice. Data are means, and error bars represent SEM.



Supplementary Figure S5. Leukemias arising in *Btg1* and *Ikzf1* knockout animals are (oligo)clonal and transplantable into syngeneic mice. (**A**) Schematic overview of the transplantation experiments. Leukemias (L) derived from either *Ikzf1*$^{+/-}$, *Btg1*$^{-/-}$, and *Ikzf1*$^{+/-}$;*Btg1*$^{-/-}$ mice were transplanted intravenously into syngeneic mice at a concentration of 2x10^5 cells (transplant-1; T1) or 5x10^5 cells (transplant-2; T2). T-cell leukemias were observed 4 weeks after transplantation. (**B**) T-cell receptor beta 2 (Trb2) gene rearrangements were determined by PCR on DNA isolated from non-lymphoid tissue (earclip) as control for germline configuration, and DNA derived from L, T1 and T2 tumor tissues of *Ikzf1*$^{+/-}$, *Btg1*$^{-/-}$, and *Ikzf1*$^{+/-}$;*Btg1*$^{-/-}$ mice. (**C**) Flow cytometry was performed on the original leukemia (L) and two independent serial transplantations (T1 and T2). The upper panel shows the side scatter (SSC) and CD3 profile. The CD3-positive fraction was subsequent analyzed for CD4 and CD8 staining (second panel) and gated for the CD4$^-$CD8$^-$ fraction to analyze CD44 and CD25 expression (third panel) as well as on the complete CD3$^+$ fraction (fourth panel).



Supplementary Figure S6. Chemotherapy response in primary lymphoid cells isolated from *Ikzf1*$^{+/-}$, *Btg1*$^{-/-}$ and *Btg1*$^{-/-}$*; Ikzf1*$^{+/-}$ mice. (**A**) AnnexinV/7-AAD staining of WT, *Ikzf1*$^{+/-}$, *Btg1*$^{-/-}$ and *Btg1*$^{-/-}$*;Ikzf1*$^{+/-}$ murine thymocytes after treatment with increasing concentrations of prednisolone (left panel) or dexamethasone (right panel) for 12 hours (n=4) or left untreated (UT). The fraction of AnnexinV-positive cells was determined. Data are means, and error bars represent SEM. Differences were statistically non-significant (ns) as assessed by two-sided t-test. (**B**) Splenocytes isolated from wild-type (WT), *Ikzf1*$^{+/-}$, *Btg1*$^{-/-}$ and *Btg1*$^{-/-}$*; Ikzf1*$^{+/-}$ mice were stimulated with lipopolysaccharide (LPS) for 48 hrs. Ficoll-purified splenic B-cells were treated with increasing concentrations of common single chemotherapeutic agents used in ALL treatment. Cells were treated with 6-mercaptopurine, daunorubicine, vincristine or doxorubicine for 48 hours, or for 72 hours with asparaginase, and analyzed by MTS assay. All values were normalized to untreated B-cells. Error bars represent ± standard error of the mean(SEM) and a two-sided ANOVA was performed for statistical analysis (n=2).