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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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ABSTRACT

Deletions and mutations affecting lymphoid transcription factor IKZF1 (IKAROS) are associated with an increased relapse risk and poor outcome in B-cell precursor acute lymphoblastic leukemia. However, additional genetic events may either enhance or negate the effects of *IKZF1* deletions on prognosis. In a large discovery cohort of 533 childhood B-cell precursor acute lymphoblastic leukemia patients, we observed that single-copy losses of *BTG1* were significantly enriched in *IKZF1*-deleted B-cell precursor acute lymphoblastic leukemia (P=0.007). While *BTG1* deletions alone had no impact on prognosis, the combined presence of *BTG1* and *IKZF1* deletions was associated with a significantly lower 5-year event-free survival (P=0.0003) and a higher 5-year cumulative incidence of relapse (P=0.005), when compared with *IKZF1*-deleted cases without *BTG1* aberrations. In contrast, other copy number losses commonly observed in B-cell precursor acute lymphoblastic leukemia, such as *CDKN2A/B, PAX5, EBF1* or *RB1*, did not affect the outcome of *IKZF1*-deleted acute lymphoblastic leukemia patients. To establish whether the combined loss of IKZF1 and BTG1 function cooperate in leukemogenesis, *Btg1*-deficient mice were crossed onto an *Ikzf1* heterozygous background. We observed that loss of *Btg1* increased the tumor incidence of *Ikzf1*$^{+/-}$ mice in a dose-dependent manner. Moreover, murine B cells deficient for *Btg1* and *Ikzf1*$^{+/-}$ displayed increased resistance to glucocorticoids, but not to other chemotherapeutic drugs. Together, our results identify *BTG1* as a tumor suppressor in leukemia that, when deleted, strongly enhances the risk of relapse in *IKZF1*-deleted B-cell precursor acute lymphoblastic leukemia, and augments the glucocorticoid resistance phenotype mediated by the loss of IKZF1 function.

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Introduction

Acute lymphoblastic leukemia (ALL) is the most common form of cancer in children and is characterized by recurrent genetic aberrations and chromosomal abnormalities, which represent distinct genetic subtypes that are used for risk stratification.[1] In the past, we and others have demonstrated that genomic alterations affecting the lymphoid transcription factor gene IKZF1 represent a strong prognostic factor associated with relapse in childhood B-cell precursor acute lymphoblastic leukemia (BCP-ALL).[2-5] Recently, we established that loss of IKZF1 affects glucocorticoid (GC)-mediated gene regulation and confers GC resistance in BCP-ALL.[6] Deletion of *IKZF1* is a hallmark of *BCR-ABL1*-positive BCP-ALL,[7] and within this high-risk cytogenetic subtype, *IKZF1* loss is associated with an even worse outcome.[8] *IKZF1* gene lesions are also frequently present in high-risk leukemias with a Philadelphia- or *BCR-ABL1*-like expression signature,[2,5,9] which often carry activated tyrosine kinases (i.e., *ABL1, PDGFRB, JAK2, FLT3*) or mutations targeting the *RAS* pathway.[10-12] Mouse studies have confirmed that two of these genomic alterations, namely *BCR-ABL1* and mutant RAS, cooperate with loss of IKZF1 during leukemic transformation.[13,14] On the other hand, *ERG* gene deletions constitute a specific subtype of BCP-ALL with favorable outcome, despite the frequent co-occurrence of *IKZF1* deletions.[15,16] Thus, specific genetic interactions may modulate the tumor suppressive functions of IKZF1 during leukemia development at initial diagnosis and at relapse after chemotherapy treatment.

Besides *IKZF1*, many other recurrent genetic aberrations have been observed in BCP-ALL, which include deletions affecting *BTG1, CDKN2A/B, EBF1, ETV6, PAX5, RAG1, RB1* and *TCF3*.[17-19] For some of these co-occurring genetic lesions, synergistic effects have been reported on leukemia development. For example, loss of *Rag1* was shown to accelerate the onset of B-cell lymphoblastic leukemia in *Cdkn2a/p19Arf*-deficient mice,[20] while increasing the incidence of T-cell lymphomas in *Tcf3*$^{-/-}$ mice.[21] Furthermore, combined heterozygosity for *Ebf1* and *Pax5* results in a strong increase in the frequency of pro-B-cell leukemia in mice.[22]

Herein we describe the impact of deletions affecting the transcriptional coregulator B-cell translocation gene 1 (*BTG1*), in co-occurrence with *IKZF1* loss on both leukemogenesis as well as outcome. We demonstrate that *BTG1* deletions are enriched in *IKZF1*-deleted pediatric BCP-ALL cases, and correlate with increased relapse risk in this patient group. Using mouse knockout models, we further demonstrate that loss of *Btg1* cooperates with *Ikzf1*$^{+/-}$ in the onset of ALL. Finally, our data indicate that both BTG1 and IKZF1 are important determinants of the GC therapy response, and that combined loss of these tumor suppressors enhances GC resistance.

Methods

Clinical samples

The discovery cohort comprised of 533 pediatric patients with newly diagnosed BCP-ALL from three consecutive Dutch Childhood Oncology Group trials (DCOG ALL-8, ALL-9 and ALL-10) and two German Cooperative ALL trials (COALL 06-97 and 07-03). The validation cohort consisted of 515 pediatric patients enrolled in the Australian and New Zealand Children's Haematology and Oncology Group (ANZCHOG) ALL8 protocol. In accordance with the Declaration of Helsinki, written informed consent was obtained from parents or legal guardians, and institutional review boards approved the use of excess diagnostic material for research purposes. Details on the patient cohorts, treatment regimens and outcomes, were described previously.[5,23,24]

Statistics patient data

Cumulative incidence of relapse (CIR) was estimated using a competing risks model, equality of CIRs was tested with Gray's test. Relapse and non-response to induction chemotherapy were considered as events, with death and secondary malignancy as competing events. Event-free survival (EFS) was calculated with non-response, relapse, secondary malignancy and death considered as events. EFS probabilities were estimated using the Kaplan-Meier method, and survival data between groups were compared using univariate and multivariate Cox regression analyses. The proportions of patients with *IKZF1* and other B-cell development gene deletions as well as other categorical variables were compared using the Fisher's exact test. All *P*-values are two-sided, and a significance level of 0.05 or less was considered to be significant. Analyses were performed in R 3.0.1 (2013-05-16), using the packages cmprsk version 2.2-7, mstate version 0.2.7, and survival version 2.37-4.[25-27]

Mice

Btg1 and *Ikzf1* (Ik^{Neo}) knockout lines have been described previously,[28,29] and were intercrossed on a *C57Bl/6J* background. Mice were maintained under specific pathogen-free conditions at our Central Animal Laboratory facility. Genotyping of the offspring was performed by polymerase chain reaction (PCR) (primer sequences are listed in the *Online Supplementary Table S1*). 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.

Functional characterization of murine lymphocytes

Detailed information on functional characterization of normal and leukemic lymphocytes by flow cytometry, immunohistochemistry, immunoglobulin (IG)/T-cell receptor (TR) PCR and cell viability assays can be found in the *Online Supplementary Methods* section. To analyze the glucocorticoid response in B lymphocytes, mononuclear splenocytes were isolated from wild-type and the different knockout mice, and stimulated *in vitro* with 5 μg/mL lipopolysaccharide (LPS) for 48 hours. The obtained activated B lymphocytes (≥ 80% B220^{+}) were isolated by ficoll gradient and cultured for another 48 hours in the absence or presence of the synthetic glucocorticoids prednisolone or dexamethasone. Thereafter, relative cell viability was assessed by MTS assay and AnnexinV/7-AAD staining.

Results

BTG1 deletions are enriched in IKZF1-deleted pediatric BCP-ALL

Gene deletion of the tumor suppressor *IKZF1*, creating either dominant-negative IKZF1 isoforms or haploinsufficiency, is an important predictor of poor outcome in BCP-ALL,[2-5] but to what extent other additional common single gene deletions, such as *CDKN2A/B, PAX5, BTG1, ETV6, EBF1* or *RB1* impact the prognostic value of *IKZF1* has not been clearly established. To address this question, we studied a previously described childhood BCP-ALL cohort

Table 1. Co-occurrence of *IKZF1* deletion with other common gene deletions in BCP-ALL.

	IKZF1 no deletion		*IKZF1* deletion		Fisher's Test	
	n	%	n	%	*P*	Odds ratio
BTG1 no deletion	397	0.93	88	0.84	0.0071	2.5
BTG1 deletion	31	0.07	17	0.16		
PAX5 no deletion	330	0.77	55	0.52	1.40E-06	3.1
PAX5 deletion	98	0.23	50	0.48		
CDKN2A/B no deletion	298	0.70	53	0.50	3.40E-04	2.2
CDKN2A/B deletion	130	0.30	52	0.50		
EBF1 no deletion	405	0.95	96	0.91	0.25	1.6
EBF1 deletion	23	0.05	9	0.09		
RB1 no deletion	400	0.93	91	0.87	0.026	2.2
RB1 deletion	28	0.07	14	0.13		
ETV6 no deletion	296	0.69	80	0.76	0.19	0.7
ETV6 deletion	132	0.31	25	0.24		

of 533 cases enrolled in consecutive DCOG and COALL trials.[5] The representation of the different BCP-ALL subtypes was similar between the DCOG and COALL cohorts, and comparable to that described in the literature (*Online Supplementary Table S2*). We identified 105 BCP-ALL patient samples containing an *IKZF1* deletion. Within the *IKZF1*-deleted group, we observed a significant enrichment for *BTG1* deletions (P=0.007), where 17 of the 105 *IKZF1*-deleted cases (16%) harbored *BTG1* deletions as compared to 31 of the 428 *IKZF1* wild-type cases (7%) (Table 1). These focal *BTG1* deletions mainly covered the second exon of *BTG1* and downstream adjacent sequences, as described previously.[30] Similarly, deletions affecting *PAX5* (P<0.0001), *CDKN2A/B* (P=0.0003) and *RB1* (P=0.026) were present at higher frequencies in *IKZF1*-deleted cases, whilst this was not observed for *EBF1* or *ETV6* deletions (Table 1). To the contrary, in the *BTG1*-deleted group we observed significant enrichment for *IKZF1* (P=0.007), *EBF1* (P=0.0011), *RB1* (P=0.042), and *ETV6* (P=0.0046) deletions (*Online Supplementary Table S3*), which is in agreement with our previous findings.[30] Previously, it has been shown that both *IKZF1* and *BTG1* deletions are strongly enriched in the cytogenetic *BCR-ABL1* subtype.[7,31] Consistent with this notion, we observed that of 24 *BCR-ABL1*-positive cases, 7 (29%) harbored deletions in both *IKZF1* and *BTG1* (Table 2), which represents 47% of *IKZF1*-deleted and 100% of the *BTG1*-deleted cases found in the *BCR-ABL1*-positive group (Table 2).

Combined deletions of BTG1 *and* IKZF1 *predict inferior outcome in BCP-ALL*

We next compared clinical characteristics of *BTG1;IKZF1* double-deleted cases, sole deletion of *IKZF1* and sole deletion of *BTG1* cases, and cases without *IKZF1* or *BTG1* deletion in our complete childhood BCP-ALL cohort. The characteristics of these four groups were similar to the total cohort with respect to gender and treatment protocol (Table 2). The double-deleted and sole deletion of *IKZF1* groups contained more patients over 10 years of age and increased white blood cell counts, and hence more National Cancer Institute (NCI)-Rome criteria high-risk cases[32] (Table 2). We compared the EFS and CIR between cases with both *IKZF1* and *BTG1* deletions (*BTG1*-del;*IKZF1*-del, n=17) with the cases of sole deletion of IKZF1 (*BTG1*-wt;*IKZF1*-del, n=88) (Figure 1A,B). The 5-year CIR in *IKZF1*- plus *BTG1*-deleted cases was 53% ± 13% compared with 28% ± 5% in the cases of sole deletion of *IKZF1* (P=0.005; Table 3A). Similarly, the 5-year EFS was lower in double-deleted cases compared with the cases of sole deletion of *IKZF1* (HR 3.5, P=0.0003; Table 3A). In contrast, the cases of sole deletion of *BTG1* (*BTG1*-del;*IKZF1*-wt, n=31) showed a similar outcome (5-year CIR:10% ± 6%) to the reference cases without *IKZF1* or *BTG1* deletions (n=397; 5-year CIR:13% ± 2%). The synergistic effect of loss of *BTG1* and *IKZF1* on outcome remained after correction for subtype in the Cox model (Table 3A), and after leaving out the *BCR-ABL1*-positive cases (Figure 1C,D; Table 3B). For the *BCR-ABL1*-positive cases, *BTG1* deletions did not further impact the poor treatment outcome as observed for the cases of sole deletion of *IKZF1* (Figure 1E,F; Table 3C).

As deletions of *PAX5*, *CDKN2A/B* and *RB1* were similarly enriched in *IKZF1*-deleted BCP-ALL, we examined the impact of these deletions on the outcome of *IKZF1*-deleted cases. In contrast to *BTG1;IKZF1* double-deleted patients, the outcome of patients with co-occurring *PAX5, CDKN2A/B* or *RB1* deletions did not differ from cases of sole deletion of *IKZF1* (Figure 2; *Online Supplementary Table S4*). Similarly, co-occurrences of *IKZF1* deletions with either *EBF1* or *ETV6* deletions did not affect outcome compared with the sole deletion of *IKZF1*. To validate our findings, we analyzed the Australian and New Zealand ANZCHOG ALL8 cohort (n=515)[23,24] to assess the prognostic value of *BTG1;IKZF1* double-deletions. In this cohort, 6 out of 11 *BTG1*-del;*IKZF1*-del patients developed a relapse (*Online Supplementary Figure S1*). The 5-year CIR in the *BTG1;IKZF1* double-deleted group was 61% ± 19% *versus* 35% ± 6% in the group with the sole deletion of *IKZF1* (P=0.19; Table 3D). Hence, the same trend was observed in this independent validation cohort, albeit statistically non-significant. Together, these data indicate that *BTG1* deletions in an unselected leukemia population have no prognostic value, but *BTG1* copy number losses specifically exacerbate the effects of *IKZF1* deletion on inferior outcome in BCP-ALL.

Table 2. Pediatric BCP-ALL patient characteristics in the *BTG1* and *IKZF1* sole and *BTG1;IKZF1* double-deleted groups.

Patients' characteristics	*BTG1*-del; *IKZF1*-del; n=17 (3%)		*BTG1*-wt *IKZF1*-del n=88 (20%)		*BTG1*-del; *IKZF1*-wt n=31 (6%)		*BTG1*-wt; *IKZF1*-wt n=397 (74%)		Total n=533		Fisher's *P*
	n	%	n	%	n	%	n	%	n	%	
Sex											0.3
Female	5	29%	44	50%	11	35%	183	46%	243	46%	
Male	11	71%	44	50%	20	65%	214	54%	290	54%	
Age (years)											0.0054
< 10	11	65%	60	68%	26	84%	330	83%	427	80%	
≥ 10	6	35%	28	32%	5	16%	67	17%	106	20%	
WBC (cells/nl)											0.066
< 50	11	65%	55	62.5%	23	74%	299	76%	388	73%	
≥ 50	6	35%	33	37.5%	8	26%	96	24%	143	27%	
NCI-Rome											0.016
SR	8	47%	39	44%	19	61%	245	62%	311	58%	
HR	9	53%	49	56%	12	39%	151	38%	221	42%	
Protocol											0.54
DCOG	14	82%	63	72%	25	81%	311	78%	413	77%	
COALL	3	18%	25	28%	6	19%	86	22%	120	23%	
Subtype											
ETV6-RUNX1	2	12%	2	2%	24	77%	126	32%	154	29%	<0.0001
HeH	1	6%	16	18%	2	7%	107	27%	126	24%	0.0055
B-other	4	24%	23	26%	5	16%	75	19%	107	20%	0.41
BCR-ABL1-like	3	18%	38	43%	0	0%	51	13%	92	17%	<0.0001
TCF3r	0	0%	0	0%	0	0%	19	5%	19	4%	0.093
BCR-ABL1	7	41%	8	9%	0	0%	9	2%	24	5%	<0.0001
MLLr	0	0%	1	1%	0	0%	10	3%	11	2%	0.89

WBC count for 2 cases missing, NCI Risk for 1 case missing, both in the *BTG1*-wt ;*IKZF1*-wt group. del: deletion; WBC: white blood cell; NCI-Rome HR: high-risk defined by age at diagnosis ≥ 10 years and/or WBC ≥ 50 cells/nl; HeH: high hyperdiploid (51-65 chromosomes); *P*-value: Fisher's exact test across the four deletions groups; wt: wild-type; DCOG: Dutch Childhood Oncology Group; COALL: German Cooperative ALL; SR: standard-risk.

Leukemia predisposition in Btg1 *knockout mice*

Although deletions affecting the *BTG1* gene are a frequent event in BCP-ALL, a direct role for BTG1 in leukemia development has not been reported. Therefore, we first examined the tumor suppressive function of BTG1 using a constitutive *Btg1* knockout line harboring a Neo-cassette in the first coding exon.[28] We previously reported that these *Btg1* knockout mice display defective B-cell development with a 25% reduction in the amount of progenitor B cells within the bone marrow compartment, mainly affecting the pre-B and immature B-cell stage.[33] Furthermore, *Btg1* is required for optimal proliferative expansion of early progenitor B cells in methylcellulose in response to interleukin-7. At the same time, there was no obvious defect in the development of myeloid and T-lymphoid cells in these *Btg1*-deficient animals.[33] In the study herein, mice that carried either one or two copies of the *Btg1* knockout allele were followed over a period of 18 months, along with control littermates. About 6% (n=3/49) of the wild-type *C57BL6/J* mice developed B-cell lymphomas between the age of 14 and 18 months (Table 4), which is consistent with previous observations.[34] Within the same time period 6% of the $Btg1^{+/-}$ (n=2/34) and 18% of the $Btg1^{-/-}$ (n=6/33) mice developed T-cell leukemia exclusively (Table 4), characterized by enlarged primary lymphoid organs, such as the spleen and lymph nodes, and focal infiltration of leukemic T cells into peripheral organs, such as the lungs and liver. These *Btg1*-deficient T-cell leukemias expressed the T-cell surface marker CD3, and displayed clonal T-cell receptor (TR) rearrangements (Figure 3B). In addition, these $CD3^+$ T-cell leukemias not only showed increased expression of the T-cell activation marker CD44, but also large numbers of $B220^+$ cells within the infiltrated areas of tissues, such as the liver or lungs, and affected lymph nodes (*Online Supplementary Figure S2*). There was no evidence for clonal immunoglobulin gene rearrangements in these $Btg1^{-/-}$ T-cell leukemias (*Online Supplementary Figure S2*), suggesting the presence of a substantial number of non-malignant B lymphocytes in proximity to these leukemic T cells. These data show that, although somatic *BTG1* deletions predominantly occur in BCP-ALL, *Btg1*-deficiency in the mouse germline predisposes exclusively to T-cell malignancies. This predilection for T-lineage leukemias is also observed in other knockout mouse models targeting genes commonly deleted in BCP-ALL, such as *Ikzf1* mutant mice.[35,36]

Loss of Btg1 *increases leukemia incidence in* $Ikzf1^{+/-}$ *mice*

To investigate cooperation between *BTG1* and *IKZF1* during leukemogenesis, we intercrossed *Btg1*-deficient mice with haplodeficient *Ikzf1* mice using the Ik^{Neo} mouse line,[29] which harbors a Neo-floxed knock-in allele combined with a Pax5-IRES-GFP complementary DNA (cDNA) at the first coding exon of *Ikzf1*, thereby creating an *Ikzf1* null allele.[29] These mice are only viable as a heterozygous knockout line ($Ikzf1^{+/-}$). First, we analyzed the phenotype of young animals (age 6-12 weeks) to assess the effect of the $Ikzf1^{+/-}$ allele on *Btg1*-deficiency in B- and T-lymphoid development. $Ikzf1^{+/-}$mice, like $Btg1^{-/-}$ mice, displayed a moderate reduction in the fraction of $B220^+$ cells in bone marrow (BM) and the spleen (*Online*

Table 3. CIR and EFS analysis of *BTG1;IKZF1* double-deleted pediatric BCP-ALL cases compared with cases of sole deleted *IKZF1*.

A BCP-ALL[1] (n=533)				Univariate				Multivariate	
Deletion	Total	Relapse	Death	5-yr CIR (SE)	Gray *P*	EFS HR (95% CI)	Cox *P*	EFS HR (95% CI)	Cox *P*
BTG1;IKZF1	17	10	2	53% (13%)	0.005	3.5 (1.8-6.8)	0.0003	2.5 (1.0-5.9)	0.043
IKZF1	88	25	5	28% (5%)					
BTG1	31	3	0	10% (6%)					
none	397	52	12	13% (2%)					

B BCP-ALL[1] without *BCR-ABL1* (n=509)				Univariate				Multivariate	
Deletion	Total	Relapse	Death	5-yr CIR (SE)	Gray *P*	EFS HR (95% CI)	Cox *P*	EFS HR (95% CI)	Cox *P*
BTG1;IKZF1	10	6	1	50% (17%)	0.011	4.4 (1.9-10.3)	0.0007	6.7 (2.7-16)	0.00003
IKZF1	80	21	2	26% (5%)					
BTG1	31	3	0	10% (6%)					
none	388	52	10	13% (2%)					

C BCP-ALL[1] *BCR-ABL1* only (n=24)				Univariate				Multivariate	
Deletion	Total	Relapse	Death	5-yr CIR (SE)	Gray *P*	EFS HR (95% CI)	Cox *P*	EFS HR (95% CI)	Cox *P*
BTG1;IKZF1	7	4	1	57% (22%)	0.7	0.8 (0.3-2.5)	0.7	ND	ND
IKZF1	8	4	3	50% (21%)					
BTG1	0	0	0						
none	9	0	2	0% (0%)					

D BCP-ALL[2] validation cohort (n=515)				Univariate				Multivariate	
Deletion	Total	Relapse	Death	5-yr CIR (SE)	Gray *P*	EFS HR (95% CI)	Cox *P*	EFS HR (95% CI)	Cox *P*
*BTG1;IKZF1**	11	6	0	61% (19%)	0.19	01.83 (0.8-4.2)	0.16	ND	ND
IKZF1	69	23	4	35% (6%)					
*BTG1**	45	13	0	27% (7%)					
none**	390	55	4	13% (2%)					

[1]Discovery cohort: pediatric patients from consecutive DCOG/COALL trials. [2]Validation cohort: pediatric patients from ANZCHOG ALL8 trial. * Report of 1 secondary malignancy in these groups; ** Report of 6 independent secondary malignancies in this group. 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); none: neither *BTG1* nor *IKZF1* deletion: BCP-ALL: B-cell precursor acute lymphoblastic leukemia; ND: not determined.

Supplementary Figure S3). This correlated with a partial block at the pre-pro-B-cell stage (Hardy fraction A) and the pre-B-cell stage (Hardy fraction D) in *Ikzf1*$^{+/-}$ mice (*Online Supplementary Figure S3*). *Btg1*$^{-/-}$*;Ikzf1*$^{+/-}$ mice showed an even stronger reduction in B220+ cells, with additive effects at both B220^{+}CD43^{+} and B220^{+}CD43^{-} differentiation stages in BM (Hardy fractions A to E) (*Online Supplementary Figure S3*). In contrast, *Btg1*$^{-/-}$*;Ikzf1*$^{+/-}$ mice, similar to *Btg1*$^{-/-}$ and *Ikzf1*$^{+/-}$ single knockout animals, showed no major defects in postnatal thymic T-cell development (*Online Supplementary Figure S4*).

Next, we followed *Ikzf1*$^{+/-}$, *Btg1*$^{-/-}$*;Ikzf1*$^{+/-}$ and *Btg1*$^{-/-}$*;Ikzf1*$^{+/-}$ mice for a period of 18 months. In our cohort, 14% (n=5/36) of the *Ikzf1*$^{+/-}$ mice developed T-cell leukemia between 3 and 18 months of age, with a median age of 12 months (Figure 3A; Table 4). Similar to *Btg1*-deficient leukemia, the *Ikzf1*$^{+/-}$ T-cell leukemias showed infiltration of CD3^{+} leukemic blasts into distant organs and clonal TR rearrangements (Figure 3). Interestingly, we observed an increased leukemia incidence upon combined loss of *Btg1* and *Ikzf1* (Figure 3A), which is consistent with our hypothesis that *BTG1* deletions cooperate with *IKZF1* aberration to induce human BCP-ALL. We found that 26% of the *Btg1*$^{+/-}$*;Ikzf1*$^{+/-}$ mice (n=10/35) and 40% of the *Btg1*$^{-/-}$*;Ikzf1*$^{+/-}$ (n=12/30) animals developed T-cell leukemia, while leukemias in *Btg1*$^{-/-}$*;Ikzf1*$^{+/-}$ appeared with a slightly shorter latency (9.4 months) relative to *Ikzf1*$^{+/-}$ mice (12.4 months) (*P*=0.011) (Table 4). Tumors in the *Btg1*$^{-/-}$*;Ikzf1*$^{+/-}$ compound mice were characterized by significantly higher leukocyte counts in peripheral blood compared to single knockout animals (Figure 3B,C), and strong infiltration of leukemic cells into the liver and lungs (Figure 3B,D), as well as clonal TR rearrangements (Figure 3E). Flow cytometric analysis of the different T-cell leukemias revealed

that most of the *Btg1*$^{-/-}$;*Ikzf1*$^{+/-}$ leukemias were CD4^{+}CD8^{+} double positive T cells with ongoing differentiation towards CD4 or CD8 single positive stage (Figure 3F, *Online Supplementary Table S5*). Moreover, isolated leukemic T cells, derived from all the different genetic backgrounds included in our studies (*Ikzf1*$^{+/-}$, *Btg1*$^{-/-}$ and *Btg1*$^{-/-}$;*Ikzf1*$^{+/-}$), could be serially transplanted into syngeneic C57BL6/J mice giving rise to similar (oligo)clonal T-cell leukemias (*Online Supplementary Figure S5*). Taken together, our data demonstrate that loss of *Btg1* cooperates with haplodeficiency for *Ikzf1* during mouse leukemia development in a dose-dependent manner.

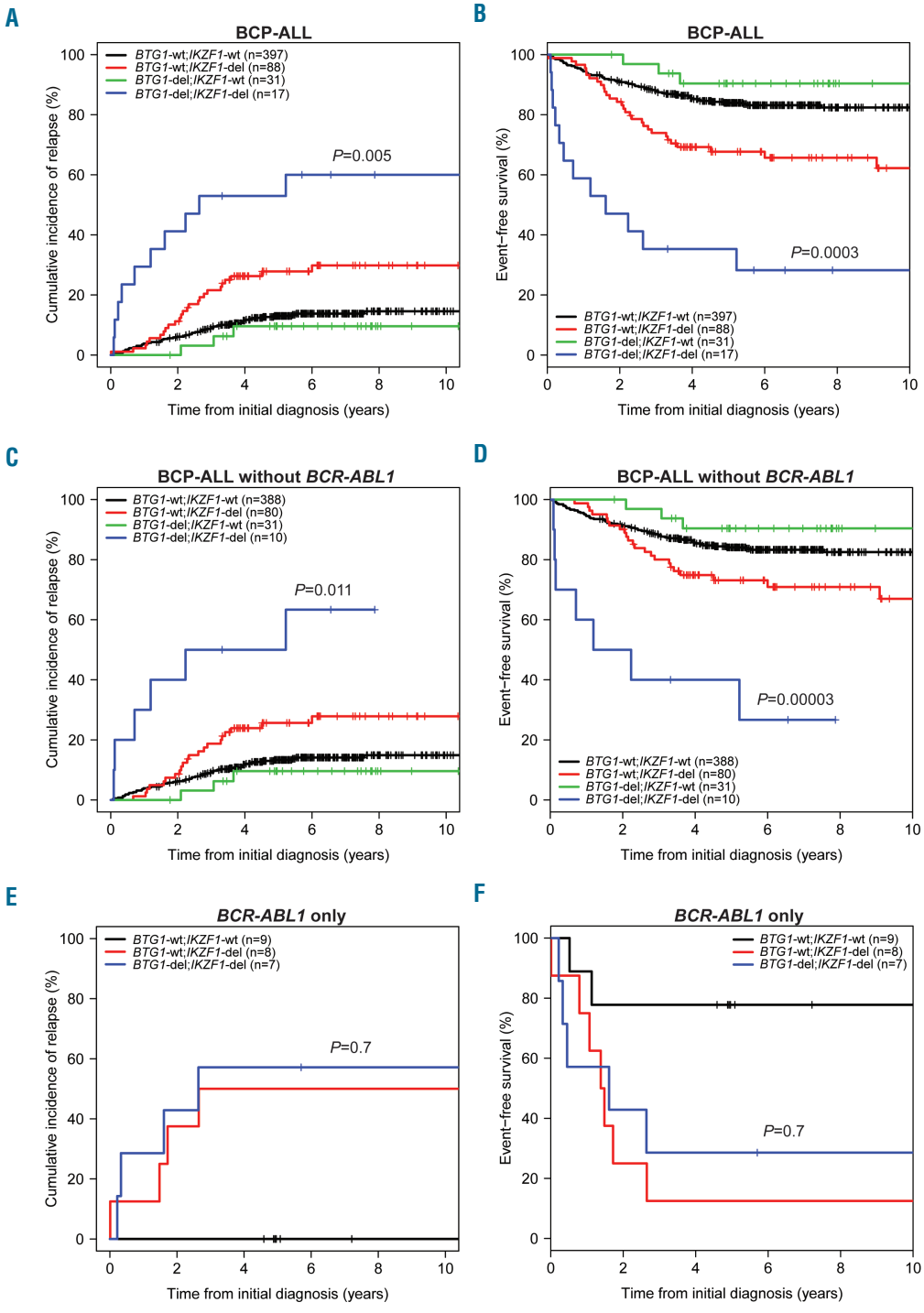

Figure 1. Cumulative incidence of relapse (CIR) and event-free survival (EFS) curves for pediatric BCP-ALL cases with or without *IKZF1* and *BTG1* deletions. (A) CIR and (B) EFS curves for total BCP-ALL cohort (n=533), *IKZF1* plus *BTG1* deletion, (C) CIR and (D) EFS curves for BCP-ALL without *BCR-ABL1*-positive cases (n=509), *IKZF1* plus *BTG1* deletion, (E) CIR (F) EFS curves for *BCR-ABL1*-positive cases (n=24), *IKZF1* plus *BTG1* deletion. Colors: black, *IKZF1* and *BTG1* wild-type; green, *IKZF1* wild-type, *BTG1*-deleted; red, *IKZF1*-deleted, *BTG1* wild-type; blue, both *BTG1*- and *IKZF1*-deleted. For CIR graphs (A,C,E) the Gray *P*-value and for the EFS graphs (B,D,F) the Cox *P*-value is indicated comparing *BTG1*-del;*IKZF1*-del with *BTG1*-wt;*IKZF1*-del. wt: wild-type; del: deletion; n: total number; BCP-ALL: B-cell precursor acute lymphoblastic leukemia.

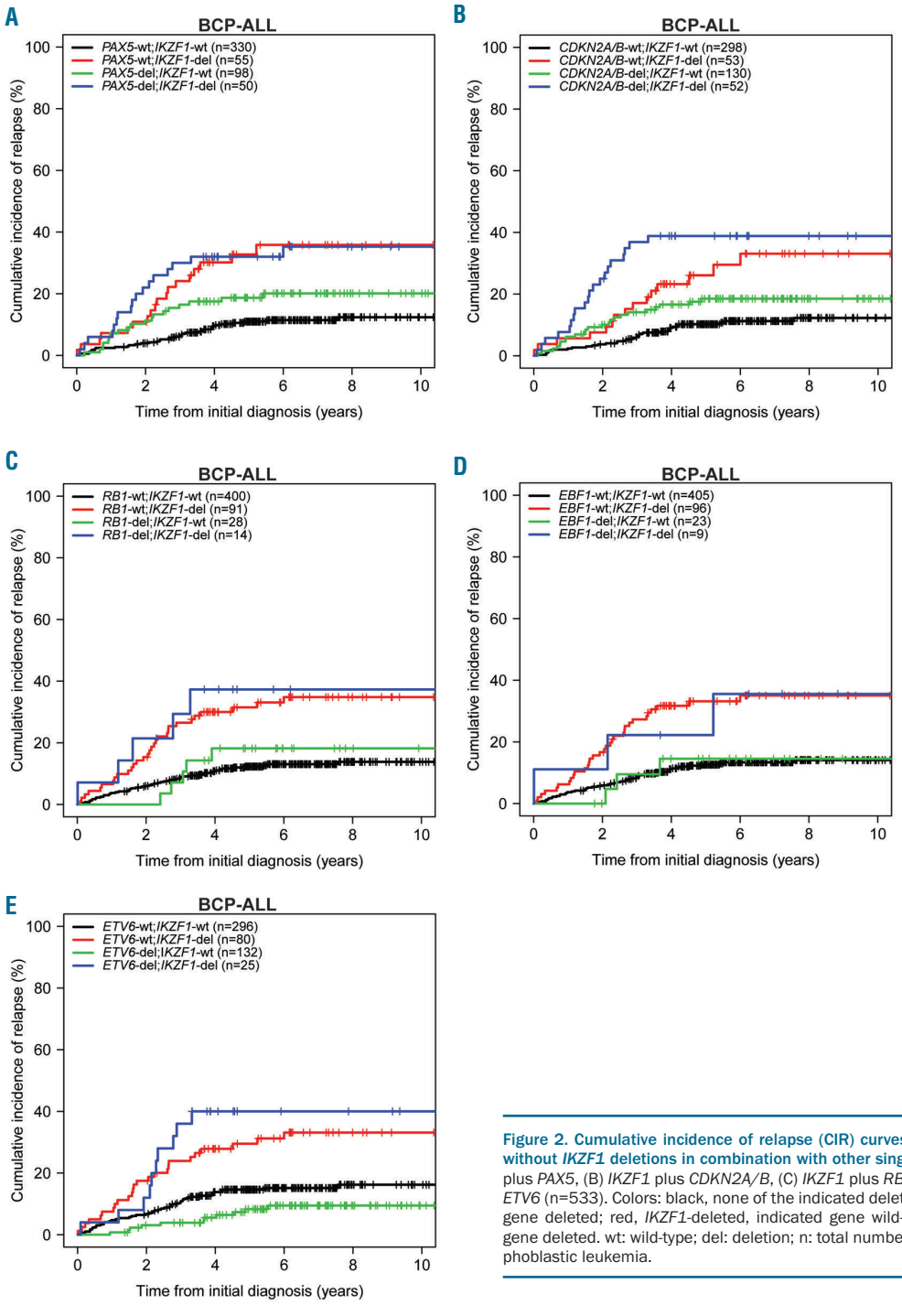

Figure 2. Cumulative incidence of relapse (CIR) curves for pediatric BCP-ALL cases with or without *IKZF1* deletions in combination with other single common gene deletions. (A) *IKZF1* plus *PAX5*, (B) *IKZF1* plus *CDKN2A/B*, (C) *IKZF1* plus *RB1*, (D) *IKZF1* plus *EBF1*, (E) *IKZF1* plus *ETV6* (n=533). Colors: black, none of the indicated deletions; green, *IKZF1* wild-type, indicated gene deleted; red, *IKZF1*-deleted, indicated gene wild-type; blue, both *IKZF1* and indicated gene deleted. wt: wild-type; del: deletion; n: total number; BCP-ALL: B-cell precursor acute lymphoblastic leukemia.

Table 4. Characteristics of lymphoid tumors derived from single and intercrossed *Btg1* and *Ikzf1* knockout lines.

Genotype	Tumor incidence (% mice)	Mean age (months)	Tumor phenotype	*P*-value[a]		
				Ikzf1$^{+/-}$	*Btg1*$^{+/-}$	*Btg1*$^{-/-}$
Wild-type	3/49 (6%)	16.4	B-cell lymphoma	–	–	–
Ikzf1$^{+/-}$	5/36 (14%)	12.4	T-cell leukemia	–	0.271	0.703
Btg1$^{+/-}$	2/34 (6%)	15.8	T-cell leukemia	0.271	–	0.143
Btg1$^{+/-}$;*Ikzf1*$^{+/-}$	10/35 (29%)	13.6	T-cell leukemia	0.134	0.013	0.231
Btg1$^{-/-}$	6/33 (18%)	17.4	T-cell leukemia	0.703	0.143	–
Btg1$^{-/-}$;*Ikzf1*$^{+/-}$	12/30 (40%)	9.4	T-cell leukemia	0.011	0.001	0.026

[a]Chi-square analysis comparing incidence of T-cell leukemia to indicated genotype at 18 months.

BTG1 modifies glucocorticoid resistance mediated by loss of IKZF1

While these experiments confirm the genetic interaction between *BTG1* deletions and *IKZF1* aberrations during leukemogenesis, they do not explain the poor outcome observed in patients showing a combined loss of BTG1 and IKZF1. Recently, we established that inferior outcome related to *IKZF1* deletions in BCP-ALL is correlated with an attenuated *in vivo* day 8 prednisolone response and increased GC resistance in *IKZF1*-deleted primary leukemic cells, as determined by *in vitro* MTT assays.[6] These results could be recapitulated using primary splenic B cells isolated from *Ikzf1*$^{+/-}$ mice, which revealed that non-leukemic *Ikzf1*$^{+/-}$ B cells are also less sensitive towards GC-induced apoptosis.[6] Based on our previous findings that *BTG1* regulates glucocorticoid receptor activation,[37] we

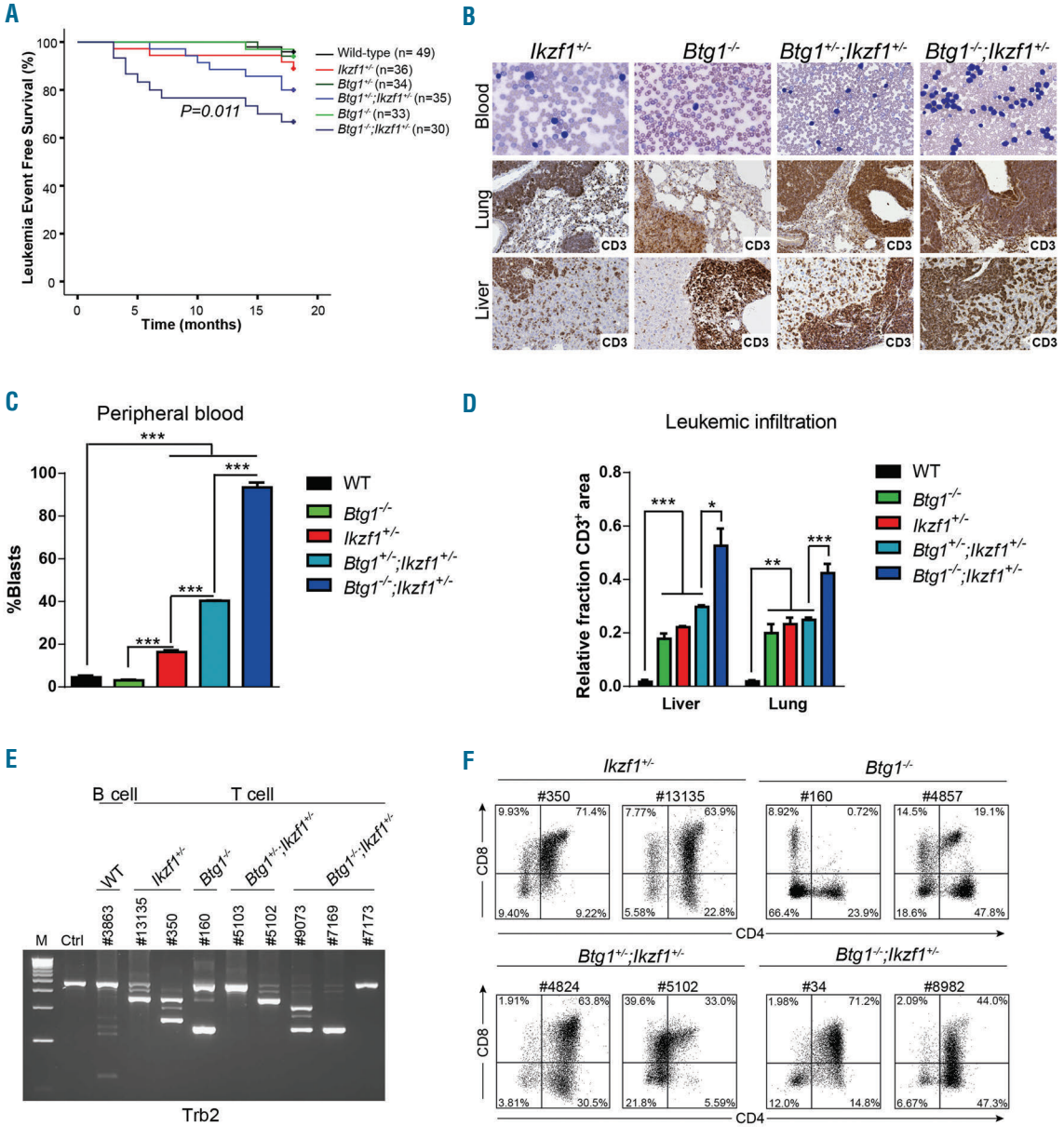

Figure 3. Leukemia incidence and phenotype of *Btg1* knockout mice intercrossed with haplodeficient *Ikzf1* animals. (A) Kaplan-Meier survival curve indicates the leukemia event-free survival in wild-type, *Ikzf1*$^{+/-}$, *Btg1*$^{+/-}$, *Btg1*$^{+/-}$;*Ikzf1*$^{+/-}$, *Btg1*$^{-/-}$, and *Btg1*$^{-/-}$;*Ikzf1*$^{+/-}$ mice over a time period of 17 months. Leukemia incidence is significantly increased in *Btg1*$^{-/-}$;*Ikzf1*$^{+/-}$ mice as compared to *Ikzf1*$^{+/-}$ mice (P=0.011). (B) Peripheral blood smear stained with Giemsa and immunohistochemistry for CD3 on the lung and liver tissues of diseased *Ikzf1*$^{+/-}$, *Btg1*$^{-/-}$, *Btg1*$^{+/-}$;*Ikzf1*$^{+/-}$, and *Btg1*$^{-/-}$;*Ikzf1*$^{+/-}$ mice. (C) Quantification of blast counts in peripheral blood smear stained with Giemsa of diseased wild-type, *Ikzf1*$^{+/-}$, *Btg1*$^{-/-}$, *Btg1*$^{+/-}$;*Ikzf1*$^{+/-}$ and *Btg1*$^{-/-}$;*Ikzf1*$^{+/-}$ mice (n=3). The percentage of leukemic blasts is indicated. ***P<0.001. (D) Quantification of T-cell (CD3) infiltration into the liver and lungs of diseased wild-type, *Ikzf1*$^{+/-}$, *Btg1*$^{-/-}$, *Btg1*$^{+/-}$;*Ikzf1*$^{+/-}$ and *Btg1*$^{-/-}$;*Ikzf1*$^{+/-}$ mice (n=3) using FIJI software. Data are represented as the positively stained area divided by the total area measured, with standard errors of the mean P-values (two-sided t-test). *P<0.05, **P<0.01, and ***P<0.001. (E) T-cell receptor beta 2 (Trb2) gene rearrangement analysis by PCR on control tissue, B-cell lymphoma derived from wild-type mice (#3863), and T-cell leukemias derived from *Ikzf1*$^{+/-}$ (#13135, #350), *Btg1*$^{-/-}$ (#160), *Btg1*$^{+/-}$;*Ikzf1*$^{+/-}$ (#5103 and #5102) and *Btg1*$^{-/-}$;*Ikzf1*$^{+/-}$ mice (#9073, #7169, #7173). (F) Flow cytometry analyzing CD4 and CD8 expression on T-cell leukemia samples of two *Ikzf1*$^{+/-}$, *Btg1*$^{-/-}$, *Btg1*$^{+/-}$;*Ikzf1*$^{+/-}$ and *Btg1*$^{-/-}$;*Ikzf1*$^{+/-}$ mice. WT: wild-type; M: 1 kb DNA ladder marker; Ctrl: control DNA.

investigated whether loss of *BTG1* would impact the GC response in primary murine B cells. To this end, B cells isolated from WT, *Btg1*$^{-/-}$, *Ikzf1*$^{-/-}$ and *Btg1*$^{-/-}$;*Ikzf1*$^{+/-}$ mice, and obtained after lipopolysaccharide activation, were stimulated for 48 hours with increasing concentrations of prednisolone or dexamethasone and subjected to MTS assays to assess relative cell survival. While *Btg1*-deficiency alone had no effect on GC-induced apoptosis, *Ikzf1*-haplodeficient B cells showed enhanced cell survival as compared to WT (Figure 4A), similar to our previous findings.[6] Importantly, *Btg1*$^{-/-}$;*Ikzf1*$^{+/-}$ B cells showed an even stronger resistance to GC-induced apoptosis when compared to *Ikzf1*$^{+/-}$ B cells (P<0.001). These findings were confirmed by AnnexinV staining, demonstrating a significantly smaller apoptotic fraction in *Btg1*$^{-/-}$;*Ikzf1*$^{+/-}$ B cells relative to *Ikzf1*$^{+/-}$ B cells (P<0.001) (Figure 4B). Analyses of primary *Btg1*$^{-/-}$, *Ikzf1*$^{+/-}$ and *Btg1*$^{-/-}$;*Ikzf1*$^{+/-}$ thymocytes revealed no differential sensitivity to GC-induced apoptosis as compared to WT (*Online Supplementary Figure S6A*). Next, we assessed whether loss of *Btg1* and *Ikzf1* would promote resistance in B cells to other chemotherapeutic drugs commonly used in the treatment of BCP-ALL patients, including 6-mercaptopurine, doxorubicin, vincristine and asparaginase. However, *Btg1*$^{-/-}$;*Ikzf1*$^{+/-}$ and *Btg1*$^{-/-}$;*Ikzf1*$^{+/-}$ B cells showed similar cell survival in comparison to control cells (*Online Supplementary Figure S6B*).

Together, these data argue that loss of tumor suppressor BTG1 enhances GC resistance in the context of *IKZF1* deletions, which may explain the inferior treatment outcome observed in patients showing combined loss of BTG1 and IKZF1.

Discussion

BCP-ALL is a heterogeneous disease characterized by recurrent deletions enriched in specific genetic subtypes.[1] For instance, it is known that deletions affecting the transcriptional co-regulator BTG1 are unevenly distributed among cytogenetic subgroups, as we and others have shown that *BTG1* deletions are strongly enriched in *ETV6-RUNX1*-positive leukemia as well as *BCR-ABL1*-positive ALL.[30,31,38] The presence of these lesions in such distinct BCP-ALL subgroups may relate to the fact that deletions of *IKZF1* and *BTG1* appear to be the result of illegitimate RAG recombination,[30] as is the case for several of the other

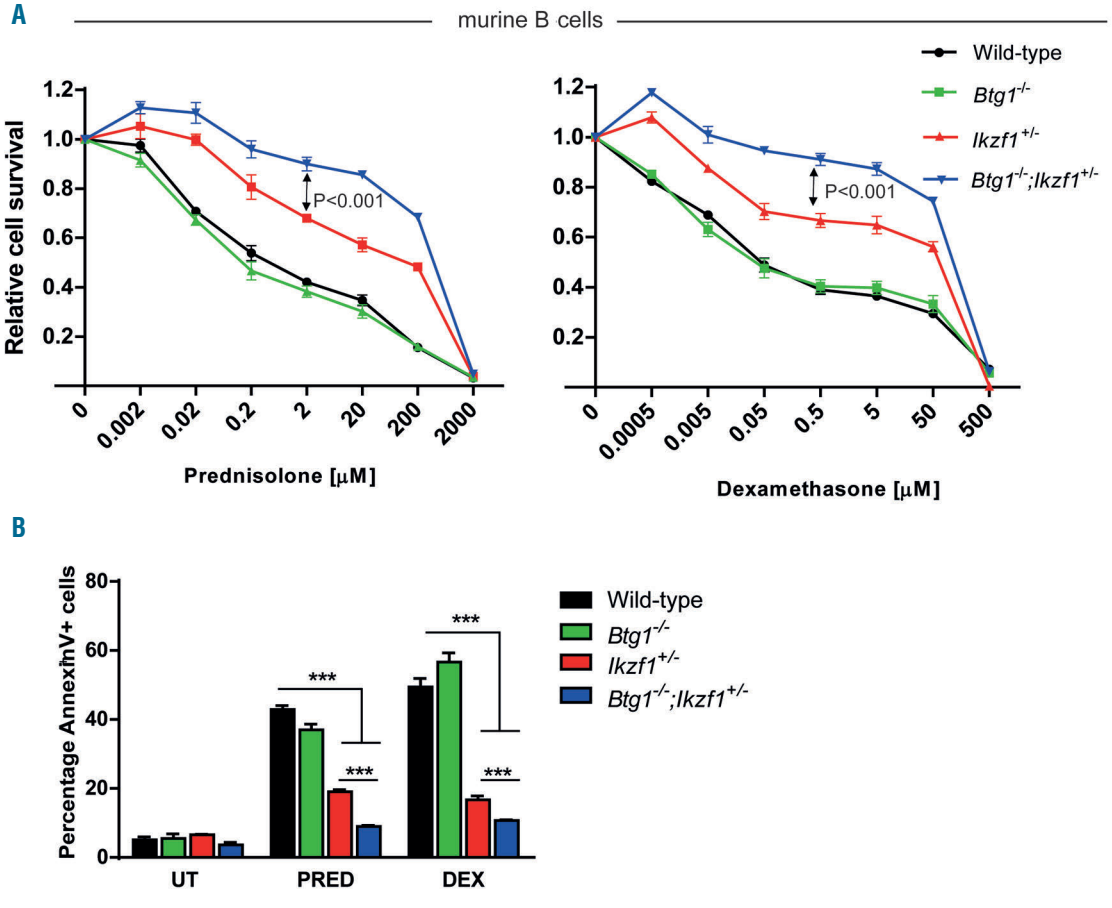

Figure 4. Glucocorticoid resistance of B cells isolated from *Btg1* knockout mice intercrossed with haplodeficient *Ikzf1* animals. (A) Splenic B cells isolated from wild-type, *Ikzf1*$^{+/-}$, *Btg1*$^{-/-}$ and *Btg1*$^{-/-}$;*Ikzf1*$^{+/-}$ mice were activated by LPS for 48 hours and subsequently treated *in vitro* for 48 hours with increasing concentrations of prednisolone (PRED, left panel) or dexamethasone (DEX, right panel) and analyzed by MTS assay (n=6). All values were normalized to untreated (UT) B cells. Error bars represent ± standard error of the mean (SEM). *P*-values were calculated based on the differences of the best-fit curve using two-way ANOVA. (B) AnnexinV/7-AAD staining of WT, *Ikzf1*$^{+/-}$, *Btg1*$^{-/-}$ and *Btg1*$^{-/-}$;*Ikzf1*$^{+/-}$ B-lymphocytes after 2 μM prednisolone or 5 μM dexamethasone treatment for 48 hours (n=4). The fraction of AnnexinV-positive cells was determined. Data represent means, and error bars represent SEM. *P*-values (two-sided *t*-test) are indicated. ***P<0.001.

commonly deleted genes, such as *EBF1* and *PAX5*. In addition to these earlier observations, we find a specific co-occurrence of *BTG1* and *IKZF1* gene deletions across cytogenetic subtypes, suggesting that the combined loss of *BTG1* and *IKZF1* may actively contribute to leukemogenesis. Previous studies, mostly carried out in mouse models, revealed that *Ikzf1* and *Arf* alterations in *BCR-ABL1*-positive ALL synergize to promote the development of leukemia by conferring stem cell-like properties.[39] This leads us to hypothesize that the preponderance of *BTG1;IKZF1* double-deletions in this particular subgroup may have similar consequences, although this remains to be assessed in well-established *BCR-ABL1*-positive mouse models. As *BTG1* and *IKZF1* deletions also (co)occur in lymphoid blast crises of chronic myeloid leukemia (CML),[7,31] it will be interesting to study if and how the combined loss of BTG1 and IKZF1 drive the progression of this disease. Of all the common copy number losses analyzed in our study, including *CDKN2A/B, PAX5, EBF1*, and *RB1*, only loss of *BTG1* appears to worsen the outcome of *IKZF1*-deleted ALL. Our data are consistent with the findings of Moorman *et al*. showing that specific combinations of different deletions impact the outcome in BCP-ALL.[40]

A number of different knockout mouse models have provided insight into the role of commonly deleted transcription factors in early hematopoiesis and spontaneous tumor incidence. It is evident that several of these transcriptional regulators play an important role as lymphoid specification factors and are essential for normal lymphopoiesis.[41-44] However, in the mouse, loss of these early B-cell transcription factors affected in BCP-ALL, such as E2A[45] and IKZF1,[46] gives rise to T-cell malignancies. E2a-deficient tumors are characterized by a strong increase in c-Myc expression,[45] an oncogene known to promote the development of T-cell lymphomas.[47] Similarly, while *IKZF1* deletions predominantly occur in human BCP-ALL, heterozygous *Ikzf1* knockout and dominant-negative *Ikzf1* mice develop T-cell malignancies exclusively, which has been attributed to activation of the Notch pathway.[36,48] In our studies we observed a lower incidence of T-cell leukemia in mice as compared to what has been reported for some other genetically engineered *Ikzf1* mouse models, where expression of dominant-negative isoforms or hypomorphic knockout alleles of *Ikzf1* yielded a higher susceptibility to T-cell malignancies.[35,36,48] Similar to mice heterozygous knockout for *Ikzf1*, *Btg1* knockout mice develop T-cell leukemia, while *BTG1* deletions are almost exclusively found in human BCP-ALL.[30] However, consistent with our finding that monoallelic *BTG1* deletions are enriched in human BCP-ALL cases with *IKZF1* aberrations, $Btg1^{+/-}$;$Ikzf1^{+/-}$ mice are more prone to develop leukemia relative to $Btg1^{+/-}$ single knockout mice (P=0.013). In addition, we observed a significant acceleration in the onset of disease in $Btg1^{-/-}$;$Ikzf1^{+/-}$ mice as compared to $Btg1^{-/-}$ (P=0.026) or $Ikzf1^{+/-}$ mice (P=0.011), indicating that loss of these tumor suppressor genes cooperates during leukemogenesis. Genomic DNA analyses further indicate that both the wild-type *Btg1* allele and *Ikzf1* allele are maintained in the $Btg1^{+/-}$;$Ikzf1^{+/-}$ leukemias (*data not shown*), arguing that *Btg1* and *Ikzf1* dosage contribute to leukemia development. These data confirm that *BTG1* acts as a tumor suppressor gene that cooperates with *IKZF1* loss during leukemia development.

Another important finding in this study is that *BTG1* deletions define a high-risk group within the *IKZF1*-deleted subtype. Our finding that loss of BTG1 specifically enhances GC resistance mediated by *Ikzf1*-haplodeficiency implies that the prognostic value of *BTG1* and *IKZF1* deletions could be dependent on the upfront treatment and dose of synthetic glucocorticoids used. However, this remains to be established in future studies. The relation between *BTG1* deletions and inferior outcome was recently confirmed with the analyses of a relapsed BCP-ALL cohort, showing that *BTG1* and *NR3C1* deletions were associated with a higher risk of disease progression.[49] Collectively, our data demonstrate that BTG1 is a prognostic factor and regulator of the GC response, particularly in the context of *IKZF1*-deletions.

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