Ikaros deficiency is associated with aggressive BCR-ABL1 B-cell precursor acute lymphoblastic leukemia independent of the lineage and developmental origin

The B-cell lineage is established from waves of differentiation that begin in the fetal liver (FL) and continue in the postnatal bone marrow (BM). In mice, FL lymphopoiesis gives rise to B1 cells, which produce natural polyreactive immunoglobulin M (IgM) antibodies and are implicated in innate immunity. Conversely, BM lymphopoiesis favors conventional B2-cell development over B1, and is essential for adaptive immunity.[1,2] The features that distinguish B1- and B2-cell differentiation remain partially understood. Ikaros, encoded by *IKZF1*, is a key transcriptional regulator of B lymphopoiesis in mice and humans.[3] It promotes B2-cell development at multiple stages, including large pre-B-cell (Hardy fraction C') differentiation where it antagonizes IL-7/STAT5 signaling.[4] Ikaros is also a tumor suppressor in B2 progenitors, and deleterious *IKZF1* alterations are prominent in BCR-ABL1$^+$ B-cell precursor acute lymphoblastic leukemias (BCP-ALL) with constitutively active STAT5.[5] While Ikaros is likewise important for fetal and adult B1-cell development, the exact stage where it is required has remained unclear, due to a dearth of in-depth knowledge about B1-cell differentiation. Whether Ikaros acts as a tumor suppressor in B1 cells, or in fetal B cells in general, is currently unknown. Here we show that B1-cell differentiation parallels that of B2 cells in terms of phenotype and cell cycle status. We show that Ikaros promotes B1-cell differentiation at a stage equivalent to fraction C' of B2 cells, by modulating the expression of genes involved in cell proliferation and migration, pre-B-cell receptor (BCR) signaling, Ig-HC rearrangement and IL-7/STAT5 signaling. We also show that Ikaros inhibits the expansion of murine BCR-ABL1$^+$ B1 progenitors from the FL and BM, and suggest that this function might be conserved in human FL-derived B cells.

In order to determine the role of Ikaros in adult B1-cell differentiation, we analyzed the BM of Ik$^{f/f}$Mb-1-Cre$^+$ conditional knockout (cKO) and Ik$^{f/f}$Mb-1-Cre$^-$ wild-type (WT) mice (Figure 1A), where the Cre recombinase is controlled by the endogenous *Cd79a* promoter.[4] While the percentage of WT B1 progenitors (Lin$^-$IgM$^-$CD93$^+$CD19$^+$B220$^{-/lo}$) remained low with age, the same population in the cKO mice was ~20x higher until 4 weeks of age, before decreasing to WT levels by week 16 (Figure 1B). This increase was not due to a compensatory decrease in B2 progenitors, as the percentage of BM B2 progenitors (B220$^+$CD19$^+$CD43$^+$) was stable over time (*Online Supplementary Figure S1A*). Ikaros loss in B1 progenitors was confirmed by intracellular staining (*Online Supplementary Figure S1B*). These results showed that Ikaros is required for differentiation in B1 progenitors from an early pro-B1 cell stage.

In order to pinpoint the stage where Ikaros promotes B1-cell differentiation, we dissected the above B1 progenitor cell population, by analyzing Ig-HC rearrangement, and evaluating CD24 and BP-1 expression, typically used to define B2-cell development.[6] WT or cKO B1 progenitors were heterogenous with unrearranged, germline HC genes, and rearranged DJ as well as proximal and distal VDJ genes (Figure 2A). WT B1 progenitors were mostly CD24$^-$BP-1$^-$ and CD24$^+$BP-1$^-$, resembling Hardy fractions A and B among B2 progenitors (Figure 2B). Interestingly, cKO cells were mainly CD24$^+$BP-1$^+$ and CD24^{++}BP-1$^+$, and resembled B2 fractions C and C'. Hence, B1 progenitors are more heterogenous than pre-

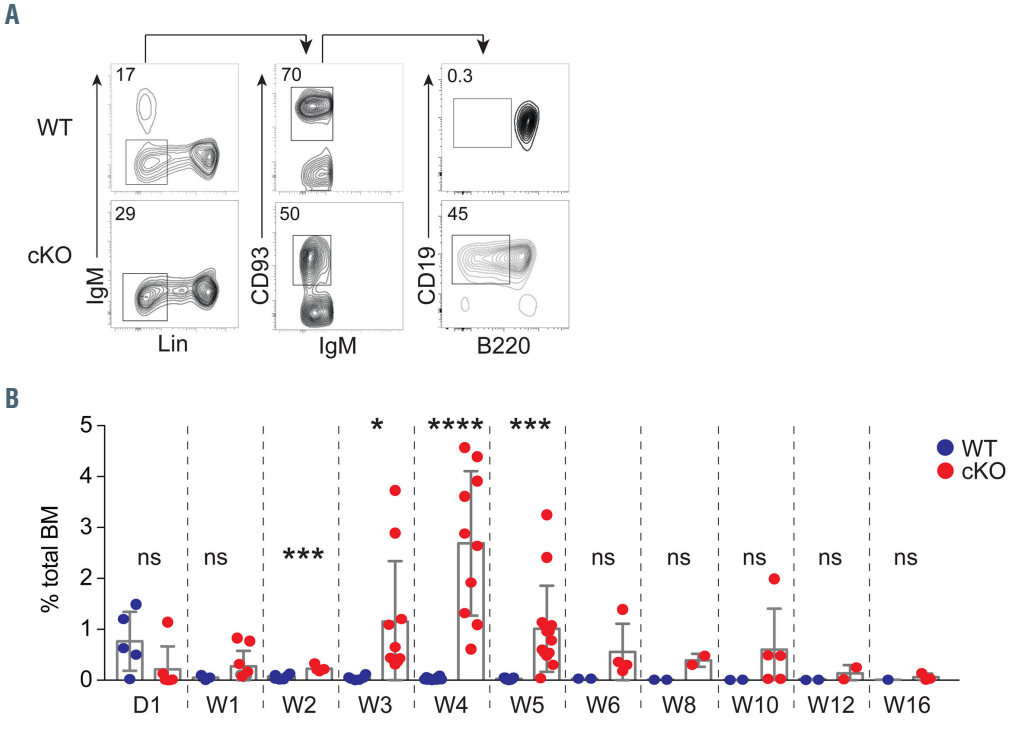


Figure 1. B1 progenitors accumulate in Ikaros conditional knockout bone marrow. (A) Representative flow cytometry analysis of bone marrow (BM) B1 progenitors (Lin$^-$IgM$^-$CD93$^+$CD19$^+$B220$^{-/lo}$) from 4-week-old mice. Numbers correspond to % of parental population. (B) % BM B1 progenitors in wild-type (WT) or conditional knockout (cKO) mice with age (D: days; W: weeks after birth). Significance was calculated using unpaired Student's *t*-test. ns: not significant; *P<0.05; ***P<0.001; ****P<0.0001. Ik$^{f/f}$Mb-1-Cre mice[4] were housed in specific pathogen-free (SPF) conditions or kept in single-ventilated cages. IgM: immunoglobulin M.

viously described, and correspond to phenotypic stages like those of B2 fractions.[7] These results suggested that Ikaros deficiency blocks B1-cell differentiation at the fraction C' stage, similar to its effect on B2-cell development, using the same Cre transgene.[4]

In order to evaluate the molecular pathways regulated by Ikaros, we analyzed the gene expression changes between WT and cKO BM B1 progenitors by RNA sequencing (RNA-seq) (*Online Supplementary Figure S2*). Pathway enrichment and gene set enrichment analyses (GSEA) associated Ikaros with cell migration, adhesion, proliferation, and B-cell differentiation and maturation (Figure 2C and D). As Ikaros antagonizes STAT5 binding to DNA in pre-B2 cells, we asked if Ikaros has a similar

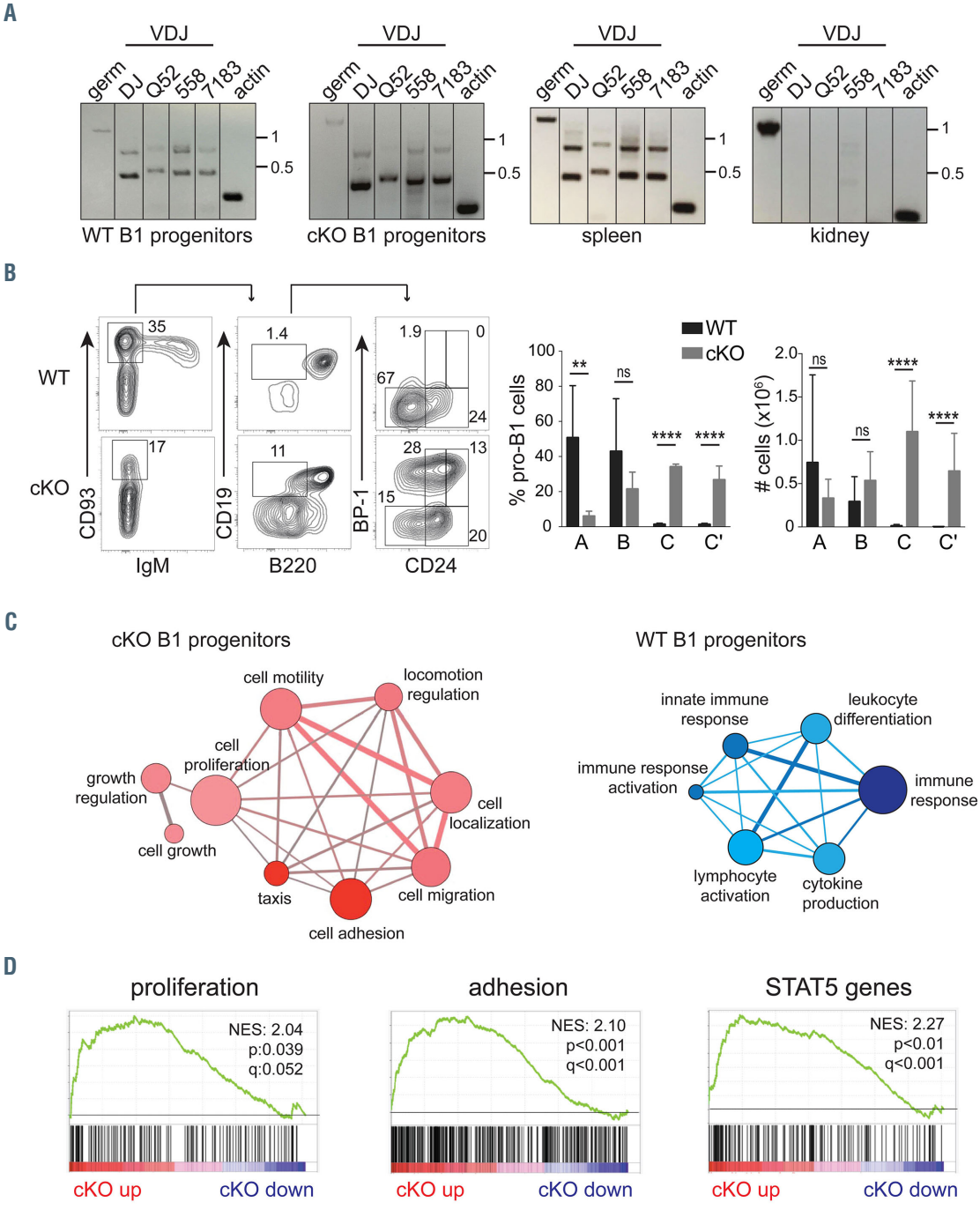


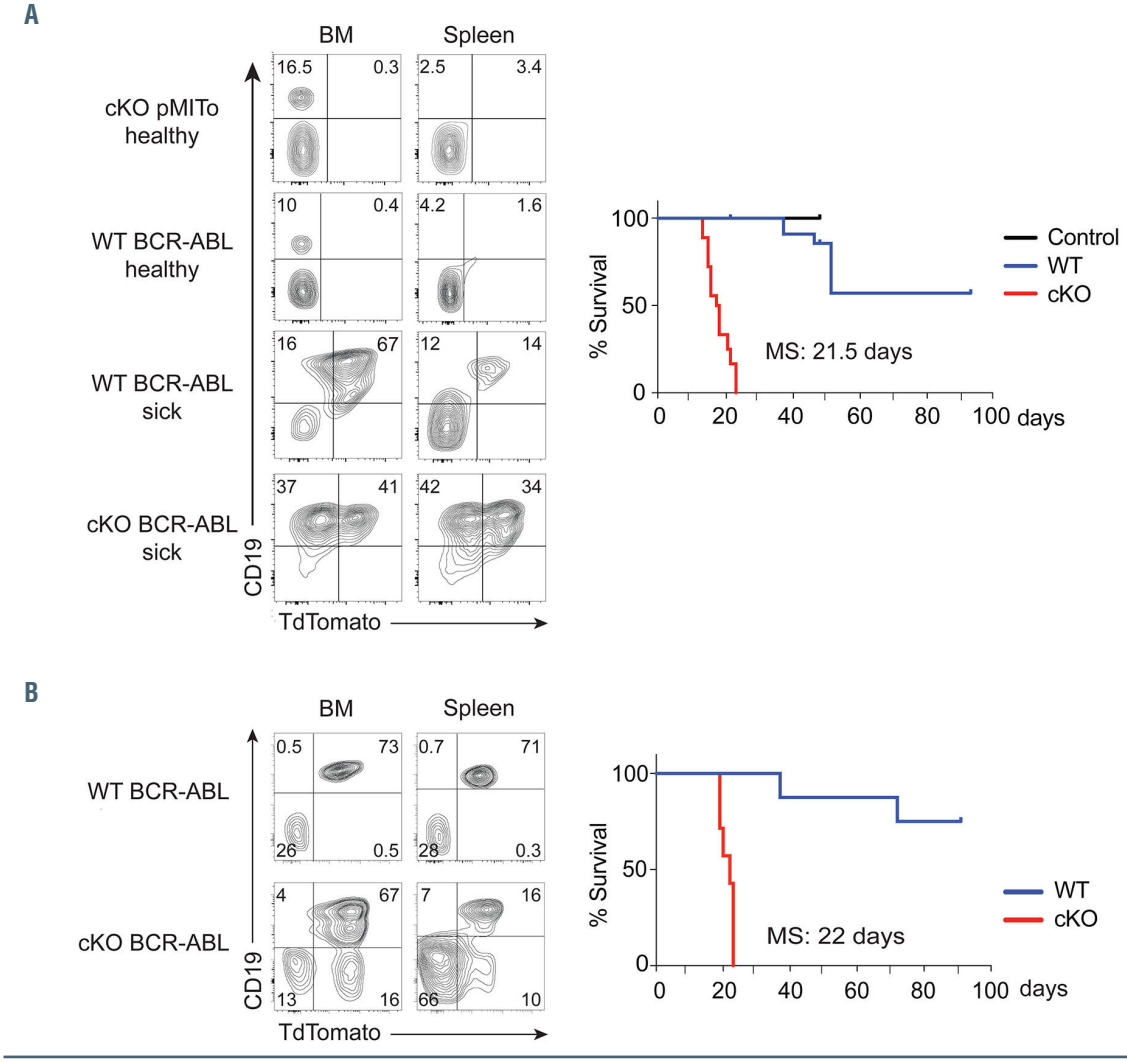
Figure 2. Ikaros deficiency in B1 progenitors induces a differentiation block at fraction C' and deregulation of genes involved in B-cell differentiation. (A) VDJ rearrangement as indicated, following published protocols.[4] Spleen and kidney cells were used as positive or negative controls, respectively. Germ: germline configuration. Numbers correspond to kilobases. (B) Bone marrow (BM) cells were analyzed for different B-cell stages by flow cytometry: B1 progenitors (IgM$^-$ CD93$^+$CD19$^+$B220$^{-/lo}$) were further gated on CD24 and BP-1 to give fraction (Fr.) A (CD24$^-$BP-1$^-$), Fr. B (CD24$^+$BP-1$^-$), Fr. C (CD24$^+$BP-1$^+$), Fr. C' (CD24$^+$BP-1^{++}). Graphs show frequencies (left) and absolute numbers (right) of B progenitor fractions as indicated. Data from 3 independent experiments with 5 mice for wild-type (WT) and 7 for conditional knockout (cKO). ns: not significant; **P<0.01; ****P<0.0001 (unpaired Student's t-test). (C) RNA sequencing (RNA-Seq) data of BM B1 progenitors from 3 WT and 3 cKO mice (4 weeks old) were evaluated by pathway enrichment analysis (http://metascape.org)[15] for genes upregulated in cKO (left) or WT B1 progenitors (right). (D) Gene set enrichment analysis (GSEA) of 3 gene sets as indicated, compared with genes upregulated (red) or down-regulated (blue) in cKO *vs.* WT B1 progenitors. The STAT5 gene set corresponds to STAT5 genes antagonized by Ikaros.[8] NES: normalized enrichment score; *P*: *P*-value; q: false discovery rate (FDR) q-value.

function in B1 progenitors.[8] GSEA of STAT5-induced genes antagonized by Ikaros in pre-B2 cells revealed their enrichment among the upregulated genes in Ikaros deficient B1 cells, suggesting that Ikaros also antagonizes STAT5 gene activation in B1 progenitors. Thus, Ikaros appears to affect similar biological pathways in B1 and B2 progenitors.

That Ikaros deficiency correlates with increased STAT5-activated gene expression suggests that Ikaros may act as a tumor suppressor in B1 cells. We transduced BM WT and cKO B1 progenitors to express the BCR-ABL1 oncoprotein, or an empty vector (pMITo), carrying a TdTomato (TdTo) reporter. Transduction efficiency was assessed and BCR-ABL1 expression was confirmed by western blot (*Online Supplementary Figure S3A and B*). TdTo$^+$ cells were injected into NSG mice and the health status was monitored over time. All mice that received cKO B1 BCR-ABL1$^+$ cells developed BCP-ALL, as characterized by BM failure (anemia, thrombocytopenia, splenomegaly) (*Online Supplementary Figure S3C*), which was sometimes associated with hepatomegaly and neurological impairment. Blast cell (CD19$^+$TdTo$^+$) infiltration was observed in the BM and spleen (Figure 3A). In contrast, only 25% of mice transplanted with WT B1 BCR-ABL1$^+$ cells developed BCP-ALL, with slower kinetics and fewer symptoms (Figure 3B). We also analyzed symptom-free mice that received WT B1 BCR-ABL1$^+$ or cKO B1 pMITo$^+$ cells, but did not detect CD19$^+$TdTo$^+$ cells in the BM or spleen (Figure 3A). The fact that not all mice that received WT B1 BCR-ABL1$^+$ cells developed leukemia, as previously described, may be due to the limiting numbers of cells injected here.[9] Our results therefore indicated that Ikaros limits the ability of BM B1 progenitors to develop BCR-ABL1-induced BCP-ALL.

In order to determine if Ikaros also functions as a tumor suppressor in fetal-derived B1 cells, we transduced WT and cKO FL B1 progenitors (from E17-E18 organs) to express BCR-ABL1 and injected them into NSG mice. Fetal B1 progenitors gave rise to BCP-ALL similar to BM B1 cells. Ikaros deficiency was associated with a higher tumor burden and decreased survival (Figure 3B). These results demonstrated that Ikaros reduces BCP-ALL development in B1 progenitors.

Lastly, we asked if Ikaros loss is implicated in human B1-like BCP-ALL. Because human B1 cells are ill-defined, we first compared a published set of genes, differentially expressed between human B1- and non-B1-like BCP-ALL, with our available human BCP-ALL RNA-seq dataset, but this did not identify clear subgroups among samples.[10] We then separated a collection of 370 pediatric and 102 adult BCP-ALL samples into B1-like and non-B1-like groups according to Vh usage, and further



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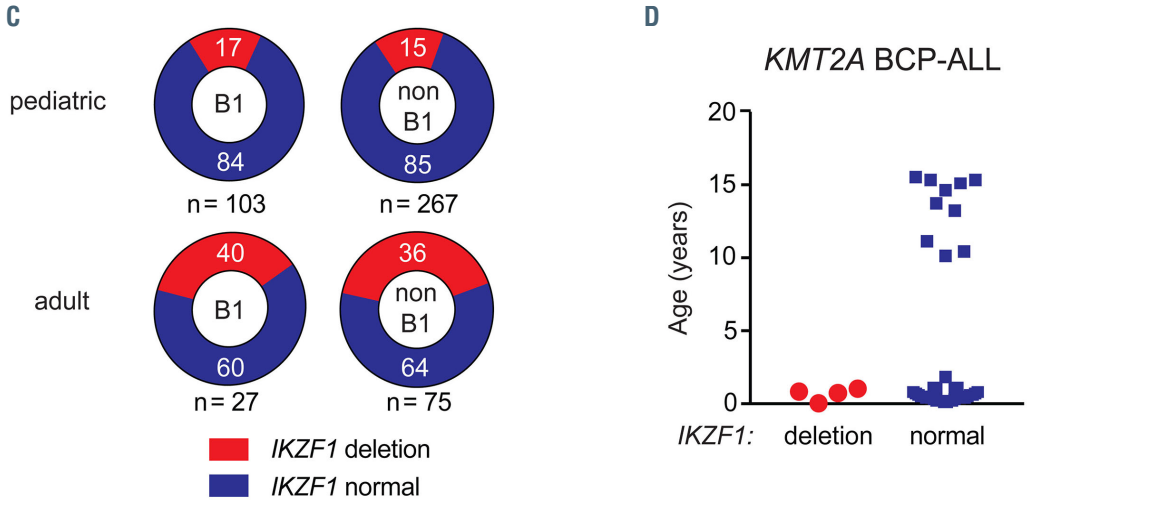

Figure 3. Loss of Ikaros in BCR-ABL1 transformed B1 progenitors of adult and fetal origin results in aggressive leukemia. (A) Bone marrow (BM) B1 progenitor cells were retrovirally transduced with BCR-ABL1-IRES-TdTomato (BCR-ABL) or the empty vector (pMITo). NSG mice were intraveously injected with an equivalent of 10^4 TdTo$^+$ cells as indicated. Recipient BM and spleen cells were analyzed by flow cytometry for CD19$^+$TdTo$^+$ cells when the mice showed signs of sickness ("sick" conditional knockout [cKO] BCR-ABL=29 days, sick wild-type [WT] BCR-ABL=56 days after transplantation) or at the corresponding "healthy" timepoints. The graph on the right shows the survival of NSG mice transplanted with cKO B1 progenitors pMITo$^+$ (control), WT B1 progenitors BCR-ABL1$^+$ (WT) or cKO B1 progenitors BCR-ABL1$^+$ (cKO). The graph represents the results of 4 independent experiments with 16 WT, 14 cKO and 3 control mice. MS: median survival. (B) FL B1 progenitors were transduced with a BCR-ABL1-IRES-TdTomato construct. NSG mice were injected with an equivalent of 10^4 TdTo$^+$ cells. BM and spleen cells from sick mice were analyzed for CD19$^+$TdTo$^+$ cells. The survival curve of 2 independent experiments with 8 WT and 7 cKO mice is shown on the right. MS: median survival. Ik$^{f/f}$Mb-1-Cre and NSG mice were housed in specific pathogen-free (SPF) conditions or kept in single-ventilated cages. All experiments were approved (APAFIS reference #9742-2017042718317841 v6T). (C) Vh usage of pediatric (upper graphs) and adult (lower graphs) B-cell precursor acute lymphoblastic leukemia (BCP-ALL) samples were analyzed according to Griffin *et al*[11] and classified into B1-like (B1) and non-B1-like (nonB1), and further divided into samples with normal or deleted *IKZF1*. Numbers in the bars represent the % patients with the indicated *IKZF1* status. Numbers at the bottom of the bars indicate the numbers of patients analyzed. (D) Pediatric BCP-ALL samples with *KMT2A* rearrangements were grouped according to *IKZF1* status and displayed as a function of patient age. Pediatric data were obtained from the Genetics Department of the Robert Debré Hospital (Paris, France), and adult data from the Genome and Cancer Department of the Saint Louis Hospital (Paris, France). Informed consent was obtained according to the Helsinki Declaration.

analyzed them for *IKZF1* deletions (gross chromosomal deletions of chromosome 7, -7p, intragenic deletions) (Figure 3C). In both children and adult samples, the ratio of mutant to functional *IKZF1* samples was similar between B1- and non-B1-like BCP-ALL.[11] Since *IKZF1* alterations are frequent in BCR-ABL1$^+$ BCP-ALL, we checked if BCR-ABL1$^+$ leukemias were enriched in B1-like cases. Interestingly, there was a significant enrichment of BCR-ABL1$^+$ cases within the B1-like group when compared with the non-B1 group in both pediatric (21% *vs*. 13.4%) and adult (67% *vs*. 45%) patients (P<0.05 in both cases; hypergeometric test). Among the pediatric samples, 35 came from infants <1 year of age, and most (34/35) contained *KMT2A* rearrangements, considered to occur *in utero*;[12] four of these samples exhibited an *IKZF1* deletion (Figure 3D). Thus, our results in mice and humans showed that Ikaros loss is associated with BCP-ALL development in B cells of both fetal and adult origin.

In conclusion, our study indicates that murine B1-cell development can be divided into phenotypically discrete stages that resemble the Hardy fractions A-C' currently used to evaluate B2-cell differentiation.[6] These newly identified populations will allow further investigation into the requirements of B1-cell development and the factors involved. We show that Ikaros is required at the fraction C' stage of B1-cell differentiation as observed for B2 progenitors, suggesting similar regulation of gene expression, particularly in antagonizing genes regulated by IL-7/STAT5.[8] Our results also suggest that Ikaros functions as a tumor suppressor by repressing STAT5 activity in B-cell progenitors of fetal and adult origin. Whether the B1-cell equivalent exists in humans is still unclear. However, human fetal B cells and murine B1 progenitors are alike in some ways. Infant and pediatric BCP-ALL cells often co-express lymphoid and myeloid markers;[13] similarly, murine B1 cells have been reported to be bipotent and can develop into both B cells and macrophages.[14] While our results do not address the validity of human B1 cells, we found *IKZF1* alterations in both B1- and non-B1-like leukemias of children and adults, including those with fetus-associated ETV6-RUNX1 translocations and *KMT2A* rearrangements. Thus, Ikaros loss-of-function is associated with BCP-ALL development in patients of all ages. It will be interesting in future studies to determine if B1- and non-B1-like cases are associated with different outcomes, and if this parameter could improve the risk classification of BCP-ALL patients.

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