Infection Exposure Is a Causal Factor in B-cell Precursor Acute Lymphoblastic Leukemia as a Result of Pax5-Inherited Susceptibility

Alberto Martín-Lorenzo1,2, Julia Hauer3, Carolina Vicente-Dueñas1,2, Franziska Auer3, Inés González-Herrero1,2, Idioia García-Ramírez1,2, Sebastian Ginzel1,4, Ralf Thiele4, Stefan N. Constantinescu5, Christoph Bartenhagen6, Martin Dugas6, Michael Gombert3, Daniel Schäfer3, Oscar Blanco8, Andrea Mayado6, Alberto Orfao9, Diego Alonso-López9, Javier De Las Rivas9,10, César Cobaleda11, María Begoña García-Cenador12, Francisco Javier García-Criado12, Isidro Sánchez-García1,2, and Arndt Borkhardt3

ABSTRACT

Earlier in the past century, infections were regarded as the most likely cause of childhood B-cell precursor acute lymphoblastic leukemia (pB-ALL). However, there is a lack of relevant biologic evidence supporting this hypothesis. We present in vivo genetic evidence mechanistically connecting inherited susceptibility to pB-ALL and postnatal infections by showing that pB-ALL was initiated in Pax5 heterozygous mice only when they were exposed to common pathogens. Strikingly, these murine pB-ALLs closely resemble the human disease. Tumor exome sequencing revealed activating somatic, nonsynonymous mutations of Jak3 as a second hit. Transplantation experiments and deep sequencing suggest that inactivating mutations in Pax5 promote leukemogenesis by creating an aberrant progenitor compartment that is susceptible to malignant transformation through accumulation of secondary Jak3 mutations. Thus, treatment of Pax5+/− leukemic cells with specific JAK1/3 inhibitors resulted in increased apoptosis. These results uncover the causal role of infection in pB-ALL development.

SIGNIFICANCE: These results demonstrate that delayed infection exposure is a causal factor in pB-ALL. Therefore, these findings have critical implications for the understanding of the pathogenesis of leukemia and for the development of novel therapies for this disease. Cancer Discov; 5(12); 1328–43. ©2015 AACR.

See related commentary by Greaves and Müschen, p. 1244.
INTRODUCTION

Childhood B-cell precursor acute lymphoblastic leukemia (pB-ALL) is the most common cancer in childhood. The overall cure rate is excellent (approximately 90%); however, treatment is associated with severe toxic side effects and long-term sequelae, and 20% of children still relapse and may later succumb to their disease. Preventional strategies are clearly superior to any therapy improvement. The prerequisite to develop these strategies is to discover the etiology of pB-ALL (1). Somatic alterations of the lymphoid transcription factor gene PAX5 are a hallmark of pB-ALL (2–4), and recent discoveries of inherited mutations of PAX5 in a new syndrome of susceptibility to pre–B-cell neoplasia have extended the role of PAX5 alterations in the pathogenesis of pB-ALL (5, 6). The presence of the inherited mutations of PAX5 seems to produce a persistent and hidden preleukemic clone that may convert to pB-ALL in only 30% of the family members who carry the mutation (5). Thus, the PAX5 syndrome shows incomplete penetrance, and the mechanisms responsible for the conversion of the preleukemic clone into full-blown pB-ALL are not known.

Over the past century, infections have been regarded as the most likely cause of childhood leukemia (7). In 1988, two specific hypotheses were proposed that implicated infection as an important causal factor in childhood leukemia: the Kinlen “population-mixing” hypothesis (8) and the “delayed-infection” hypothesis (9). Although the two hypotheses differ in detail and hypothetical mechanism, they do share common ground, as both postulate that childhood leukemia is a consequence of a rare response to common infections (1). These studies suggested that the lack of timely exposure to infections in postnatal life in the clean environments of developed societies might predispose the immune system to aberrant or pathologic responses following subsequent or “delayed” exposure to common pathogens. Descriptive epidemiologic studies support infection as a causal factor in childhood leukemia (10, 11). However, there is no evidence supporting delayed exposure to infection as a second hit related to the natural history of the disease with prenatal initiation (1). Integrating these conflicting data has proven challenging.

In this work, we have explored the role of infections in the development of pB-ALL using a Pax5 heterozygous model, as recently germline mutations in PAX5 have been described as conferring an inherited risk to pB-ALL in three kindreds from different ethnic backgrounds (5, 6). We present in vivo genetic evidence mechanistically connecting inherited susceptibility to pB-ALL and postnatal infections by showing that pB-ALL was initiated in Pax5+/- mice only when they were exposed to common pathogens. Strikingly, these pB-ALLs closely resemble the human disease in pathology, genomic lesions, and leukemia-associated transcripts. Transplantation experiments and deep sequencing suggest that inactivating mutations in Pax5 promote leukemogenesis by creating an aberrant progenitor compartment that is susceptible to malignant transformation through the accumulation of secondary Jak3 mutations. Therefore, this model proves for the
first time that delayed exposure to infection triggers pB-ALL development.

RESULTS
Exposure to Common Pathogens Generates pB-ALL in Pax5⁻/⁻ Mice

Recently, germline mutations in PAX5 have been described as conferring an inherited risk for pB-ALL in three kindreds from different ethnic backgrounds (5, 6). Pax5 is required for normal B-cell development (12–15), although Pax5 heterozygous mice do not spontaneously develop pB-ALL (16). Thus, we initially asked if we could provoke pB-ALL development in Pax5⁻/⁻ mice by exposing them to common pathogens. To test this hypothesis, the first group was composed of Pax5⁻/⁻ mice and control littermate wild-type (WT) mice born and kept in a specific pathogen-free (SPF) environment during their lifespan. These Pax5⁻/⁻ mice did not spontaneously develop pB-ALL after 2 years, corroborating previous results (16, 17). The second group was composed of Pax5⁻/⁻ mice and control littermate WT mice born and kept in the SPF environment until moved to a common infectious environment (Fig. 1A). The microbiologic status in the conventional facility was defined and controlled for 2 years (Supplementary Table S1; Supplementary Fig. S1). Under this scenario, specific pB-ALL development was observed in 22% (9 of 41) of Pax5⁻/⁻ animals but not in WT mice (Fig. 1B, C, and D), closely resembling the low penetrance of pB-ALL development in the families with the heterozygous Pax5 c.547G>A mutation (5, 6). The appearance of leukemia in Pax5⁻/⁻ mice occurred between 6 and 16 months of age. This is independent of a crucial time point of exposure to infection, as pB-ALL appeared in mice that were transferred to a conventional animal facility between 2 and 5 months of age. These pB-ALLs became manifest with splenomegaly, disruption of splenic architecture due to blast infiltration, and appearance of blast cells in the peripheral blood (PB; Fig. 1C). FACS analysis revealed a cell surface phenotype of CD19⁻/⁺ B220⁻/⁺ IgM⁻/⁺ cKit⁺/− CD25⁻/⁺ for tumor cells that extended through bone marrow (BM), PB, spleen, and lymph nodes (Fig. 1D; Supplementary Fig. S2A and S2B) and infiltrated nonlymphoid tissues like liver, kidney, and lung (Supplementary Fig. S2C). All pB-ALLs displayed clonal immature B-cell receptor (BCR) rearrangement (Fig. 1E). To explore the relevance of our findings for human leukemia, we next identified 4,511 significantly repressed and 3,929 significantly induced genes (FDR = 0.005) in tumor-bearing BM from Pax5⁻/⁻ mice compared with BM-derived B220⁺ cells from WT mice (Fig. 2A, left; Supplementary Table S2), including increased expression of genes with roles in progenitor and stem cell compartments of BM and with significant enrichment in human B-ALL gene sets (refs. 18, 19; Gene Set Enrichment Analysis (GSEA) FDR = 0.000; FDR = 0.000) as well as significant alignment with the pro-B-cell stage of differentiation (refs. 20, 21; GSEA up genes FDR = 0.000; down genes FDR = 0.000; Supplementary Fig. S2D). In order to rule out that the heterogeneity of the control B cells could also contribute to the observed differential gene expression, we characterized the global expression signature of purified WT pro/pre-B cells and compared with the expression signature of Pax5⁻/⁻ leukemias. The analysis showed a similar differential gene expression profile (Fig. 2A, right).

The universal finding of deletion of the WT PAX5 allele and retention of the hypomorphic PAX5 c.547G>A allele in human pB-ALL suggests that very reduced activity of WT PAX5 is required to establish the leukemic clone (6). Thus, we next investigated the status of Pax5 in mouse pB-ALL. The majority of the murine Pax5⁻/⁻ pB-ALL (5/9; 55.6%) did not express CD19 (Table 1), suggesting loss or marked reduction of Pax5 activity, which is in agreement with gene expression analysis of murine Pax5⁻/⁻ tumors showing reduced transcriptional activity of PAX5, similar to what is found in human leukemia (refs. 5, 6; Supplementary Fig. S2E; Supplementary Tables S2 and S3). In this sense, whole-exome sequencing analysis of 8 of 9 Pax5⁻/⁻ pB-ALL identified two cases involving the acquisition of an additional Pax5 mutation, which were the presence of a Pax5 variant, p.Pro80Arg (2), causing reduced Pax5 transcriptional activity in humans and a second Pax5 variant, p.Pro80Leu. However, our finding that 4 murine pB-ALL tumor cells expressed CD19 (Table 1) suggests that a complete loss of PAX5 activity is not required to establish the leukemic clone in a significant percentage of cases. In addition, in 100% of cases these human pB-ALLs displayed homozygous loss of the CDKN2A (p19ARF/INK4A) tumor suppressor locus (5, 6). In 2 of 5 murine pB-ALLs, p19ARf expression was absent (Fig. 2B), correlating in one mouse (O361) with genomic loss of the Cdkn2a locus (Fig. 2C). Overall, these results provide evidence that this murine model closely reproduces the human disease phenotype with respect to clinical and molecular/genetic aspects. It represents the first proof that delayed exposure to infection can induce human-like pB-ALL in mice with an inherited genetic predisposition at reduced penetrance.

Exposure to Infection in Pax5⁻/⁻ pB-ALL Development

To identify the types of pathogens in the conventional facility, we monitored the health status of the respective animals. In the SPF facility, animals are pathogen free according to the serological analysis carried out (Supplementary Table S1). When transferred to the conventional animal facility, mice are exposed to a variety of pathogens, among them murine norovirus, mouse hepatitis virus, helicobacter species, and trichomonas muris (Supplementary Fig. S1), inciting a significant immune response in the animals. In order to exclude insertional mutagenesis and leukemia development on the basis of viral integration, we carried out whole-genome sequencing of three Pax5⁻/⁻ pB-ALLs. This experimental approach was very sensitive as it was able to detect a single 127 bp integration into exon 2 of the Pax5 locus in all three tumor samples and control mice. This is the SV40 nuclear localization signal inserted together with the targeting vector into Pax5 exon 2 for generation of the Pax5⁻/⁻ mice (15). The sequence serves as positive control to prove this approach is able to identify a small single virus copy within the genome. In addition, we did not observe viral integration close to proto-oncogenes ruling out pB-ALL development based on insertional mutagenesis, providing evidence against direct viral transformation in agreement with observations in human pB-ALL (22).
Figure 1. Pax5 heterozygosity and exposure to infectious stimuli cooperate in pB-ALL development. A, experimental setup. Mice are born under SPF conditions and are moved to a non-SPF facility where they are exposed to common infectious pathogens between 5 and 21 weeks after birth. B, pB-ALL-specific survival curve of Pax5+/- animals in SPF conditions (red, n = 14) compared with WT control mice (black, n = 15), close to pB-ALL-specific survival curve of Pax5+/- animals in non-SPF conditions (red, n = 41) compared with WT control mice (black, n = 20), showing a significantly (log-rank P value 0.0098) shortened life span (right). C, example of splenomegaly observed in 55% (5 of 9) of Pax5+/- mice. WT mouse spleen is shown for reference. Hematoxylin and eosin staining of spleen sections from WT- and tumor-bearing Pax5+/- mice shows infiltrating blast cells. Scale bar, 10 μm (400×; n = 9). D, flow cytometric analysis of hematopoietic subsets in diseased Pax5+/- mice. Representative plots of cell subsets from the BM and PB show accumulation of blast B cells in Pax5+/- mice (n = 9; age, 6–16 months) compared with control littermate age-matched WT mice (n = 4; age, 8–16 months). E, immunoglobulin clonality in Pax5+/- tissues infiltrated with blast B cells. PCR analysis of immunoglobulin heavy-chain gene rearrangements in infiltrated spleens and lymph nodes of diseased Pax5+/- mice. Thymocytes (T cells) were included as a negative control, and sorted CD19+ B cells (B cells) from the spleens of healthy mice serve as a control for polyclonal BCR rearrangements (indicated by numbers 1–4). Infiltrated tissues show an increased clonality within their immunoglobulin repertoire (red squares; n = 2).
Figure 2. pB-ALLs from Pax5−/+ mice resemble human pB-ALL. A, differential gene expression analysis of tumor-bearing BMs of three Pax5−/+ mice (O361, W634, and S748 CD19+ phenotype; S665, O388, and W362 CD19− phenotype) compared with B220+ BM B cells from five WT mice shows significant differences (left). In the right plot, differential gene expression analysis of tumor-bearing BMs of six Pax5−/+ mice compared with BM pro-B and pre-B cells from three WT mice shows significant differences (FDR = 0.01). B, relative expression of p19Arf from BM tumor cells of Pax5−/+ mice (samples 1–5). Total BM of a SCA1-BCR–ABL-p210 mouse was used as a positive control (sample 6). Blast cells of Pax5−/+ mice 1, 2, and 3 are CD19−, and blast cells of Pax5−/+ mice 4 and 5 are CD19+. Error bars represent the mean ± SD of three replicates. C, loss of Cdkn2a locus from all tumor/reference pairs.

Copy-number variation profiles were calculated using Free copy number and genotype caller (FREEC). Copy-number profiles displayed a loss of heterozygosity at 4:89274500-89276999 in mouse O361.

Table 1. Genotype and phenotype of pB-ALL–bearing Pax5−/+ mice

<table>
<thead>
<tr>
<th>Mouse number</th>
<th>Mouse age at disease (months)</th>
<th>CD19 phenotype</th>
<th>Jak3 mutation in mouse*</th>
<th>Jak3 mutation (allele frequency)b</th>
<th>IAK3 human homolog</th>
<th>Pax5 mutation in mouse*</th>
<th>PAX5 human homolog</th>
<th>Jak1 mutation in mouse*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>O361</td>
<td>CD19+</td>
<td>R653H</td>
<td>(50%)</td>
<td>R653H</td>
<td>R653H</td>
<td>S645P</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>O388</td>
<td>CD19−</td>
<td>R653H</td>
<td>(11%)</td>
<td>R657Q</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>S665</td>
<td>CD19−</td>
<td>R563H</td>
<td>(2%)</td>
<td>R657Q</td>
<td>P80R</td>
<td>V657F</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>S663</td>
<td>CD19−</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>W495</td>
<td>CD19−</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>S748</td>
<td>CD19+</td>
<td>V670A</td>
<td>(27%)</td>
<td>V674A</td>
<td>P80L</td>
<td>Not described</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>W893</td>
<td>CD19−</td>
<td>R653H</td>
<td>(49%)</td>
<td>R653H</td>
<td>R657Q</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>S767</td>
<td>CD19−</td>
<td>R653C</td>
<td>(63%)</td>
<td>R657Q</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>W634</td>
<td>CD19−</td>
<td>R653H</td>
<td>(35%)</td>
<td>R657Q</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a Mutations confirmed by Sanger sequencing.

*b Mutations confirmed by deep sequencing.
**Pax5**<sup>+/−</sup> Creates an Aberrant, IL7-Sensitive Precursor B-cell Compartment

We next aimed to explain the mechanism of this specific pB-ALL susceptibility due to Pax5 heterozygosity under exposure to infections. To this end, the different B-cell developmental stages were analyzed in WT and preleukemic Pax5<sup>+/−</sup> littermates of the same breeding. Pax5<sup>−/−</sup> mice presented a significantly reduced amount of total B cells in the PB when compared with WT mice (Fig. 3A; Supplementary Table S4). This decrease was not induced by the exposure to common pathogens, as a similar decrease in PB B cells was observed in Pax5<sup>−/−</sup> mice housed in an SPF environment (Fig. 3B). On the other hand, we observed significantly higher proportions of (pro-pre)-B and immature B cells in the BM of Pax5<sup>−/−</sup> mice compared with age-matched WT littermates (Fig. 3A; Supplementary Table S4), but no differences were detected in earlier developmental compartments of the BM (data not shown). These data therefore suggest that Pax5 heterozygosity favors the appearance of an aberrant B-cell precursor compartment in the BM and that differentiation to mature PB B cells is impaired in vivo in keeping with observations in Pax5-null mice (13) and in a stable in vivo Pax5 knockdown model (23). These observations are in agreement with reports of preleukemic activity in mice bearing pB-ALL-associated oncogenes, e.g., the **ETV6–RUNX1** fusion (24, 25). The murine precursor B cells are dependent on intact IL7/IL7R signaling and subsequent activation of JAK3 and STAT5 phosphorylation. Thus, we next explored the response of Pax5<sup>+/−</sup> pro-B cells to IL7 withdrawal and showed that Pax5<sup>+/−</sup> pro-B cells are more sensitive to IL7 withdrawal than their WT counterparts (Fig. 3C). Similar observations were obtained when preleukemic Pax5<sup>+/−</sup> pro-B cells were exposed to specific JAK1/3 inhibitors (Fig. 3D). Taken together, these results imply that aberrant precursors present in Pax5<sup>+/−</sup> mice are extremely sensitive to IL7 withdrawal and susceptible for second hits leading to leukemia development.

**Mouse Tumor Exome Sequencing Data Identify the Second Hit within the IL7R/JAK3/STAT5 Axis**

In order to identify the second hit related to pB-ALL disease with prenatal initiation triggered by infection exposure in the context of impaired B-cell development, we next performed whole-exome sequencing of three Pax5<sup>−/−</sup> tumors and corresponding germline samples on a HiSeq 2500 (Illumina) platform. Pax5<sup>−/−</sup> tumor cells were derived from BM of diseased mice. We identified between 28,638 and 31,105 single-nucleotide variations (SNV) in tumor samples and between 27,141 and 31,151 SNVs in germline samples,
resulting in 31 (O361; CD19+), 17 (O388; CD19+), and 9 (S665; CD19+) somatic SNVs in the three mice. Finally, we detected Jak3 c.1958G>A (p. R653H) as one recurrent heterozygous nonsynonymous somatic SNV in the pseudokinase domain of JAK3 in two mice (Fig. 4A; Supplementary Table S5). The corresponding human homolog (JAK3R657G) is recorded in the cancer gene consensus database (ref. 26; Table 1). Next, we applied Sanger sequencing to the remaining tumors and, surprisingly, we identified nonsynonymous Jak3 mutations in 6 of 9 mice causing Jak3R653H (4/9; human homolog JAK3R667Q), Jak3R653C (1/9; human homolog JAK3R667Q), and Jak3V670A (1/9; human homolog JAK3V674A; data not shown). This result and the fact that all Jak3 variants (R653H, R653C, and V670A) are located in a region highly conserved across different species together indicate that these variants are relevant for leukemia evolution (Table 1). We next performed deep sequencing with a depth between 600,000 and 2.5 × 10^6 reads per Jak3 variant and observed the nonsynonymous Jak3 variant only in tumor samples but not in BM cells of healthy Pax5−/− or WT mice (Fig. 4B). In addition, in mice S748 (Jak3V670A), W634 (Jak3R653H), and S767 (Jak3R665C), we were able to monitor leukemia evolution over time by collecting peripheral blood mononuclear cells (PBMC) at routine intervals after birth using deep sequencing at the same depth as described above. All Jak3 variants were first detectable by deep sequencing and Sanger sequencing when the mouse appeared clinically ill with pB-ALL (Fig. 4C). This result is incompatible with a model in which Jak3 mutations are present in few cells from birth on and become selected over time. Instead, they are de novo mutations occurring on the Pax5−/− background as a consequence of environmentally driven selection pressure with a very short latency to leukemia outgrowth.

**SomaticJak3 Mutations Rescue IL7-Sensitive Pax5−/− Precursor B Cells and Cause Leukemia Outgrowth**

The corresponding human JAK3R667Q and JAK3V674A variants result in constitutive kinase activity of JAK3 and downstream STAT5 phosphorylation (27, 28). Hence, we transfected IL3-dependent Ba/F3 cells with the murine JAK3R665C variant and observed cytokine-independent growth (Fig. 4D), consistent with phosphorylation of STAT5 in the absence of IL3 (Fig. 4E). Similarly, we observed STAT5 phosphorylation in the mouse leukemic cells carrying Jak3R6670A (Fig. 4F). Consistently, tumor pro-B cells harboring Jak3R6670A and Jak3R6653H grow independent of IL7 (Fig. 4G, H, and I). In two pB-ALLs (O361 and S665), we additionally identified Jak1 mutations (Table 1). JAK3 pseudokinase mutants are dependent on JAK1 kinase activity for cellular transformation (29). This is consistent with the increase in cell death after administration of the Jak1/2 inhibitor ruxolitinib in vitro (Fig. 4f) as well as the decrease in leukemia cell burden in vivo (see below).
Causal Role of Infection in pB-ALL Development

To identify the tumor repopulating cells of the murine Pax5+/− pB-ALL, we transplanted primary myeloid cells, Pax5+/− pro-B cells harboring WT Jak3, and Pax5+/− pro-B cells harboring Jak3V670A, respectively, into sublethally irradiated syngeneic recipient mice. Each of the mice transplanted (n = 9) with 10^5 Pax5+/− pro-B cells harboring Jak3V670A cells developed a pB-ALL with a latency of 13 ± 3 days. The disease was phenotypically identical to the primary disease. In contrast, when recipient mice were transplanted with myeloid cells (n = 7; 10^5 cells per mouse) and Pax5+/− pro-B cells (n = 10; 10^5 cells per mouse), they were incapable of inducing pB-ALL in secondary recipients (Fig. 5A–C). This indicates that infection-induced Pax5+/− pB-ALL in this model is propagated by transformed Pax5+/− pro-B cells harboring Jak3V670A but not Pax5+/− pro-B cells harboring WT Jak3, and confirms that Jak3V670A can induce pB-ALL in mice. These results suggested that the Pax5 heterozygous condition can establish the environment for the generation of the Jak3 mutation and is not playing a role in the pathogenesis of the final leukemia. Thus, we next examined the PAX5-independent potential role of the identified Jak3 mutations in leukemogenesis by injecting 10^6 Ba/F3 cells expressing either Jak3V670A (n = 5) or Jak3R653H (n = 4) mutants in nude mice, and we monitored the pB-ALL development by periodic analysis of the presence of blast cells in PB. The results show that both mutants are able to generate pB-ALL in mice with a disease latency of 27 ± 3 days (Supplementary Fig. S3A–S3C), suggesting that inactivating mutations in Pax5 promote leukemogenesis by simply creating an aberrant IL7-sensitive progenitor compartment that is susceptible to malignant transformation through accumulation of secondary Jak3 mutations as a rescue mechanism of IL7/IL7R/STAT5 signaling.

Successful In Vivo Treatment of Pax5+/− pB-ALL Using JAK Inhibitors

These results suggest that JAK1/3 inhibitors may be useful therapeutic options for pB-ALL in patients with germ-line PAX5 mutations. Thus, we treated tumor pro-B cells (Jak3V670A) with three different selective JAK inhibitors: tofacitinib (JAK1/3), TG101348 (JAK2), and ruxolitinib (JAK1/2). Tumor pro-B cells were sensitive to the JAK1/3 and the JAK1/2 inhibitors but not to the JAK2 inhibitor in vitro (Fig. 4J).

We next tested the efficacy of the JAK inhibitors in vivo. To this end, mice infused with transformed Pax5+/− pro-B cells harboring Jak3V670A were randomized to treatment at day 12 when the disease was confirmed by the presence of

Figure 4. Mouse tumor exome sequencing data identify the second hit within the IL7R/JAK3/STAT5 axis. A, exome sequencing analysis yielded around 30,000 SNVs genome wide; 1 to 4 were deemed tumor-specific by MUTECT analysis with one recurring Jak3 variant. B, the allele frequency of the Jak3 variants V670A, R653C, and R653H is shown for 9 Pax5+/− mice. Healthy Pax5 heterozygous and WT mice serve as negative controls. Variants confirmed by Sanger sequencing are marked +. C, backtracking of the mutations Jak3V670Al, Jak3V670A, and Jak3R653H by Sanger sequencing and deep sequencing in the PB of Pax5+/− mice W634, S748, and S665 (n = 2). D, Ba/F3 cells were transfected with pMC3 empty vector, pMC3-JAK3WT, or pMC3-JAK3R653H, and hygromycin-resistant cells were selected. Cells were cultured in media without IL3, and their proliferation was measured every day using Trypan Blue. Values represent the mean (n = 2) with essentially identical datasets. (continued on next page)
blasts in the PB of the reconstituted mice (Fig. 6A). Mice were treated with ruxolitinib for only 5 days. FACS analysis of the PB was used to verify disease remission after therapy. Although ruxolitinib provided no significant survival advantage, all mice treated (n = 5) showed a transient decrease in the blast cells (Fig. 6B). However, disseminated leukemia, documented by clinical criteria and FACS (Fig. 6C and D), invariably ensued, except in 1 of 5 mice that was alive and healthy 33 days after discontinuation of treatment. In this setting, PAX5 seems to retain driver functions in established leukemia because restoring endogenous PAX5 expression triggers disease remission (23).

Recent discoveries of inherited mutations of PAX5 in a new syndrome of susceptibility to pB-ALL have extended the role of PAX5 alterations in the pathogenesis of pB-ALL (5, 6). The presence of the inherited mutations of PAX5 seems to produce a persistent and hidden preleukemic clone that may convert to pB-ALL in only a fraction of the family members (5, 6). The patients lose the WT PAX5 allele related to a secondary structural aberration of chromosome 9p. In mice, we face the same scenario, but the sequence is reverse. The mice have lost one WT Pax5 allele and acquire Pax5 point mutations as secondary events. However, the final common path in both species is reduced transcriptional activity of PAX5. The mechanisms responsible for the conversion of the preleukemic clone, carrying the inherited mutations of PAX5,
Causal Role of Infection in pB-ALL Development

Figure 5. pB-ALL is transplantable to secondary recipients. A, experimental setup. A total of 100,000 leukemic Pax5<sup>−/+</sup> pro-B cells harboring Jak3<sup>V670A</sup> mutation were injected into sublethally irradiated WT syngeneic mice. Regular bleedings were performed in order to monitor the development of the pB-ALL. B, representative flow cytometric analysis of mice injected with leukemic Pax5<sup>−/+</sup> pro-B cells harboring Jak3<sup>V670A</sup> mutation. Cytometric analyses at different time points show that leukemic pB-ALL cells (B220<sup>lo</sup> IgM<sup>−</sup>) were able to grow in secondary recipients. BMT, bone marrow transplant. C, representative flow cytometric analysis of mice injected with leukemic Pax5<sup>−/+</sup> pro-B cells harboring Jak3<sup>V670A</sup> mutation shows the accumulation of pB-ALL cells (B220<sup>lo</sup> IgM<sup>−</sup>) in BM. FSC-A, forward scatter-area; SSC-A, side scatter-area.

into pB-ALL are not understood yet. However, recent results suggest that the B-cell-specific enzyme AID is supposed to be the predominant driver of clonal evolution in human ETV6–RUNX1 pB-ALL (30–32) and B-cell lymphoma (33). These observations are in line with previous studies showing that AID is capable of initiating aberrant genomic alterations in precursor B cells (34) The pB-ALL that originated as a result of delayed infection exposure in Pax5<sup>−/+</sup> mice offers a unique possibility to confirm if the proposed mechanisms are involved in the conversion of the preleukemic clone into a full-blown leukemia (35, 36). The assumption of a functional interaction between inherited susceptibility and postnatal infection is a highly attractive explanation in the natural history of childhood ALL. Our results bring strong functional proof in support of the “infectious” hypothesis that posits that exposure to infection represents an oncogenic environment. Hence, this observation closely mimics the human situation. Children encounter a plethora of different infectious agents at the ages of 2 to 5 years, but all attempts to identify a single consistent infectious agent have failed in humans with pB-ALL (11). This indicates that it is a sequence of infections and broad immune responses in an unprimed immune system, which is important for leukemia development, rather than exposure to a single pathogen. Likewise, several months prior to ALL, children show a marked deterioration of immune response and profound liability to a variety of infections (35).

In our model, inactivating mutations in Pax5 promote leukemogenesis by creating an aberrant IL7-sensitive progenitor compartment. This progenitor population is susceptible to malignant transformation through accumulation of secondary Jak3 mutations on the basis of a selection pressure triggered by the increased and recurrent exposure to delayed infection in conventional housing facilities. These somatic Jak3 mutations induce leukemia with a phenotype very similar to the human pB-ALL linked to germline mutations.
in PAX5 with respect to clinical, immunophenotypic, and molecular genetic characteristics. In vivo transplantation experiments demonstrate that the activating Jak3 mutations per se are sufficient to drive leukemia. Thus, targeting the deregulated JAK–STAT pathway can be a promising therapy for this disease, as we have shown in vivo.

Strikingly, this model parallels very well the incomplete penetrance observed in human PAX5−/− leukemia. Further studies with Pax5−/− mice exposed to the same infectious conditions in utero and in the first month of their life will reveal if the timing and pattern of infectious exposure are indeed relevant for pB-ALL development. This suggestion was rendered moot over 70 years ago (37). Toward this development, our findings are important not only for endorsing the credibility of the "infectious" hypothesis, but also for encouraging the prospect of novel interventions that might help to prevent a significant proportion of childhood leukemia.

**Figure 6.** In vivo efficacy of JAK inhibition in transformed Pax5−/− pro-B cells harboring Jak3V670A. A, experimental setup. A total of 100,000 leukemic Pax5−/− pro-B cells harboring Jak3V670A mutation were injected into sublethally irradiated WT syngeneic mice. Regular bleedings were performed in order to check the development of the pB-ALL. When pB-ALL cells (B220lo IgM−) were detected in the PB, the mouse treatment with ruxolitinib started. Mice were treated with ruxolitinib for only 5 days. FACS analysis of the PB was used to verify disease remission after therapy. B, percentage of PB pB-ALL cells (B220lo IgM−) was monitored by flow cytometry at different time points in mice treated with ruxolitinib (ruxo). C, follow-up of leukemic cells (B220lo IgM−) by PB flow cytometry in mice before and after ruxolitinib treatment. Mice treated with ruxolitinib showed a transient decrease in the blast cells except in mouse D009, which was alive and healthy 33 days after discontinuation of treatment. D, representative disseminated leukemia documented by FACS analysis of the BM of mice 5 days after starting treatment with ruxolitinib.
METHODS

Heterozygous Pax5+/- Mice

The heterozygous Pax5+/- mice (15) have been described previously. Pax5+/- and control WT mice were born and kept at the SPF facility until exposed to a common infectious environment. All animal work was conducted according to relevant national and international guidelines, and it has been approved by the Bioethics Committee of the University of Salamanca and by the Bioethics Subcommittee of Consejo Superior de Investigaciones Científicas (CSIC). Both male and female WT and Pax5+/- mice of a mixed C57BL/6 × CBA background were included in the study. We used WT and Pax5+/- littermates of the same breeding. Upon signs of disease, mice were sacrificed and subjected to standard necropsy procedures. All major organs were examined under the dissecting microscope. Tissue samples were taken from homogenous portions of the resected organ and fixed immediately after excision. Differences in Kaplan–Meier survival plots of transgenic and WT mice were analyzed using the log-rank (Mantel–Cox) test.

FACS Analysis

Nucleated cells were obtained from total mouse BM (flushing from the long bones), PB, thymus, or spleen. Contaminating red blood cells were lysed with red cell lysis buffer, and the remaining cells were washed in PBS with 1% fetal calf serum (FCS). After staining, all cells were washed once in PBS and were resuspended in PBS with 1% FCS containing 2 mg/mL propidium iodide (PI) to allow dead cells to be excluded from both analyses and sorting procedures. The samples and the data were acquired in an AccuriC6 Flow Cytometer and analyzed using FlowJo software. Specific fluorescence of FITC, Phycoerythrin, PI, and Allophycocyanin excited at 488 nm (0.4 W) and 633 nm (30 mW), respectively, as well as known forward and orthogonal light scattering properties of mouse cells, were used to establish gates. Nonspecific antibody binding was suppressed by preincubation of cells with CD16/CD32 Fc-block solution (BD Biosciences). For each analysis, a total of at least 50,000 viable (PI−) cells was assessed.

The following antibodies were used for flow cytometry: anti-B220 (RA3-6B2), CD3e (145-2C11), CD4 (RM4-5; 1:500), CD8α (53-6.7; 1:500), CD11b/MAC1 (M1/70; 1:200), CD19 (1D3), CD117/c-KIT (Mantle; 1:500), CD127/IL7R (2B8; 1:200), CD127/IL7Rα (A7R34; 1:50), Ly-6G/Gr1 (RB6-8C5), IgM (R6-60.2), SCA1/Ly-6A/E (E13-161.7; 1:50), CD25 (PC61), CD48 (H4-M1), and CD150 (TC15-12F12.2). Unspecific antibody binding was suppressed by preincubation with CD16/CD32 (2.4G2) Fc-block solution (PharMingen). The different hematopoietic progenitors and B-cell stages were defined by flow cytometry as shown in Supplementary Fig. S2A and S2B. All antibodies were purchased from BD Biosciences and used at a 1:100 dilution unless otherwise indicated.

Histology

Animals were sacrificed by cervical dislocation; tissue samples were formalin-fixed and embedded in paraffin. Pathology assessment was performed on hematoxylin–eosin-stained sections under the supervision of Dr. Oscar Blanco, an expert pathologist at the Salamanca University Hospital.

V(D)J Recombination

Immunoglobulin rearrangements were amplified by PCR using the primers below. Cycling conditions consisted of an initial heat activation at 95°C followed by 31 to 37 cycles of denaturation for 1 minute at 95°C, annealing for 1 minute at 65°C, and elongation for 1 minute 45 seconds at 72°C. This was followed by a final elongation for 10 minutes at 72°C. To determine the DNA sequences of individual V(D)J rearrangements, the PCR fragments were isolated from the agarose gel and cloned into the pGEM-Teasy vector (Promega); the DNA inserts of at least ten clones corresponding to the same PCR fragment were then sequenced. The following primer pairs were used: Vj5′J58: forward CGAGCTTCTCACACAGGCWTCATGTACRC, reverse GTCATTAGCTCTCAAGAGCTCGAGTTACCGTTC; Vj7183: forward CGGTACCAGAASACCTCGTCTGGCTACTG, reverse GTCTAGGTCACCTCAAGAGCTCGAGTTACCGTTC; VjQ52: forward CGGT ACCAGACGTCARCTCASCAGAGGACAAATCTC, reverse GTCTAGATTCTACAAAGATGCTCGAGTTACCGTTC; GH: forward TCTCAAGACGTAATGCGCCGC; reverse GCTCTAGCTAGCTAGCTCACACAGAGGAAGA.

Microarray Data Analysis

Total RNA was isolated in two steps using TRIzol (Life Technologies) followed by RNeasy Mini-Kit (Qiagen) purification, following the manufacturer’s RNA Clean-up Protocol with the optional On-Column DNase treatment. The integrity and the quality of the RNA were verified by electrophoresis, and its concentration was measured. Samples were analyzed using Affymetrix Mouse Gene 1.0 ST arrays.

Briefly, the robust microarray analysis algorithm was used for background correction, intra- and intermicroarray normalization, and expression signal calculation (38–40). Once the absolute expression signal for each gene (i.e., the signal value for each probe set) was calculated in each microarray, a method called significance analysis of microarray (41) was applied to calculate significant differential expression and find the gene probe sets that characterized the tumor-bearing BM from Pax5+/- mice compared with BM-derived B220+ cells from WT mice. The method uses permutations to provide robust statistical inference of the most significant genes and provides P values adjusted to multiple testing using FDR (42). A cutoff of FDR < 0.05 was used for the differential expression calculations. We applied all these methods using R and Bioconductor (43).

The data discussed in this publication have been deposited in NCBi’s Gene Expression Omnibus (GEO; ref. 44) and are accessible through GEO Series accession number GSE62529.

Enrichment Analysis

Differentially expressed genes were tested for enrichment using GSEA from MSigDB (21). Gene expression signatures that are specifically upregulated or downregulated in human B-ALL gene sets (18, 19) were also assessed for their overlap with those that were upregulated or downregulated within tumor specimens using GSEA. We also carried out more enrichment analysis for the specific normal murine B-cell stage signatures (20). Finally, we carried out one more enrichment analysis for the specific Pax5+/- gene signatures described in refs. 23 and 45–47.

Cdkn2a/Arf Expression

cDNA was synthesized from BM tumor cells of Pax5+/- mice using the High Capacity RNA-to-cDNA Kit and used for TaqMan gene expression assays. For quantitative PCR, the following TaqMan Gene Expression Assay (Applied Biosystems) was used: Cdkn2a/Arf (Mm.PT.56a.8388138). Gdph (Mm.PT.39a.1) was used as an internal control.

Detection of Viral Integration

The Genome Information Broker for Viruses (GIB-V) was screened for viral sequences. The database comprises the genomes of 25,525 clones and strains of DNA and RNA viruses (mouse and human). The virus detection can be divided into three steps: retrieve potential viral sequences, filter low-quality detections, and analyze paired-end reads for viral integration. Reads, which could not be mapped against the mouse reference genome (GRCm38) with Burrows-Wheeler Aligner
Reads could be mapped to multiple viruses or strains of the same virus to avoid missing alignments due to sequence similarity between the viral sequences. The viral genomes were analyzed for low complexity and repetitive regions with RepeatMasker and compared with the mouse reference genome with the Nucmer algorithm in the MUMmer package. Low-quality alignments within repeats or regions with high sequence homology with the mouse genome were discarded as well as multiple alignments of one read against the same viral genome. Paired-end reads, where one mate mapped to the mouse genome and the other mate to a virus, hinted at possible viral integration spots. Candidates, like the integration on Pax5, were investigated manually with the Integrative Genomics Viewer to pinpoint the exact breakpoints.

Mouse Exome Library Preparation and Next-Generation Sequencing

Sample Acquisition The AllPrep DNA/RNA Mini Kit (Qiagen) was used to purify DNA according to the manufacturer’s instructions.

Exome Library Preparation and Next-Generation Sequencing Exome library preparation was performed using the Agilent SureSelectXT Mouse All Exon Kit with modifications. Targeted capture by hybridization to an RNA library was performed according to the manufacturer’s protocol. Purification and enrichment of the captured library were achieved by binding to MyOne Streptavidin T1 Dynabeads (Life Technologies). To examine the PAX5-independent potential role of the identified Jak3 mutations in leukemogenesis, we injected Ba/F3 cells expressing either Jak3 WT, Jak3 R653H/C, or the empty vector control were washed twice with medium without IL7, before they were cultured in the absence of IL7 for 10 days. Pax5−/− (Jak3 WT), Pax5−/−, and WT pro-B cells (1 × 10^6) were washed twice with medium without IL7, before they were cultured in the absence of IL7 for 7 days. Proliferation was measured by counting the cells every day using Trypan Blue (Sigma-Aldrich).

Immunoblot Analysis Whole-cell extracts were obtained as previously described (28). Spleen samples were prepared using a 100-μm cell strainer and depleted of erythrocytes using NH4Cl, before lymphocytes were lysed in RIPA buffer (50 mmol/L Tris, pH 8.0, 150 mmol/L NaCl, 0.5% Sodiumdeoxycholate, 1% NP-40 substrate, 0.1% SDS) containing protease and phosphatase inhibitors (Roche Diagnostics). Whole protein (20 μg) was separated on SDS-PAGE and transferred to Hybond-C Extra membranes (Amersham Biosciences). Immunoblotting was carried out using the following antibodies: anti–phospho-STAT5 (1:1,000), anti–STAT5 (1:1,000; Cell Signaling), and anti–β-actin clone AC-74 (1:10,000; Sigma-Aldrich). Detection was
done using anti-rabbit or anti-mouse horseradish peroxidase conjugates (Santa Cruz Biotechnology), respectively, with an enhanced chemiluminescence system (Thermo Scientific).

**Pro-B-cell Culture**

Iscove’s Modified Dulbecco’s Medium (IMDM) supplemented with 50 μmol/L β-mercaptoethanol, 1 mmol/L L-glutamine, 2% heat-inactivated FCS, 1 mmol/L penicillin–streptomycin (BioWhittaker), and 0.03% (v/v) primatone RL (Sigma) was used for pro-B-cell culture experiments. Pro-B cells isolated by magnetic-activated cell sorting for activated FCS, 1 mmol/L penicillin–streptomycin (BioWhittaker), and 0.03% (v/v) primatone RL (Sigma) was used for pro-B-cell culture experiments. Pro-B cells isolated by magnetic-activated cell sorting for B220+ (Milety Biotec) from BM were cultured on Matrigel–C–treated ST2 cells in IMDM medium containing IL7 (R&D Systems). IL7-independent tumor pro-B cells were grown in the same medium without IL7.

**Inhibitor Experiments**

WT, Pax5+/−, and tumor pro-B cells were seeded at 10^5/3 mL/well in 6-well plates and treated with or without (vehicle) tofacitinib (JAK1/3 inhibitor; 1 μmol/L), TG101348 (JAK2 inhibitor; 1 μmol/L), and ruxolitinib (JAK1/2 inhibitor; 1 μmol/L) for 24 hours before being subjected to cell viability assay. Experiments were performed at the same time under the same experimental conditions.

**Apoptosis Assays**

Aliquots (200 μL) containing 10^6 cells in 10 mmol/L Hepes/NaOH, pH 7.4, 140 mmol/L NaCl, and 2.5 mmol/L CaCl2 were incubated with Annexin V–FITC (BD Biosciences) to a final concentration of 1 μg/mL for 10 minutes, at room temperature in the dark, and then labeled with PI to a final concentration of 2 μg/mL (PI in binding buffer). Flow cytometry was performed within an hour of labeling, and data were analyzed using FlowJo software. The difference between experimental variables was determined using the Student \( t \) test.

**BM Transplantation Experiments**

BM myeloid cells, Pax5+/− pro-B cells with intact Jak3, and Pax5−/− pro-B cells harboring the Jak3V670A mutation were injected intravenously into sublethally irradiated (4 Gy) secondary recipient 12-week-old male syngenic mice (C57BL/6 J). The authors monitored the recipient mice was monitored by periodic PB analysis (CBA). Disease development in the recipient mouse was monitored by periodic PB analysis until blast cells were detected. Then, they were sacrificed and assessed for PB-ALL development.

**Preclinical Therapeutics**

Ruxolitinib prepared in PBS was administered at 180 mg/kg, orally, twice a day, when animals had developed a significant leukemic burden as documented by FACS analysis of PB. Disease development in the recipient mouse was monitored by periodic PB analysis until blast cells were detected. Then, they were sacrificed and assessed for PB-ALL development.

**Disclosure of Potential Conflicts of Interest**

S.N. Constantinescu has received speakers bureau honoraria from Novartis and Shire New Horizons in Hematology and is a consultant/advisory board member for Dafla, Novartis, Personal Genetics, and Shire. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**

Conception and design: J. Hauer, C. Vicente-Dueñas, I. Sánchez-García, A. Borkhardt


Acquisition of data (provided animals, entered clinical data, collected and assembled data): A. Martín-Lorenzo, J. Hauer, C. Vicente-Dueñas, F. Auer, I. González-Herrero, I. García-Ramírez, M. Gombert, D. Schäfer, O. Blanco, A. Orfao, A. Borkhardt


**Writing, review, and/or revision of the manuscript:** A. Martin-Lorenzo, J. Hauer, C. Vicente-Dueñas, F. Auer, I. González-Herrero, I. García-Ramírez, S.N. Constantinescu, D. Schäfer, A. Mayado, A. Orfao, D. Alonso-López, J. De Las Rivas, C. Cobaleda, I. Sánchez-García

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** M. Gombert, A. Borkhardt

**Study supervision:** J. Hauer, C. Vicente-Dueñas, I. Sánchez-García, A. Borkhardt

**Acknowledgments**

The authors are indebted to all members of their groups for useful discussions and for their critical reading of the manuscript. They are grateful to Dr. Meinrad Busslinger for the Pax5+/− mice. The authors also thank Lorraine Springuel and Jean-Christophe Renauld, Ludwig Cancer Research Brussels, for tofacitinib.

**Grant Support**

J. Hauer has been supported by the German Children’s Cancer Foundation and from the “Forschungskommission” of the medical faculty of the Heinrich Heine University and the “Strategischer Forschungsfond” of the Heinrich Heine University. S. Ginzel has been supported by a scholarship from the Hochschule Bonn-Rhein-Sieg. A. Borkhardt has been supported by the German Children’s Cancer Foundation and the Federal Ministry of Education and Research, Bonn, Germany. Funding to S.N. Constantinescu from Salus Sanguinis, Fondation contre le cancer Belgium; Interuniversity Attraction Poles IAP, and ARC 10/15-027 is acknowledged. Research in the L. Sánchez-García group is partially supported by FEDER and MINECO (SAF2012-32810 and Red de Excelencia Consolidador Oncología BíoSAF2014-57991-R-EDC), Instituto de Salud Carlos III (PIE14/00066), Junta de Castilla y León (BIO/SA32/14, BIO/SA51/15, and CSI01U14), Fundacion Inocente Inocente, and the ARIMMORA project [European Union’s Seventh Framework Programme (FP7/2007-2013) under grant agreement no. 282891]. The L. Sánchez-García lab is a member of the EuroSysTem and the DECIDE Network funded by the European Union under the FP7 program. A. Borkhardt and I. Sánchez-García have been supported by the German Carreras Foundation (DJCLS R13/26). Research in the C. Vicente-Dueñas group is partially supported by a “Miguel Server” Grant (CP14/00082 - AES 2013-2016) from the Instituto de Salud Carlos III (Ministerio de Economía y Competitividad). Research at C. Cobaleda’s lab was partially supported by FEDER, Fondo de Investigaciones Sanitarias (PI13/0160 and PI14/00025), and from an institutional grant from the Fundacion Ramon Areces. A. Martin-Lorenzo was supported by FSE-Conserjería de Educación de la Junta de Castilla y León (CS001-13).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received July 23, 2015; revised September 15, 2015; accepted September 17, 2015; published OnlineFirst September 25, 2015.

**REFERENCES**


Causal Role of Infection in pB-ALL Development

Infection Exposure Is a Causal Factor in B-cell Precursor Acute Lymphoblastic Leukemia as a Result of Pax5-Inherited Susceptibility

Alberto Martín-Lorenzo, Julia Hauer, Carolina Vicente-Dueñas, et al.