IRF4 deficiency vulnerates B progeny for leukemogenesis via Jak3 mutations resembling Ph-like B-ALL in humans

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IRF4 deficiency vulnerates B progeny for leukemogenesis via *Jak3* mutations resembling Ph-like B-ALL in humans

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Abstract

How disturbances of B cell development provoke adult B acute lymphoblastic leukemia (B-ALL) remains poorly understood. Here we describe *Irf4*^{-/-} mice as prone to developing B-ALL with age. *Irf4*^{-/-} pro/preB cells exhibited impaired differentiative but enhanced proliferative potential *in vitro* and accumulated in spleens of healthy *Irf4*^{-/-} mice, suggesting reduced adherence to the IL-7 providing bone marrow niche. Thus selected, pro/preB cells transformed acquiring proliferative IL-7 independency through *Jak3* gain-of-function mutations. Targeting JAK signaling with Ruxolitinib *in vivo* prolonged survival of mice bearing established *Irf4*^{-/-} leukemia. Intriguingly, organ infiltration including leukemic meningeosis was selectively reduced without affecting blood blast counts. As low IRF4 expression and *JAK3* mutations also characterize a subpopulation of Ph-like B-ALL in adult humans, our results imply *Irf4*^{-/-} mice as a suitable model for investigating preleukemic conditions in adults. Using this model, we identified an unexpected effect of Ruxolitinib treatment in B-ALL.

Introduction

Two counteracting signaling pathways via the Interleukin-7 receptor (IL-7R) and the preB cell receptor (preBCR) ensure an orderly progression from proB to preB cells during B lymphopoiesis.¹⁻³ ProB cells adhere to bone marrow (BM) stromal cells (SCs) expressing CXCL12 and VCAM-1 through CXCR4 and VLA-4, respectively.⁴⁻⁷ IL-7 from SCs secures proliferation and further promotes adherence of proB cells via upregulation of focal adhesion kinase (FAK).⁵ The formation of the preBCR composed of Ig μ protein (resulting from the productive rearrangement of the IgH locus) and the surrogate light chain CD179b, marks the entrance to the preB cell stage. Signaling via the preBCR in turn induces the transcription factor interferon regulatory factor 4 (IRF4) which is also highly important during T cell differentiation.^{8,9} Upon induction in preB cells, IRF4 halts cycling, induces migration away from the IL-7 rich niche, and facilitates recombination of the Igk or Ig λ locus by the RAG1/2 recombinases.¹ Despite this central role, *Irf4*^{-/-} mice still have effective, albeit lowered, development of membrane-bound (m)IgM⁺ mature B cells,¹⁰ likely due to a partially redundant function of IRF8. Accordingly, *Irf4,8*^{-/-} B cell progenitors are completely arrested at the preB cell stage.¹¹

Importantly, disruption of this developmental track can provoke progenitor B acute lymphoblastic leukemia (pB-ALL). In humans, this disease preferentially affects children (age 0-19) with a four times higher incidence when compared to adults.¹² Most deaths however occur in the adult population, with the relative survival rate decreasing progressively with the age at diagnosis.¹² Not surprisingly, leukemia biology also differs between children and adults. Some driver mutations like the ETV6-RUX1 rearrangement are almost exclusively found in ALL of children. Furthermore, clonal expression of drivers like MLL rearrangements has even been traced back to birth in a pediatric ALL patients cohort.¹³ In contrast, these typical hallmarks of ALL in children are rarely encountered in adults. Instead, cases affecting adolescents and young adults (AYA) display more acquired mutations per tumour cell and a

different set of driver mutations.^{14–17} A famous driver in AYA-patients is the Philadelphia-chromosome (Ph), a t(9;22) translocation generating the fusion protein BCR-ABL1.^{18–20} Ph-like- (or BCR-ABL1 like) ALL also preferentially affects AYA-patients and is marked by transcriptomic changes similar to Ph⁺-B-ALLs without expressing the fusion protein.^{21–27} Instead, driver mutations often target *CRLF2*, *EPOR* or *JAK2*, while some mutations categorized as “other JAK-STAT” are involved in IL-7 signaling.²⁸ Importantly, preleukemic changes leading to AYA-B-ALL are unclear and animal models scarce.

Herein, we report for the first time that adult *Irf4*^{-/-} mice spontaneously develop pB-ALL with similarities to AYA B-ALL. A previous report described early onset B-ALL in *Irf4/Irf8*^{-/-} co-deficient mice and used *Irf4*^{-/-} mice as negative controls.²⁹ Our findings indicate that IRF4/8 double deficiency merely speeds up a process, which can be initiated by single IRF4 deficiency. Because one preleukemic alteration might better reflect the human situation and because *Irf4*^{-/-} (but not *Irf4/Irf8*^{-/-}) mice productively develop B cells, they are better suited to examine spontaneous B-leukemogenesis. We herein delineate the steps from disturbed *Irf4*^{-/-} B lymphopoiesis to overt pB-ALL, while comparing it with human AYA-B-ALL and deriving targeted drugs for treating established disease *in vivo*.

Results

***lrf4*^{-/-} mice spontaneously develop preB cell leukemia**

Following the serendipitous finding, that some aged *lrf4*^{-/-} mice developed swellings and succumbed to a consuming disease, we systematically observed 80 *lrf4*^{-/-} mice with an age > 150 days. We detected 14 rapidly progressing tumours (4 in female and 10 in male mice, incidence thus: 17.5 %), that spontaneously appeared in lymph node (LN) areas (mean age at diagnosis: 268 days, median: 238 days, Fig.1a). Tumours were neither detected in mice younger than 150 days nor in C57BL/6 wild-type (wt) mice housed in the same room of our facility. Incidence and delayed disease onset are reminiscent of other spontaneous genetic mouse tumour models such as the KC pancreatic cancer model.³⁰

A representative tumor *in situ* (asterisk) is depicted in Fig.1b. All tumors were accompanied by lymphadenopathy (arrow heads) and increased spleen size, compared to healthy *lrf4*^{-/-} or wt mice (Fig.1c). Suspected lymphomatous origin was corroborated microscopically (Fig.1b), with dense infiltration of mononucleated cells also in the BM, lung, and liver (Fig.1d). Due to the known impaired maturation of *lrf4*^{-/-} preB cells,¹⁰ we reasoned that eruption of preB-leukemia might be the explanation. In a representative spleen section (Fig.1e), infiltrating cells stained positive for B220 and Igμ, (but less than untransformed “follicle” B cells), and highly positive for the proliferation marker Ki67. By flow cytometry, tumour cells infiltrating the spleen (bottom panels, marked with asterisk) expressed no mIgM and low amounts of both B220 (confirming histology) and CD2 (Fig.1f). Further, all tumours expressed λ5 (CD179b; Fig.1f) and CD19 (not shown). In addition, Igμ, but not Igκ or Igλ were found intracellularly (not shown). Flow cytometry also demonstrated tumour cells in peripheral blood (pB), BM and LN (Fig.1g; two exemplary tumours “T8” and “TD2”). Considering the BM infiltration and the devastating progression, these attributes characterize the disease as preB-ALL.

To prove clonality, we performed PCR on the VDJ junctions of the IgH region in three tumours, cloned the amplicons and sequenced multiple clones independently (Table 1). Almost all sequences per tumour were identical, demonstrating clonality. Few detectable alternative sequences probably originated from contaminating non-transformed B cells.

As further characteristic of malignancy, the tumours (two examples) displayed copy number variations (CNV) (Fig.1h), i.e. subchromosomal alterations. Finally, only 500 transferred tumour cells readily elicited disease in wt mice already after two weeks, as tested for tumour 8 (T8) and three other tumours.

Preleukemic alterations affecting *Irf4*^{-/-} B lymphopoiesis

The uniform appearance of preB-ALL in *Irf4*^{-/-} mice suggested a defined preleukemic pro/preB cell vulnerability to immortalization, possibly related to the partial maturation defect. Most likely, a second, possibly again uniform, acquired genetic alteration was necessary for *bona fide* leukemia development and arose with low frequency per time, explaining the affected age and relatively low penetrance.

To identify this hypothetical preleukemic state, we thoroughly analyzed *Irf4*^{-/-} B lymphopoiesis. Dimensional reduction of BM cells stained for four B cell differentiation markers (B220, mIgM, CD2, IL-7Rα), identified a B220^{dim} population (“G1” in Fig.2a) with higher abundance in *Irf4*^{-/-} (5.99 %) than in wt (1.75 %) BM. In turn, another population (“G2”; B220^{hi}) was more prominent in wt mice (7.75 vs. 3.08 %). Marker expressions classified G1 cells as pro/preB cells (B220^{dim}mIgM⁻IL-7Rα⁺CD2^{-dim}) and G2 as more mature B cells (B220^{hi}mIgM⁺IL-7Rα⁻CD2^{hi}). Thus, IRF4 deficiency skews B lymphopoiesis towards less differentiated cells, while still principally allowing maturation, because *Irf4*^{-/-} G2 cells remained detectable (Fig.2a). This disturbed, but nevertheless productive B cell maturation confirms and extends previous reports.¹⁰

Following the Hardy classification of B cell progeny (Fig. 2b) based on B220 and mIgM expression^{31,32} we found the expected decrease in recirculating mature *Irf4*^{-/-} B cells (Fig.2b, “F”), accompanied by a relative increase in pro/preB cells (Fig.2b, “A-D”). To pinpoint the exact developmental hindrance, we measured expression of IL-7R α and of CD2, which accompanies cytosolic Ig μ expression (Fig.2c).³³ Importantly, we noted an increased frequency of preproB and CD2^{-dim}IL-7R α ⁺B220⁺mIgM⁻ proB cells in *Irf4*^{-/-} mice. The respective definition of cell state was corroborated by comparing cell size measurements per subpopulation (Fig.2d). These results characterize the proB to large preB cell step as the definitive bottleneck state in *Irf4*^{-/-} B lymphopoiesis.

Cells with proliferative potential and stagnated differentiation are vulnerable for malignant transformation. To directly demonstrate selective proliferation of IL-7R α ⁺ *Irf4*^{-/-} proB cells without further differentiation, purified BM B220⁺ cells from *Irf4*^{-/-} and wt mice were cultured with IL-7 (Fig.2e,f). After three days, *Irf4*^{-/-} cells had expanded three-fold, whereas wt cell numbers remained stable. A substantial part of the surviving wt B cells had matured under IL-7 influence, shown by a population coexpressing mIgM and B220 (Fig.2e, bottom right). Part of the wt B cells remained in an immature cell state, characterized by surface λ 5 expression (Fig.2f). In contrast, all expanding *Irf4*^{-/-} B220⁺ cells remained mIgM⁻ and therefore more immature, also supported by a greatly increased fraction expressing λ 5 (Fig.2f). The observed relative increase in λ 5⁺ cells (Fig.2f) between *Irf4*^{-/-} and wt after culturing with IL-7 adds to the observed absolute three-fold increase in cell numbers. Thus, IL-7 culturing further accentuated the differentiation impairment beyond the proB/large preB checkpoint (Fig.2c). High IL-7 driven *Irf4*^{-/-} B cell proliferation was corroborated by Ki67 expression (sFig.1a), cluster formation and larger size compared to wt B cells (sFig.1b). Notably, IL-7 dependent *Irf4*^{-/-} pro/preB cell proliferation was fully blocked by NIBR3049,³⁴ a selective inhibitor of JAK3 – associated with γ -chain cytokine signaling downstream of the IL-7R (sFig.1a).³⁵

These data indicate particular significance of *Irf4*^{-/-} pro/preB cell vicinity to IL-7 producing SCs in the BM, which *in vivo* is secured via interaction of CXCR4 on pro/preB cell with the SC-derived chemokine CXCL12.^{6,36} Notably, while *Irf4*^{-/-} and wt pro/preB cells expressed CXCR4, the intensity on *Irf4*^{-/-} cells was markedly lower (Fig. 2g), suggesting reduced adherence to SCs. Together with increased IL-7Rα⁺ *Irf4*^{-/-} pro/preB cell number, these results implied that some cells might lose access to BMSCs and drain into peripheral organs. Indeed, we detected B220⁺mIgM⁻CD2^{-dim} pro/preB cells at strongly increased frequencies within spleens of healthy *Irf4*^{-/-} compared to wt mice (Fig.2h). Interestingly, the frequency of pro/preB cells in the spleen also increased with age in *Irf4*^{-/-} mice.

Recurrent signaling pathway alterations in *Irf4*^{-/-} and human B-ALL

Importantly, peripheral pro/preB cells should face a strong survival stress due to the distance from IL-7 producing SCs. Perhaps, this circumstance was the decisive selection pressure for *bona fide* leukemogenesis, whereby genetic alterations providing IL-7-independency would ascertain aggressive preB cell proliferation. To test for mutations in the IL-7R pathway, we performed whole exome sequencing (WES) of three independent tumours (T8, T10, T11) compared to sorted B220⁺mIgM⁻ cells from *Irf4*^{-/-} BM. Comparing the single nucleotide polymorphisms (SNPs) between the three samples, eleven genes were affected in all three tumour samples (Fig.3a). Among these, *Jak3* is directly involved in mediating signaling via gamma-chain cytokines such as IL-7. All three tumours carried *Jak3* mutations with a frequency of 0.5, probably because the mutations affected one allele in all bulk cells (Table 2). Using Sanger sequencing of *Jak3*, we found two additional mutations in tumour samples TD1 and TD2 (Fig.3b, Table 2). Thus, Five out of five tested primary tumour samples carried *Jak3* mutations. Interestingly, although different SNVs occurred, all targeted either the active kinase domain or the pseudokinase domain regulating JAK3 activity. Some of these SNVs are already known from other leukemia mouse models.³⁷

To clarify the significance of the mutations for IL-7 signaling, we cloned the JAK3^{R653H} and JAK3^{T844M} mutants into a retroviral (RV) expression plasmid, which labels transduced cells with Thy1.1. We then transduced IL-7 dependent, Eμ-Myc transgene driven murine preB-leukemia cells^{38,39} with these RVs and cultured them with or without IL-7. Without IL-7, mutated *Jak3* transferred a clear growth advantage for Thy1.1⁺ compared to Thy1.1⁻ cells not seen after transduction with wt *Jak3* or empty vector (EV-)RV (Fig.3c). Strikingly, this growth advantage was lost in the presence of IL-7. Thus, the *Jak3* mutations are gain of function mutations (GOFs) providing IL-7 independency. Furthermore, selection of JAK3-mutated cells is supported by the lack of IL-7 *in vitro* and therefore potentially by distance from BMSCs *in vivo*.

***Aicda* is upregulated in *Irf4*^{-/-} preB cells by LPS and lack of IL-7**

Next, we focused on the mechanistic pathways of tumourigenesis. Because five out of six *Jak3* mutations were produced by cytosine to thymine exchanges (Table 2), we suspected a more specific mutagenic cause. Many DNA editing enzymes including the APOBEC family member AID can introduce single nucleotide mutations.⁴⁰ AID transforms C:T base pairs into U:T mismatches,^{41,42} e.g. during somatic hypermutation in germinal center B cells.^{43–45} Thereafter, repair mechanisms most often ultimately cause C to T conversions.^{46,47} Notably, AID is induced in wt preB cells by IL-7 withdrawal and LPS stimulation and was implicated as a facilitator of human B-ALL.⁴⁸ Therefore, we compared *Aicda* expression in sorted *Irf4*^{-/-} and wt B220⁺mIgM⁻ pro/preB cells to wt mesenteric LN cells as positive and CD4⁺ T_H1 cells as negative control. Individual leukemia samples were also included. While mLN cells highly expressed *Aicda*, pro/preB cells also expressed readily detectable amounts (Fig.3d). Interestingly, expression was significantly higher in *Irf4*^{-/-} than in wt cells, contrasting earlier results in stimulated mature B cells.⁴⁹

Furthermore, like their wt counterpart,⁴⁸ *in vitro* expanded (see Fig.2e) *Irf4*^{-/-} pro/preB cells upregulated *Aicda* further under LPS treatment or independently with IL-7 withdrawal, while IL-7 suppressed *Aicda* (Fig.3e). This finding poses an attractive explanation as to how BM evasion and potential exposure to microbial pathogens might initiate mutagenic processes in vulnerable *Irf4*^{-/-} preB cells cooperatively.

To test if the observed *Aicda* upregulation correlated with frequent C:T/G:A transitions in *Irf4*^{-/-} leukemia on a genomic level, we performed WES comparing *Irf4*^{-/-} and wt pro/preB cells sorted from BM and T8 tumour cells with matched tail-tip samples (Fig.3f). We found a marked preponderance of C:T/G:A transitions in the T8 sample (Fig.3g), both in all detectable SNPs and when filtering on the core mutations likely reflecting mono- or bi-allelic appearance in all bulk cells. This finding further supports a specific mutagenic process, potentially involving AID, explainable by infectious stimuli and IL-7 withdrawal.

IRF4 re-expression leads to cell death and expression of differentiation markers

As our model was based on *Irf4* deletion as a prerequisite for leukemia, we examined the effect of forced IRF4 re-expression. We established cell lines (T8-1 and T8-2) from the representative, JAK3 mutation carrying, tumour 8 and produced RVs coding for GFP with IRF4 (IRF4-RV) or without (EV-RV). When re-introducing IRF4 into T8-1 (Fig.4a) and T8-2 cells (not shown), GFP⁺ IRF4 expressing cells gradually disappeared over time (Fig.4a). AnnexinV/PI stainings confirmed apoptosis (not shown). EV-RV had no such effect (Fig.4a). We then compared the transcriptome of the cells 24 h after transduction, when viability was still unaltered (Fig.4b,c). Intriguingly, before entering apoptosis, cells re-expressing IRF4 exhibited transcriptomic signs of differentiation beyond the preB checkpoint, such as downregulation of *Ccnd3* and *Igll1* (coding for CD179b), but upregulation of *Rag1* and germline *Igkv* transcripts (Fig.4b). Therefore, fully transformed leukemia driven by *Jak3* GOF mutations remained targetable by IRF4 re-expression.

***Ptk2* is upregulated in *Irf4*^{-/-} leukemia and cells depend on clustering for survival**

To investigate, whether IRF4 deletion might have further oncogenic effects apart from promoting *Jak3*-GOFs, we compared gene expression of three tumours with that of sorted *Irf4*^{-/-} B220⁺ mIgM⁻ pro/preB cells. Differentially expressed genes were then reduced to only those altered by IRF4 re-expression (Fig.3b,c) and matched with a published ChIPseq of genes bound by IRF4 in B cells.⁵⁰ The resulting genes (Fig.5a) were particularly notable for *Ptk2* (encoding focal adhesion kinase, FAK), which was upregulated in leukemia cells (Fig.5a “Tumour vs. BM”) and suppressed by IRF4 overexpression (“IRF4-RV vs. GFP-RV”). FAK integrates signals generated through cell adhesion with relevance for cancer cell survival in solid tumors.^{51,52} Interestingly, FAK also regulates the localization of pro/preB cells in their BM niche⁵ and is associated with stromal adhesion in BCR-ABL⁺ B-ALL.⁵³ We focused on FAK also, because cultured T8.1 cells (Fig.5b) and *Irf4*^{-/-} pro/preB cells formed striking cell clusters (sFig.1b). Apparently, these clusters were needed for cell survival, as demonstrated by marked reductions in viability at lower cell densities even under supply with exogenous IL-7 (Fig.5b). Higher FAK quantities in tumour versus untransformed mIgM⁻ *Irf4*^{-/-} pro/preB samples were confirmed on a protein level using Western Blot, while mature mIgM⁺ B cells of *Irf4*^{-/-} or wt genotype contained the lowest amounts (Fig.5c).

Small compound agents including inhibitors of FAK and JAKs kill *Irf4*^{-/-} leukemia cells *in vitro*

To complete our analysis of signaling pathways, we screened kinase inhibitors for their capacity to kill *Irf4*^{-/-} leukemia cells *in vitro*. We included Ruxolitinib, an inhibitor of JAK1/2 downstream of JAK3, Defactinib targeting FAK, and Dexamethasone, a cornerstone for treating lymphomatous malignancies. Furthermore, we included inhibitors of NFκB (IKK, TAK1), JNK, MEK, ERK, PP2A, GFI1 and of the Bruton tyrosine kinase (BTK) acting downstream of the BCR.

A variety of these substances potently killed tumour cells (sFig.2a). Efficacy of Ruxolitinib and Defactinib corroborated our genetic results. The strong activity of dexamethasone was also expected. Furthermore, inhibitors of GFI1 and PP2A as well as NKkB and JNK (both also active in a diffuse-large-cell-B-cell-lymphoma model)⁵⁴ were very potent. In contrast, inhibiting BTK, MEK and ERK had no impact on cell survival. We also found clear synergistic effects between Ruxolitinib and Dexamethasone (a combination successfully tested in a mouse model of T-ALL)⁵⁵ and between Defactinib and Dexamethasone or the JNK inhibitor (sFig.2b).

***Irf4*^{-/-} mouse leukemia shares resemblance with a subset of human Ph-like ALL**

Next, we wanted to address the question, whether the herein described correlation between IRF4 deletion and secondary *Jak3* GOFs is transferable to human disease. Progenitor B-ALL has been classified into six groups, five of which are defined by clear genetic drivers. Within the sixth group, hierarchical clustering identified patients with similar transcriptomic profiles as BCR-ABL1 carrying (Ph⁺) ALL. Being BCR-ABL1 negative, this disease subset has thus been termed BCR-ABL1 like or Ph-like ALL.^{22,25–28,56–58} A previous report elucidating the genetics of this subset of patients found recurrent genetic alterations in signaling kinases that partially explained the Ph-like transcriptomes.²⁸ Especially, JAK2 and CRLF2 were altered in many cases. A further subset of Ph-like ALL however, defined as “other JAK-STAT” (sFig.3a), harbored mutations affecting IL-7 receptor signaling, including alterations in JAK3 and JAK1. In the 154 patients analyzed, 2.6 % of all Ph-like patients carried JAK3 mutations and none of these carried detectable gene fusions, perhaps representing a further subgroup.²⁸ Because of the homogeneous presence of GOF *Jak3* mutations in our model and because of its clear protein kinase-dependent, but gene-fusion negative oncogenesis, we considered Ph-like-B-ALL as particularly attractive for comparison. Indeed, while B-ALL preferentially arises in children, the incidence of Ph-like-B-ALL increases from 10 % in children to above 25 % in AYA and adults (sFig.3b),^{28,57} reminiscent of the older age in affected mice. Furthermore, the Ph-

like ALL study had reported drastic reductions of IRF4 transcripts in the published dataset of 154 Ph-like-B-ALL cases, when compared to all other B-ALL subtypes (sFig.3c).²⁸

Finally, to further compare our disease model to human Ph-like ALL, we sought to apply the set of 38 genes, used to initially contrast Ph-like ALL against other forms of B-ALL in the COALL (German) and DCOG (Dutch) ALL study cohorts (referred to as “Den Boer gene set”).²⁴ Out of these 38 genes, transcription of only 15 genes was reported in the supplemental information to the Ph-like study we used as reference.²⁸ When comparing regulations of these remaining 15 genes between the two scenarios “Ph-like vs. other B-ALL”²⁸ and “*Irf4*^{-/-} leukemia vs. *Irf4*^{-/-} progenitor B cells” (our study), we found that our scenario correlated with similar expression changes compared to Ph-like ALL patients (sFig.3d); perhaps due to the homogenous presence of *Jak3* GOFs. Not all 15 genes were regulated in our scenario, but at least none of the genes showed obvious differences in regulation compared to the Ph-like ALL patients.

Taken together, these findings suggest sound relevance of our data to human B-ALL. Intriguingly, our model probably resembles only a small subset of Ph-like ALL, hinting at the quite substantial notion, that human Ph-like ALL itself encompasses a still diverse cancer biology.

***In vivo* therapy of established *Irf4*^{-/-} B-ALL**

We then attempted to apply our knowledge *in vivo*. In preliminary studies, *i.p.* rather than *s.c.* transfer of T8.1 tumour cells led to homogenous appearance of leukemia. We adoptively transferred 3 x 10⁵ T8.1 cells *i.p.* into wt mice and started treatment around day 14 thereafter, when overt leukemia (Fig.6a) and deterioration was observed. To imitate clinical protocols, we started induction therapy with Dexamethasone. Within one week, leukemic cell numbers in pB were robustly reduced, although few cells remained detectable, again with very little variability (Fig.6a). We then started maintenance therapy with Ruxolitinib or vehicle control by oral

gavage twice daily (Fig.6a-d). In a similar experiment, Defactinib was tested (sFig.4a-c). Both substances were selected from our panel because clinical experience exists for both.^{59–62} Importantly, the half-life of Ruxolitinib in mice is only 0.8 h (from the “Australian Public Assessment Report for Ruxolitinib”, by the Department of Health of the Australian Government), while oral bioavailability of Defactinib is also low in mice.⁶³ Therefore, any effectiveness in our model likely underestimates the potential in humans. To limit stress burden, we ended gavage after 12 days but further monitored mice.

Despite maintenance therapy, leukemic cells in pB reappeared, with little difference between treatment groups (Fig.6a). However, we detected a clear survival benefit for mice treated with Ruxolitinib (Fig.6b) and less so also with Defactinib (sFig.4a). Surprisingly, sick sham-treated mice showed a striking phenotype: within seconds after gavage, temporary limpness of tail and hind legs occurred, reminiscent to experimental autoimmune encephalomyelitis. Video supplements 1 and 2 show a mouse before and 30 s after treatment intervention. Initially, paraparesis was only triggered by our intervention and vanished shortly after, but during disease progression the symptom's duration increased, until mice presented permanent paraparesis and had to be sacrificed. We established a scoring system quantifying duration of paraparesis (from 0 to 3, see Methods for details). Most importantly, this symptom was almost abolished during Ruxolitinib treatment (Fig. 6c,d), and considerably relieved by Defactinib (sFig.4b,c). We reasoned that this symptom might be due to the increased intraabdominal pressure exerted during gavage. If plexus nerves or spinal nerve roots of the lumbar region were to have a preexistent lesion, this temporary increase in pressure might explain the transient paraparesis through mechanical stress induced reductions in conductivity.

To investigate the basis for this symptom, we performed high-resolution ultrasound. Strikingly, mice with score 3 displayed an echogenic and heterogeneous paravertebral mass in the psoas muscle region (Fig. 6e) which was significantly smaller in score 0-1 mice (Fig.6f) and appeared

less echogenic (Fig.6g). By histology, score 3 correlated with severe infiltration of mononucleated blasts into muscles, but more importantly also intrathecally around the spinal cord (X in Fig.6h), extending into the lumbar spinal nerve roots (arrowhead in Fig.6h). Infiltration was decisively reduced by Ruxolitinib treatment (score 0 in Fig.6h), resembling the picture of tumour-free mice (left panels). A quantification of differences in perimyeloid infiltration (X in Fig.6g) is shown in Fig.6h. As for BM, leukemia cells abolished normal hematopoiesis almost totally in sham-treated compared to tumour-free mice (CAE staining in Fig. 6g). Interestingly however, and in contrast to neural infiltration, BM infiltration was only partially resolved by Ruxolitinib.

These findings raise the possibility that Ruxolitinib and Defactinib preferentially target infiltrated solid organs and leukemic meningeosis rather than BM or pB. In support of this idea, Ruxolitinib treatment fully blocked liver infiltration as compared to tumour-free or sham-treated mice (sFig.4d,e). As tissue infiltration is regulated by homing receptors on invading cells, we treated T8.1 cells with Ruxolitinib *in vitro* and checked the expression of CD29 (integrin $\beta 1$), which pairs with various integrin alpha chains involved in multiple cell interactions and tissue adhesion.^{64,65} Notably, T8.1 cells expressed CD29 and Ruxolitinib reduced this expression dose-dependently (sFig.4f) while it even slightly increased expression of MHC I molecules (H2D^b, H2K^b), stained as specificity control. Possibly therefore, Ruxolitinib modulates the expression of adhesion molecules needed for extravasation into parenchymatous organs. If so, the compound might hinder infiltration of tumour cells into solid tissue more efficiently than into BM, thus explaining persisting blasts in the blood.

Discussion

It has been previously demonstrated that *Irf4/Irf8*^{-/-} as well as *Irf4/Spi1*^{-/-} mice early develop leukemia at high rates.^{29,66} In contrast to these reports, we report that single deficiency for IRF4 fully suffices to establish leukemia – albeit with less penetrance and only in older mice. We

found unchanged expression and gene sequence of IRF8 (not shown) and normal amounts of *Spi1* transcripts (sFig.5a) in *Irf4*^{-/-} pro/preB cells.

Similar tumours were also described in *Pax5*^{+/-} mice, a transcription factor operating at a similar B lymphopoiesis checkpoint.⁶⁷ Importantly, *Pax5* expression is even upregulated in *Irf4*^{-/-} pro/preB cells (sFig.5b), thus excluding that our model simply reflects reduced PAX5 levels. As PAX5 controls IRF4 expression in preB cells,⁶⁸ *Pax5*^{+/-} leukemia may in turn depend on reductions in IRF4. If so, two scenarios exist. First, the *Pax5*^{+/-} genotype might imitate an IRF4 knockout because regulatory complexes binding the *Irf4* locus could depend on a certain threshold amount of PAX5. For IRF4, such a scenario exists, because some regulatory programs are fully inactive even with only lowered IRF4 expression.⁵⁰ Second, *Pax5*^{+/-} could cause intermediate (not fully lacking) IRF4 expression comparable to *Irf4*^{+/-} mice. Since we found only one spontaneous *Irf4*^{+/-} leukemia, any directly linked tumorigenesis of the two factors would then attribute the higher incidence in *Pax5*^{+/-} mice to the intensely employed microbial stimuli as a difference in the two model environments.⁶⁷ Deficiency of PAX5 or IRF4 could, however, also cause tumours independently and phenotypic similarities could stem from individual activities on a related developmental checkpoint. Taken together, our study stresses the general vulnerability of pro/preB cells to immortalization. Secondary steps for mutagenesis may include infectious agents as recently found empirically in the *Pax5*^{+/-} model⁶⁹ and also supported by our finding of *Aicda* upregulation by LPS.

Why is the lack of IRF4 in B cell progenitors oncogenic at all? As mentioned above, IRF4 regulates counteracting pathways at the preBCR checkpoint and several alterations in *Irf4*^{-/-} B lymphopoiesis were described.^{1,11,66,70,71} Our study extends this knowledge to comprehensively delineate multiple preoncogenic alterations in *Irf4*^{-/-} B cell progenitors: 1) Differentiation: Disturbed differentiation beyond the preBCR checkpoint (Fig.2 and ¹⁰). 2) Adhesion: Migration away from the IL-7-providing BM niche (Fig.2h), along with lowered CXCR4 expression (Fig.2g

and 1). 3) IL-7 signaling: *Irf4*^{-/-} pro/preB cells are more sensitive to IL-7 signaling, resulting in increased proliferation *in vitro*⁶⁶ without differentiation (Fig.2f). Thus, without IRF4, IL-7R signaling overcomes the antiproliferative and prodifferentiative activity of the preBCR.⁷² Additionally, our data implies that the observed early BM evasion and withdrawal from IL-7 causes increased AID expression in *Irf4*^{-/-} preB cells, as a potential culprit for generating oncogenic mutations (Fig.3g).

Most importantly, when reviewing published data of B-ALL patients,²⁸ we found that the Ph-like B-ALL subtype presents with lowered *IRF4* (sFig.3c) and *PAX5* expression (sFig.5c) and includes mutations in *JAK3* in specific subtypes. Furthermore, we showed that within the Ph-like predictor gene set devised by Den Boer et al.,²⁴ our leukemia model shared considerable resemblance with a Ph-like patient cohort. (sFig. 2d). However, Ph-like B-ALL may encompass further subtypes, only part of which our model imitates: For instance, while we report recurrent *JAK3* and never *JAK2* GOFs, the human disease more commonly includes *CLRF2*- and *JAK2*- and less *JAK3*-alterations. Potentially, different pathways predispose for leukemia at differing pro/preB cell checkpoints. In our study, IRF4 deficiency predisposes for independence of IL-7 via *JAK3* GOFs, because the *Irf4* knock-out renders cells vulnerable to transformation at precisely that differentiation stage, where IL-7 controls cell-cycling. *JAK2* does not act downstream of the IL-7R- γ chain complex but of CD117 (c-kit) and TSLPR (encoded by *CRLF2*) instead.^{73,74} *JAK2* GOF carrying cells could therefore represent a differing subset of progenitor B cells that depends more heavily on TSLPR or CD117 signaling. Thus, a similar selection pressure as in *Irf4*^{-/-} mice but affecting a different pathway, might provoke mutations in *JAK2*. However, we also cannot rule out the possibility, that TSLPR signaling (which also depends on the IL-7R α chain⁷⁴) differs in its functions in B cell differentiation between humans and mice. Altogether, we hypothesize, that Ph-like B-ALL comprises several leukemia forms arising from the narrow pro/preB cell window, which share homologies but remain heterogeneous in their pathophysiological (e.g. IRF4 deregulation) and genetic landscape.

This hypothesis is supported by the distinct subtypes of Ph-like ALL that C. Mulligan and colleagues proposed based on mutational signatures (sFig.3a and ²⁸).

As proof of its usefulness as a disease model, the *Irf4*^{-/-} pB-ALL raised several therapeutic options. Intriguingly, forced IRF4 re-expression caused differentiation and cell death, encouraging treatments to boost its expression. Moreover, our transcriptomic survey highlights a fourth oncogenic (and targetable) role of IRF4-deficiency to those described above: 4) Proximity signaling: IRF4 loss upregulated FAK expression, securing cell survival likely via integrin mediated cell contacts. Targeting FAK is currently under investigation for treating solid tumors,^{59,60} but our preliminary data give logic to trials also in IRF4 deregulated B-ALL.

Nevertheless, our *in vivo* experiments highlight Ruxolitinib as the most suitable treatment for JAK driven leukemia. This compound represents an important therapeutic agent in myeloproliferative disease and is already considered for treatment of Ph-like-ALL.^{61,62} We found a surprising and novel preferential effect of Ruxolitinib on CNS- and organ infiltration, with less pronounced activity on leukemic cells in the BM. SCs may provide a sanctuary rendering leukemic cells less sensitive to targeting by Ruxolitinib.⁵ However, others showed clear anti-leukemic Ruxolitinib effects in a murine Ph⁺ ALL model.⁷⁵ Perhaps the more sensitive effects on organ infiltration are demasked only in generally less sensitive tumour types. Especially the effects on leptomeningeal involvement are of key translational importance because current CNS-targeted therapies for ALL remain toxic, significantly lowering quality of life for patients. We provide preliminary evidence that this Ruxolitinib effect might involve downregulation of surface integrins. Future studies should test this hypothesis mechanistically and evaluate efficacy of Ruxolitinib for leukemic organ infiltration also in other leukemia models.

Methods

Mice

C57Bl/6 mice were purchased from Charles River, Sulzfeld, Germany. *lrf4*^{-/-} mice¹⁰ were bred on the C57Bl/6 background and housed in the animal facility of the Biomedical Research Center at the university of Marburg, Germany. If not stated otherwise, all mice used in the presented experiments were 8-12 weeks old and sex-matched.

Tumor cell lines and cell culture

Stable tumor cell lines T8.1, T8.2 and T11 were established from primary *lrf4*^{-/-} leukemia cells (derived from primary tumour 8, i.e. T8 or tumour 11) by culturing them on a monolayer of irradiated (30 Gy) ST2 stromal cells⁷⁶ grown to confluency in Opti-MEM medium (31985070, ThermoFisher Scientific) supplied with 1 % cell culture supernatant from JIL-7.6 J558 cells⁷⁷ (a gift from Fritz Melchers, Berlin) as a source of IL-7. After several passages, T8 and T11 cells grew independently of ST-2 cells. For *in vitro* inhibitor experiments, 2.5 x 10⁵ T8.1 or T8.2 cells (or T11 cells, not shown) were cultured in 500 µL RPMI medium in 48 well plates in the presence of the indicated amounts of inhibitors. To determine the percentage of viable cells, samples were stained using Annexin V and propidium iodide (PI) (see below) after 48 h. Substances used include: Defactinib (S7654, Selleckchem), Oxocaenol (O9890, Sigma), GANT61 (Sigma, G9048), SP203580 (EI-286-0001, Enzo), SP600125 (EI-305-0010, Enzo), PD98059, Promega), Ibrutinib (S2680, Selleckchem), BAY11-7082 (ALX-270-219, Alexis), Dexamethasone (PZN 08704491, mibe GmbH) and Ocadaic acid (O4511, Sigma).

Murine pro/preB cell cultures

Femur and tibia bones from 8-12 weeks old mice were explanted and cleaned from adherent tissues. Cells were extracted via centrifugation at 11×10^3 RPM for 10 s. Total BM cells were enriched for B220⁺ B lineage cells using an inhouse magnetic activated cell sorting protocol. Briefly, whole bone marrow cells were stained with a mix of FITC-conjugated antibodies to CD11b, B220, Ter119, CD49b, CD4 and CD8 (all from eBioscience), followed by incubation with an anti FITC/streptavidin/biotin/magnetic bead complex (Miltenyi Biotec) and magnetic sorting using a microcentrifugation tube stand (Miltenyi Biotec).⁷⁸ Sorting efficiency, as confirmed by flow cytometry, routinely exceeded 90 %. Cells were seeded at a density of 1×10^5 cells per well in 200 μ L RPMI complete (96-well plates, Greiner). Pro/preB cell cultures were propagated with 20 ng/mL rmlL-7 (217-17, Peprotech) in RPMI-1640 medium complete (R8758, Sigma-Aldrich, supplemented with: 10% FCS (Sigma-Aldrich), 2mM L-glutamine (Biochrom), 50 μ M β -mercaptoethanole (Sigma-Aldrich), 0,03/0,05 g per 500 mL Penicillin G/Streptomycin Sulfate, 1 % non-essential amino acids (PAA Laboratories)). In some experiments, pro/preB cells (1.25×10^6 /mL medium) were treated for 24 h with LPS (Sigma, 1 μ g/ml), anti-IL-7 (BioXCell, 10 μ g/ml), rmlL-7 or respective combinations, before generating mRNA for qRT-PCR.

Flow cytometry and cell sorting

For surface staining of B lineage markers, cells were harvested, resuspended in PBS/1% FCS and stained with anti-B220 (RA3-6B2, Biolegend), anti-IgM (II/41, BD), anti-CD2 (RM2-5, Biolegend), anti-CXCR4 (L276F12, Biolegend), anti-CD127 (=IL-7R α) (A7R34, BD Bioscience), anti-CD179b (= λ 5) (LM34, BD Bioscience) as indicated (20 min at room temperature in the dark). All antibodies were employed at a dilution of

1:500. Fluorescence was recorded using either a FACS Aria III (BD) or an Attune NxT (Thermo-Fisher) analyzer. Data analysis was performed using the FlowJo V10 software (BD). For dimensional reduction we used the t-Distributed Stochastic Neighbor Embedding (t-SNE)⁷⁹ algorithm built into FlowJo V10. Epitopes on BM cells from *Irf4*^{-/-} and wt control mice used for dimensional reduction analysis comprised B220, membrane-bound (m)IgM, CD2 and CD179b. For RNA and WES analyses, BM cells were surface labeled for B220 and mIgM expression and B220⁺ mIgM⁻ cells were sorted using a FACS Aria III (BD Bioscience). Sorting efficiency was routinely above 95 %. To determine cell viability, AnnexinV/propidium iodide (PI) staining was performed using 5 μ L AnnexinV (640905, Biolegend) per 500 μ L HBSS. After 20 min of incubation at room temperature in the dark, 1 μ L propidium iodide (421301, Biolegend) was added and cells were immediately measured.

Copy number variations (CNVs) analysis

CNVs were analyzed in tumour samples 10, 11 and 14 and compared to *Irf4*^{-/-} normal tail tissue. Whole DNA was extracted from 5 x 10⁶ cells per sample using the Macherey-Nagel NucleoSpin Tissue kit (REF 740952.50) according to the manufacturer's protocol. Library preparation was performed using the Illumina Nextera DNA kit according to manufacturer's instruction. Sequencing was performed on an Illumina-HiSeq-1500 platform in rapid-run mode at the Genomics Core Facility of Philipps-University Marburg. Fastq quality control was performed using custom scripts. Raw sequenced reads were aligned to the Ensembl Mus musculus reference (revision 79) using Bowtie2 (version 2.0.0)⁸⁰ with standard parametrization. Analysis of CNVs was performed using the cn.mops (Copy Number estimation by a Mixture Of PoissonS) package (version 1.18.1)⁸¹ with the following parametrization: prior

impact = 1, lower threshold -0.9, upper threshold = 0.5 minimum width = 4. Window length was set to 10000 and the algorithm was run in unpaired mode.

Histological analyses

Tissue samples were immediately fixed in 4 % formaldehyde in PBS solution. Histological analysis was performed on 3 μ m thick sections from paraffin embedded tissue as described previously.⁸² Briefly, rehydrated paraffin sections were first blocked with 0.3 % H₂O₂ and goat normal serum. For immunohistochemical (IHC) stainings, rat antibodies against CD45R/B220 (clone RA3-6B2, BD) and KI67 (clone TEC-3, Dako) were then incubated on the tissue slices and bound antibody was detected with biotinylated goat anti-rat IgG (Southern Biotechnology). Bound antibody was visualized with the Vectastain-kit (Vector Laboratories) according to the manufacturer's protocol. Hematoxylin-Eosin (HE) stainings were performed according to standard procedures. Cells of the granulocytic lineage were stained on paraffin embedded tissues with the Naphthol AS-D Chloracetate (Specific Esterase, CAE) Kit (Ref: 91C-1KT, Sigma-Aldrich) according to the manufacturers protocol.

In the in vivo therapeutic experiments, we calculated the narrowing of the spinal cord using the equation $A_t/(A_{sca}-A_{sp})$, where A_{sca} is the area of the spinal canal, A_t that of the tumor and A_{sp} that of the spinal cord area. Two different cross sections per animal were examined. The infiltration of the liver was calculated by dividing the tumor area in the liver by the whole area of the liver section. One liver section was analyzed per animal. All measurements were performed using Fiji.⁸³

Whole Exome Sequencing and biostatistical analysis

To determine single nucleotide variants (SNV) within leukemia samples, genomic (g)DNA was extracted both from primary *Irf4*^{-/-} tumours as well as FACS-sorted control B220⁺ mIgM⁻ BM pro/preB cells using the High Pure PCR Template Preparation kit from Roche (11796828001). Integrity of resultant gDNA was confirmed in a 2 % Agarose gel. MacroGen in Seoul performed SureSelect All Exon V6 library preparation and sequenced exons on a NovaSeq platform producing 2 x 150 bp reads at a coverage of 100x (50x on-target coverage). Fastq quality control was performed using FASTQC (version 0.11.9). Raw sequenced reads were aligned to the Ensembl Mus musculus reference (revision 96) using STAR (version 2.6.1d) using default parametrization. Soft-clipped aligned reads were then subjected to variant calling analysis. Position-wise pile-up files were generated using samtools (version 1.9) with the mpileup option and a pileup quality threshold of 15, both for single sample and matched variant calling. Subsequently, variant calling was performed for SNP and InDel detection using VarScan2 (version 2.3.9) on single samples with the following parametrization: sampling depth = 100000, minimum variant frequency = 0.05, minimum coverage = 8, minimum variant reads = 2, minimum average read quality = 15 and a p-value threshold was set to 0.05. Only primary alignments were considered, the strand filter was enabled, and duplicates were removed. As a comparison, matched tumour-normal variant calling was performed with VarScan as well using identical parameter setting with the somatic p-value threshold set to 0.05.

For Fig.3H raw sequenced reads were aligned to the Ensembl Mus musculus reference (revision 96) using Burrows-Wheeler Aligner (BWA version 0.7.17) using default parametrization.⁸⁴ Prior to variant calling, aligned reads were filtered using a custom filter that excludes reads with more than 3 mismatches, more than 2 indels or

a mapping quality below 20 using pysam (version 0.16.0.1). Duplicates were marked and removed using Picard (GATK version 4.1.6.0).⁸⁵ Filtered aligned reads were then subjected to variant calling analysis. Position-wise pile-up files were generated using samtools (version 1.9) with the mpileup option and a minimal base quality threshold of 20. Subsequently, variant calling performed for SNP detection using VarScan2 (version 2.4.4) using matched tumor-normal (somatic) mode with the following parametrization: sampling depth = 100000, minimum variant frequency = 0.2, minimum coverage = 8, minimum variant supporting reads = 5, minimum average read quality = 20 and a somatic p-value threshold was set to 0.05. Only primary alignments were considered, the strand filter was enabled. SNP calls were filtered to high confidence somatic mutations using VarScan's somaticFilter method, SNPs with a variant allele frequency above 0 in the matched reference sample were excluded.

Sanger Sequencing and polymerase chain reaction

SNVs in the JAK3 gene were confirmed by Sanger sequencing of PCR fragments spanning the Jak3 pseudokinase and kinase region (primers used for PCR amplification and Sanger Sequencing: mJAK3 for, mJAK3 s. supplemental data). Sequencing services were provided by Microsynth Seqlab. To determine clonality of tumour cells, the V μ H region was amplified by PCR. Amplicons were run on an agarose gel and extracted using the QIAquick Gel Extraction Kit (Qiagen). DNA fragments were then cloned into the vector pJet1.2 (Thermo Scientific) and transformed into DH10B E. coli. The indicated numbers of clones (Fig. 1G) for each PCR amplicon were sequenced and aligned with software from IMGT/V-quest.⁸⁶

Retroviral transduction of *Jak3*-mutants and IL-7 independency assay

The coding sequence of murine *Jak3* was amplified from pCineo-Jak3 (a gift from Olli Silvennoinen from Tampere-university in Finland) and cloned into the pMSCV-Thy1.1 expression plasmid using *Bam*HI and *Sall* restriction digestion. Site directed mutagenesis was performed following the manufacturer's protocol using the Quick-Change II site-directed mutagenesis kit (Agilent Technologies; primers employed are listed in the supplemental materials). Viral supernatant from mutated pMSCV-Thy1.1-*Jak3* constructs was produced as described previously.⁷⁸ For viral transduction, 5 x 10⁵ IL-7 dependent E μ -Myc transgene driven murine 35911 preB cells^{38,39} were resuspended in 400 μ L DMEM medium (D5030, Sigma-Aldrich) with 600 μ L viral supernatant plus 1.5 μ L polybrene and spun in culture plates at 2700 RPM for 90 min at 37 °C. Cells were then replenished with conditioned medium and rested for 24 h. Transduction efficiency was measured by flow cytometry using surface staining for Thy1.1 (OX-70, Biolegend). For the IL-7 independency assay (Fig. 3 B), transduced cells. split and cultured with either 1 ng/mL recombinant murine (rm)IL-7 or 10 μ g/ml neutralizing anti-IL-7 antibody (BE0048, Bio X Cell). Cells were harvested at 1, 4, 7, 10 and 14 days post transduction and surface-stained for Thy1.1 expression.

RNA sequencing and biostatistical analysis

RNA extraction from primary tumour samples and FACS sorted B220⁺ mIgM⁻ pro/preB cells was performed using Trizol extraction. Quality control was performed using the Bioanalyzer RNA 6000 NanoChip (Agilent Technologies). Library preparation was performed at the Institute for Immunology, University Medical Center of the Johannes Gutenberg-University Mainz using the NEBNext Ultra Library Prep kit (New England Biolabs). For deep sequencing, the Illumina-HiSeq- 4000 platform was used (Beijing

Genomic Institute). Quality control on the sequencing data was performed with the FastQC tool (version 0.11.2, <https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). RNA sequencing reads were aligned to the ENSEMBL Mus_musculus.GRCm38 reference genome. The corresponding annotation (ENSEMBL v76) was also retrieved from ENSEMBL FTP website. The STAR aligner (version 2.4.0j) was used to perform mapping to the reference genome. Alignments were processed with the featureCounts function⁸⁷ of the Rsubread package, using the annotation file also used for supporting the alignment. Exploratory Data Analysis was performed with the pcaExplorer package.⁸⁸ Differential expression analysis was performed with DESeq2 package,⁸⁹ setting the false discovery rate (FDR) cutoff to 0.1. Data comparing IRF4-RV and GFP-RV samples was visualized as a volcano plot using R package ggplot2: $-\log_{10}$ of Benjamini-Hochberg adjusted P-values were plotted against $\log_2$ fold change of transcript-reads comparing T11 cells transduced according to the protocol described above for *Jak3*-mutants with either IRF4-EV or GFP-EV (transduction efficiency routinely above 85 %). Biological relevance was defined as a $\log_2$ fold change of > 1 or < -1 , while false positives were controlled with a false discovery rate (FDR) set to 0.1. Identification of genes involved in leukemogenesis and regulated by IRF4 was performed as a multistep process: first, genes were included into the analysis, that showed > 2 -fold expression change between *Irf4*^{-/-} B220⁺ mIgM⁻ pro/preB cells and leukemia samples. Second, genes were filtered to only include genes that are also differentially regulated in IRF4-RV or EV-RV transduced T11 cells. Third, resultant genes were limited to genes previously shown to be bound by IRF4 using ChIP-seq.⁵⁰

Quantitative real time (qRT-)PCR

Total RNA was extracted both from primary *Irf4*^{-/-} tumors as well as FACS-sorted control B220⁺ mIgM⁻ BM pro/preB cells of either *Irf4*^{-/-} or wt animals using the Gdansk extractme kit (EM09.1) according to the manufacturers protocol. cDNA was prepared from whole RNA samples using the RevertAid cDNA kit from Thermo Fisher (K1621). qRT-PCR for *Aicda*, *Spi1* and *Pax5* was performed using the SybrGreen MasterMix reagent (4385612, AppliedBiosystems) in a StepOnePlus cycler (AppliedBiosystems). Data was analyzed as percentage of HPRT using the formula $x = 1 / 2^{(\text{cycles}_{Aicda} - \text{cycles}_{HPRT})} * 100$.

Western blotting

To quantify FAK protein, sample cells were lysed in 100 μ L RIPA buffer (Sigma-Aldrich, supplemented with 1 μ L protease inhibitor, 1 μ L EDTA, 0.2 mM Na₃VO₄, 20 mM NaF) on ice. 20 mg of total protein were loaded per lane and proteins were detected according to standard protocols using 10 % SDS-PAGE and PVDF-blotting membranes. Antibodies used: anti-FAK (AHO0502, Invitrogen) at 1:1000 in blocking buffer (washing buffer supplemented with 5 % BSA), goat anti-rabbit IgG HRP (sc-2004, Santa Cruz) at 1:1000 in blocking buffer, anti- β -Actin (AC-15, Sigma-Aldrich) at 1:10000 in blocking buffer, goat anti-mouse HRP (sc-2055, Santa Cruz) at 1:1000 in blocking buffer. Quantification of relative signal intensities was performed in ImageJ2.⁹⁰

In vivo therapeutic studies and ultrasound imaging

Mice were injected with 3x10⁵ T8.1 cells intraperitoneally and monitored daily for clinical symptoms. When mice began showing signs of general morbidity, leukemia

was confirmed by FACS analysis of tail vein blood for B220⁺ mIgM⁺ blast cells. When blast cells in pB reached 25 (mean 50) %, therapy was initiated with oral Dexamethasone (Jenapharm) at 6 mg/L supplied *ad libitum* in the drinking water for seven days. Maintenance therapy comprised either Ruxolitinib phosphate (S5243, Sellekchem) 1 mg (in 2% DMSO, 30% PEG300 in H₂O, as proposed by the manufacturer), Defactinib (S7654, Sellekchem) 1.2 mg (in 5 % DMSO, 50 % PEG300, 5 % Tween 80 in H₂O, as proposed by the manufacturer) or vehicle control (5 % DMSO, 50 % PEG300, 5 % Tween 80 in H₂O) administered twice daily via oral gavage. During the course of disease, this treatment led to paraparesis of the hind legs and tail (video 1 before and video 2 immediately after gavage). A clinical scoring system was established according to the extent of paraparesis and mice were scored daily accordingly: Scores 0-3: 0) no paraparesis, 1) paraparesis induced by treatment intervention, resolves within 30 s, 2) paraparesis induced by treatment intervention, does not resolve within 30 s, 3) persistent paraparesis, independent of treatment intervention. Score 3 led to sacrifice of affected mice. High-resolution ultrasound imaging was performed using a Visual Sonics Vevo 2100 System (FUJIFILM VisualSonics, Toronto, Canada) with microscan transducer MS-550-D, 22-55MHz (FUJIFILM VisualSonics, Toronto, Canada) as described previously.⁹¹

Data availability

The sequencing datasets generated during the current study have been deposited in the Gene Expression Omnibus (GEO) archive and are available under the accession number GSE157958.

Statistical analysis

Statistical analysis was performed using the GraphPad 9.0 software. Data are commonly presented as mean $\pm$ SD. Prior to significance testing, normal distribution and homogeneity of variances was confirmed by Shapiro–Wilk test and Brown–Forsythe testing. Statistical significance when comparing two normally distributed groups was evaluated using two-tailed unpaired t-tests. In case of significant differences in variances between groups, Welch's correction was applied to account for non-normal distribution of data. When comparing multiple groups, one-way or two-way analysis of variance (ANOVA) was performed, depending on the number of variables that differed between compared groups. This was followed by a Tukey's HSD, or Dunnett's *post hoc* test, as indicated in figure legends. An alpha level of $P < 0.05$ was employed for significance testing. Thresholds of $P < 0.05$, 0.01 , 0.001 and 0.0001 are marked by one to four asterisks respectively (also detailed in each figure legend).

Study approval

All animal experiments were approved by the local government (Regierungspräsidium Gießen, G49/2018) and conducted according to the German animal protection law.

Primers

Sanger Sequencing	
mJAK3 for	5' CCCGTCCTGCTGTGCGCTGACAC 3'
mJAK3 rev	5' GGA CTCGGGATGCCAGGTGTGG 3'
mJAK3 mid part	5' CGATGTCTGGAGTCCATGACCTTCAG 3'
Mutagenesis and Cloning	
mJAK3 T844M for	5' CCCCCTGGGGGACAATATGGGACCCCT 3'
mJAK3 T844M rev	5' AGGGGTCCCATATTGTCCCCCAGGGGG 3'
mJAK3 R653H for	5' ATCACCCCCCTCATGAGCCAGGAGCAC 3'
mJAK3 R653H rev	5' GTGCTCCTGGCTCATGAGGGGGGTGAT 3'
mJAK3 for <i>Bam</i> HI	5' AAAGGATCCATGGCACCTCCAAGTGAGGAGACACCTC 3'
mJAK3 for <i>Sall</i>	5' AAAAGTCGACTATCCGGGTCTTCCACGCCACAGC 3'
VμH clonality PCR	
V μ H for	5' GTGCTGGGCAGGAAGTCCCG 3'
V μ H rev	5' AGGTSMARCTGCAGSAGTCWGG 3' *
qRT-PCR	
<i>Pax5</i> fwd	5' CAAGCCAGAACAGACCACAGA 3'
<i>Pax5</i> rev	5' GGCCTGTGACAATAGGGTAGG 3'
<i>Spi1</i> fwd	5' ATCAAACCTTGTCCCCAGCC 3'
<i>Spi1</i> rev	5' TTTTCTTGCTGCCTGTCTCC 3'
<i>Aicda</i> fwd	5' AAATGTCCGCTGGGCCAA 3'
<i>Aicda</i> rev	5' CATCGACTTCGTACAAGGG 3'
<i>Aicda</i> (2) fwd	5'AGTCACGCTGGAGACCGATA 3'
<i>Aicda</i> (2) rev	5' GCAGAGGTAGGTCTCATGCC 3'

*: V μ H rev represents a mixture of different primers according to a degenerate nucleotide code, for amplification of more V μ H sequences. S: C/G, M: A/C, R: A/G, W: A/T

Author contributions

D.D.G., C.P., N.S., M.B., F.H., M.L. designed experiments; D.D.G., C.P., N.S., M.B., D.S., L.M., B.C., F.H. performed experiments; D.D.G., C.P., M.B., H.R., E.R., P.D., M.W., M.M., A.N., U.M.B., F.M., F.H., M.B., H.M.J., A.N., A.B., M.K., T.B., T.S., A.P. M.L. analyzed data; D.D.G. and M.L. prepared the manuscript.

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Fig. 1: Spontaneous emergence of pB-ALL in adult *lrf4*^{-/-} mice

- A) Distribution of age at disease onset (n = 14).
- B) Macroscopic appearance of an exemplary tumour (asterisk) and LNs (arrow heads) in an *lrf4*^{-/-} mouse. Right: HE stainings of the tumour. Scale bars: top: 50 μ m, bottom: 20 μ m
- C) Longitudinal spleen axis (mm) of *lrf4*^{-/-} mice with (n = 8) or without (n = 3) tumour and control wt mice (n = 3). Bars depict the mean $\pm$ SD.

- D) HE stainings of lung (bars 50 μm), liver (bars 100 μm) and BM (bars 20 μm) of tumour mouse T8 and healthy *Irf4*^{-/-} mouse. Scale Bars: top, middle: 50 μm , bottom: 20 μm , BM = bone marrow
- E) IHC-stainings for IgM, B220 and Ki67 of spleen (“infiltrating cells”), also containing a nest (“follicle”) of non-transformed B cells. Scale bars: 100 μm
- F) Flow cytometry of spleen cells for B220, membrane-bound (m)IgM, CD2 and CD179b. Gated on live lymphocytes; the far-right column additionally sub-gated for B220⁺ (dim plus high) cells. Column headers define gates (rectangles) set in the panels in each column. Representative for three stainings of individual tumour samples.
- G) Quantification of B220^{dim} mIgM⁻ cells in tissues and blood from T8 and TD2 mice in comparison to healthy control mice. Bars depict mean $\pm$ SD of three mice or individual value.
- H) Copy number variation (CNV) analysis for T10 and T14 using the cn.mops pipeline. Examples of altered chromosomal areas are boxed and highlighted.

Fig. 2: Impaired *Irf4*^{-/-} B lymphopoiesis results in immature BM evasion and IL-7 dependent hyperproliferation

a) tSNE plot of surface stainings. Colour depicts B220 signal intensity. Histograms of the G1 (grey curves) and G2 (red curves) populations on the right for individual markers. Representative of three experiments.

- b) Flow cytometric analysis for surface B220 and mIgM identifying BM B cell populations (Hardy “A-D, E and F”, see illustration). Bars to the right denote the mean $\pm$ SD as percentage of B220⁺ cells of six (*Irf4*^{-/-}) or seven (wt) mice. * : p<0.05; ** : p<0.01; by Two-way ANOVA, Sidak corrected.
- c) Surface IL-7R α and CD2 analysis defines subpopulations of “A-D” in C). Bars present percentages within B220^{dim}/mIgM⁻ B cells as mean $\pm$ SD of six (*Irf4*^{-/-}) or seven (wt) mice per group. *** : p<0.001; **** : p<0.0001 by Two-way ANOVA, Sidak corrected. ppB = preproB.
- c) Relative sizes of cell populations in C by forward scattering. Individual cell measurements from one representative experiment, depicted as box (IQR and median) and whiskers.
- e-f) B220⁺ BM cells from *Irf4*^{-/-} or wt mice, cultured for three days (surface stained as indicated). Diagrams give the mean percentage of cells within the B220⁺ gates $\pm$ SD (n = 4). n.s. : not significant; * : p<0.05; ** : p<0.01 by one-way ANOVA, Sidak corrected.
- g) Histograms comparing CXCR4 and control isotype staining of *Irf4*^{-/-} and wt BM B220^{dim}/mIgM⁻ pro/preB cells gated according to “A-D” in C). Bars depict the mean ratio $\pm$ SD of geometric mean fluorescence intensities (MFI) of CXCR4 to isotype calculated for seven independent experiments. **** : p<0.0001 by two-tailed unpaired t test.
- h) Flow cytometric analysis of spleen cells from healthy young and old (> 100 d) *Irf4*^{-/-} and of wt mice for B220, mIgM and CD2. Bars present mean $\pm$ SD from five separate experiments. ** : p<0.01 by two-tailed unpaired t test.

Fig. 3: Recurrent *Jak3* mutations in *Irf4*^{-/-} leukemia are accompanied by elevated *Aicda* transcripts and a C:T SNV bias

- Venn diagram of mutations shared between T8, T10, T11 as detected by WES.
- Mutations mapped to the respective domains of JAK3.
- preB cell line 35911 infected with retroviruses encoding for Thy1 (EV-RV = empty, JAK3-RV = wt *Jak3*, R653H-RV and T844M-RV = *Jak3* mutants) cultured with IL-7 or anti-IL-7. Presence of Thy1.1⁺ (infected) cells was monitored by flow cytometry. Thy1.1⁺/Thy1.1⁻ ratio compared to post-transduction (= "day 0") is depicted as fold change; mean±SD of six independent experiments. n.s.: not-significant; * : p<0.05; **: p<0.01 by mixed-effects model comparing groups to JAK3-RV, Dunnett corrected.
- qRT-PCR for *Aicda* mRNA. Samples from leukemia cells, control mLN, CD4⁺ Th1 cells and FACS-sorted BM B220⁺mIgM⁻ cells of healthy *Irf4*^{-/-} and wt mice. *Aicda* expression is presented as % of HPRT. * : p<0.05; **** : p<0.0001 by one-way ANOVA, Tukey corrected.

- e) Samples from BM *Irf4*^{-/-} preB cells cultured with IL-7 for 3-4 days, then stimulated as indicated for 24 h. Quantification as % of HPRT. n.s. : not significant; ** : p<0.0021; **** : p<0.0001 by one-way ANOVA, Tukey corrected.
- f) Strategy for identification of SNPs in matched WES sample-pairs. Sorted BM BL/6 and *Irf4*^{-/-} preB, as well as T8 cells are compared to tail tips to identify somatic exome mutations. A frequency of either ≥ 0.45 plus ≤ 0.55 or ≥ 0.95 focuses on “core” SNPs, probably affecting one or both alleles in the respective bulk cells (thus “mono/bi”). Graphical representations of the resultant SNPs. Stacked bars depict percentages of SNPs per indicated cell sample, colours the types of nucleotide exchanges.

Fig. 4: IRF4 re-expression results in cell death and transcriptional changes

compatible with differentiation of blast cells

- T8 cells were transduced with retroviruses encoding for GFP alone (EV-RV) or with IRF4 and cultured in the presence of IL-7 (10 ng/ml) *in vitro*. Mean $\pm$ SD percentage of transduced among viable cells is depicted relative to 24 h after transduction (n = 3). ** : $p < 0.01$, *** : $p < 0.001$ by one-way ANOVA comparing groups per time-point.
- T11 cells transduced with RVs as described in a). Total RNA sequenced 24 h after transduction. Volcano plot: $\log_2$ of fold change between conditions (IRF4-RV vs. EV-RV) against respective $-\log_{10}$ of Benjamini-Hochberg adjusted p-values. Dotted lines mark x- and y- cutoff values.
- Heatmap depicting the 50 most significantly regulated genes of data as in b). Euclidean clustering depicted as dendrograms to the left. Two samples per group.

Fig. 5: *Ptk2* is overexpressed in leukemia samples and negatively influenced by IRF4

- a) Heatmap of gene regulated by malignant transformation and IRF4 re-expression: Colour coding: log₂ of fold change in the indicated comparison (bottom): Left: untreated pro/preB cells from BM of healthy *Irf4*^{-/-} mice compared to the compiled transcriptomes of tumours T10, T11 and T14. Right: IRF4-RV vs. EV-RV (scenario as in Fig.4b). Gene inclusion criteria: 1.) > 2 times fold change in left scencario 2.) gene expression altered by IRF4 re-expression (right scenario) 3.) only genes are presented previously shown to be a target of IRF4 by ChipSeq.⁵⁰
- b) T8.1 cells were cultured in 1:2 dilutions in the presence or absence of IL-7 or anti-IL-7. Cell viability by flow cytometry using propidium iodide exclusion after 24 h. Presented data relative to undiluted cells and depicted as the mean $\pm$ SD of three independent experiments. Dotted line for comparison of maximally diluted samples between groups * : $p < 0.05$; ** : $p < 0.01$, **** : $p < 0.0001$ by two-way ANOVA comparing treatment groups per dilution step or *vice versa*, Tukey corrected.
- c) Western blot for FAK. B cells: untreated B220⁺ cells from of *Irf4*^{-/-} or wt mice, pro/preB cells: *Irf4*^{-/-} BM B220⁺mIgM⁻ cells. The FAK/ β -Actin signal intensity ratio is given as fold change compared to wt B cells. Representative of three independent experiments.

Fig. 6: Ruxolitinib inhibits leukemic meningeosis in *lrf4*^{-/-} leukemia, prolonging survival *in vivo*

- a) 12 days after *i.p.* injection of T8.2 cells into female C57BL/6 wt mice treatment was started with dexamethasone (day “0”). Dexamethasone treatment was replaced by twice daily oral gavage with Ruxolitinib-phosphate (11 mice) or vehicle control (13 mice) on day 8 until day 21. Presence of B220⁺mIgM⁻ cells in the PB presented as mean percentage $\pm$ SD measured in all mice per respective treatment group.

- b) Survival of the mice described in a) as Kaplan-Meier plot analyzed with Log-rank test. In the Ruxolitinib group, 4 mice were excluded and censored due to intervention related adverse reactions or due to their use for to the analysis described in e-g).
- c-d) Scores, determined as described in Methods. c) Mean $\pm$ SD of the scores per indicated treatment group. ** : $p < 0.01$; *** : $p < 0.001$; **** : $p < 0.0001$ by two-way ANOVA, Sidak corrected d) Percentage of all mice per group showing any score > 0 a-d) Two replicate experiments with similar outcome.
- e-g) Ultrasound scan of the paravertebral lumbar area of tumour-affected mice. The region of the psoas muscle is highlighted as a circle, the area of which is measured. f,g) Bars show the mean $\pm$ SD size (f) of the defined area or the grey values (g) for three mice with score 0 and 3. V: vertebra; ** : $p < 0.01$ by unpaired t test.
- h) Exemplary histopathology (HE or CAE) of healthy or leukemia bearing mice (score 3, vehicle-treated or score 0, Ruxolitinib-treated). One exemplary mouse per condition. Bar size in the bottom right corners. Top panels show the lumbar vertebral area. Middle panels show enlargements from the top panels depicting leptomeningeal space (left) and spinal nerve roots (right). Asterisk: bone, X: subarachnoid space, arrowhead: nerve root area. Lower panels: CAE staining of BM including inserts at higher magnification. At least three mice per condition were analyzed. CAE: chloroacetate esterase
- i) Quantification of perimyelon invasion of tumour cells (X in G)) according to the depicted formula (A_t : area of tumour infiltration, A_{sca} : area of total spinal canal, A_{sp} : area of the spinal cord) Bars depict Mean $\pm$ SD of three (score 0) or four (score 3) mice. *** : $p < 0.001$ by unpaired t test.

	samples		
	T7	T8	T12
number of independent sequencing reactions	27	30	28
number of successful sequencing reactions	24	29	13
number of identical sequences	23	28	12

Table 1: Sequencing of VDJ junctions for IgH reveals that tumour samples are clonal

tumour	characteristics			lifespan			Jak3 mutation				
	genotype	type	sex	birth (dd/mm/yy)	death (dd/mm/yy)	age (days)	SNP	method	Reference Base	Alternative Base	frequency
T8	C57BL/6 <i>Irf4</i> ^{-/-}	primary	m	09/08/10	06/04/11	240	p.R653H	WES	G	A	0.50
T10	C57BL/6 <i>Irf4</i> ^{-/-}	primary	m	19/07/10	12/04/11	267	p.T844M	WES	C	T	0.57
T11	C57BL/6 <i>Irf4</i> ^{-/-}	primary	m	24/10/10	11/05/11	199	p.R653H	WES	G	A	0.46
T14	C57BL/6 <i>Irf4</i> ^{-/-}	primary	m	24/08/10	06/07/11	316	p.L634W	RNA	T	G	0.50
TD1	C57BL/6 <i>Irf4</i> ^{-/-}	primary	w	30/09/17	07/06/18	250	p.L627F	Sanger	G	A	0.50
TD2	C57BL/6 <i>Irf4</i> ^{-/-}	primary	w	30/09/17	08/06/18	249	p.A742V	Sanger	G	A	0.50

Table 2: Jak3 mutations identified in five out of five tested leukemia samples

Figures

Figure 1

Spontaneous emergence of pB-ALL in adult *Lrf4*^{-/-} mice A) Distribution of age at disease onset (n = 14). B) Macroscopic appearance of an exemplary tumour (asterisk) and LNs (arrow heads) in an *Lrf4*^{-/-} mouse. Right: HE stainings of the tumour. Scale bars: top: 50 µm, bottom: 20 µm C) Longitudinal spleen

axis (mm) of *Irf4*^{-/-} mice with (n = 8) or without (n = 3) tumour and control wt mice (n = 3). Bars depict the mean $\pm$ SD. D) HE stainings of lung (bars 50 μ m), liver (bars 100 μ m) and BM (bars 20 μ m) of tumour mouse T8 and healthy *Irf4*^{-/-} mouse. Scale Bars: top, middle: 50 μ m, bottom: 20 μ m, BM = bone marrow E) IHC-stainings for IgM, B220 and Ki67 of spleen ("infiltrating cells"), also containing a nest ("follicle") of non-transformed B cells. Scale bars: 100 μ m F) Flow cytometry of spleen cells for B220, membrane-bound (m)IgM, CD2 and CD179b. Gated on live lymphocytes; the far-right column additionally sub-gated for B220⁺ (dim plus high) cells. Column headers define gates (rectangles) set in the panels in each column. Representative for three stainings of individual tumour samples. G) Quantification of B220^{dim} mIgM⁻ cells in tissues and blood from T8 and TD2 mice in comparison to healthy control mice. Bars depict mean $\pm$ SD of three mice or individual value. H) Copy number variation (CNV) analysis for T10 and T14 using the cn.mops pipeline. Examples of altered chromosomal areas are boxed and highlighted.

Figure 2

Impaired *Irf4*^{-/-} B lymphopoiesis results in immature BM evasion and IL-7 dependent hyperproliferation

a) tSNE plot of surface stainings. Colour depicts B220 signal intensity. Histograms of the G1 (grey curves) and G2 (red curves) populations on the right for individual markers. Representative of three experiments. b) Flow cytometric analysis for surface B220 and mIgM identifying BM B cell populations (Hardy "A-D, E and F", see illustration). Bars to the right denote the mean ± SD as percentage of B220+ cells

of six (*Irf4*^{-/-}) or seven (wt) mice. *: $p < 0.05$; **: $p < 0.01$; by Two-way ANOVA, Sidak corrected. c) Surface IL-7Ra and CD2 analysis defines subpopulations of “A-D” in C). Bars present percentages within B220dim/mIgM⁻ B cells as mean $\pm$ SD of six (*Irf4*^{-/-}) or seven (wt) mice per group. ***: $p < 0.001$; ****: $p < 0.0001$ by Two-way ANOVA, Sidak corrected. ppB = preproB. c) Relative sizes of cell populations in C by forward scattering. Individual cell measurements from one representative experiment, depicted as box (IQR and median) and whiskers. e-f) B220⁺ BM cells from *Irf4*^{-/-} or wt mice, cultured for three days (surface stained as indicated). Diagrams give the mean percentage of cells within the B220⁺ gates $\pm$ SD ($n = 4$). n.s.: not significant; *: $p < 0.05$; **: $p < 0.01$ by one-way ANOVA, Sidak corrected. g) Histograms comparing CXCR4 and control isotype staining of *Irf4*^{-/-} and wt BM B220dim/mIgM⁻ pro/preB cells gated according to “A-D” in C). Bars depict the mean ratio $\pm$ SD of geometric mean fluorescence intensities (MFI) of CXCR4 to isotype calculated for seven independent experiments. ****: $p < 0.0001$ by two-tailed unpaired t test. h) Flow cytometric analysis of spleen cells from healthy young and old (> 100 d) *Irf4*^{-/-} and of wt mice for B220, mIgM and CD2. Bars present mean $\pm$ SD from five separate experiments. **: $p < 0.01$ by two-tailed unpaired t test.

Figure 3

Recurrent *Jak3* mutations in *Irf4*^{-/-} leukemia are accompanied by elevated *Aicda* transcripts and a C:T SNV bias a) Venn diagram of mutations shared between T8, T10, T11 as detected by WES. b) Mutations mapped to the respective domains of JAK3. c) preB cell line 35911 infected with retroviruses encoding for

Thy1 (EV-RV = empty, JAK3-RV = wt Jak3, R653H-RV and T844M-RV = Jak3 mutants) cultured with IL-7 or anti-IL-7. Presence of Thy1.1+ (infected) cells was monitored by flow cytometry. Thy1.1+/Thy1.1- ratio compared to post-transduction (= “day 0”) is depicted as fold change; mean±SD of six independent experiments. n.s.: not-significant; * : p<0.05; **: p<0.01 by mixed-effects model comparing groups to JAK3-RV, Dunnett corrected. d) qRT-PCR for *Aicda* mRNA. Samples from leukemia cells, control mLN, CD4+ Th1 cells and FACS-sorted BM B220+mIgM- cells of healthy *Irf4*-/- and wt mice. *Aicda* expression is presented as % of HPRT. * : p<0.05; **** : p<0.0001 by one-way ANOVA, Tukey corrected. e) Samples from BM *Irf4*-/- preB cells cultured with IL-7 for 3-4 days, then stimulated as indicated for 24 h. Quantification as % of HPRT. n.s. : not significant; ** : p<0.0021; **** : p<0.0001 by one-way ANOVA, Tukey corrected. f) Strategy for identification of SNPs in matched WES sample-pairs. Sorted BM BL/6 and *Irf4*-/- preB, as well as T8 cells are compared to tail tips to identify somatic exome mutations. A frequency of either ≥ 0.45 plus ≤ 0.55 or ≥ 0.95 focuses on “core” SNPs, probably affecting one or both alleles in the respective bulk cells (thus “mono/bi”). Graphical representations of the resultant SNPs. Stacked bars depict percentages of SNPs per indicated cell sample, colours the types of nucleotide exchanges.

Figure 4

IRF4 re-expression results in cell death and transcriptional changes compatible with differentiation of blast cells a) T8 cells were transduced with retroviruses encoding for GFP alone (EV-RV) or with IRF4 and cultured in the presence of IL-7 (10 ng/ml) in vitro. Mean±SD percentage of transduced among viable cells is depicted relative to 24 h after transduction (n = 3). ** : p< 0.01, *** : p<0.001 by one-way ANOVA comparing groups per time-point. b) T11 cells transduced with RVs as described in a). Total RNA sequenced 24 h after transduction. Volcano plot: log2 of fold change between conditions (IRF4-RV vs. EVRV) against respective -log10 of Benjamini-Hochberg adjusted p-values. Dotted lines mark x- and y-cutoff values. c) Heatmap depicting the 50 most significantly regulated genes of data as in b). Euclidean clustering depicted as dendrograms to the left. Two samples per group.

Figure 5

Ptk2 is overexpressed in leukemia samples and negatively influenced by IRF4 a) Heatmap of gene regulated by malignant transformation and IRF4 re-expression: Colour coding: log2 of fold change in the indicated comparison (bottom): Left: untreated pro/preB cells from BM of healthy *Irf4*^{-/-} mice compared to the compiled transcriptomes of tumours T10, T11 and T14. Right: IRF4-RV vs. EV-RV (scenario as in Fig.4b). Gene inclusion criteria: 1.) > 2 times fold change in left scencario 2.) gene expression altered by IRF4 re-expression (right scenario) 3.) only genes are presented previously shown to be a target of IRF4 by ChipSeq.50 b) T8.1 cells were cultured in 1:2 dilutions in the presence or absence of IL-7 or anti-IL-7. Cell viability by flow cytometry using propidium iodide exclusion after 24 h. Presented data relative to undiluted cells and depicted as the mean±SD of three independent experiments. Dotted line for comparison of maximally diluted samples between groups * : p<0.05; ** : p< 0.01, **** : p<0.0001 by two-way ANOVA comparing treatment groups per dilution step or vice versa, Tukey corrected. c) Western blot for FAK. B cells: untreated B220+ cells from of *Irf4*^{-/-} or wt mice, pro/preB cells: *Irf4*^{-/-}

BM B220+mIgM- cells. The FAK/ β -Actin signal intensity ratio is given as fold change compared to wt B cells. Representative of three independent experiments.

Figure 6

Ruxolitinib inhibits leukemic meningeosis in *Irf4*^{-/-} leukemia, prolonging survival in vivo a) 12 days after i.p. injection of T8.2 cells into female C57BL/6 wt mice treatment was started with dexamethasone (day “0”). Dexamethasone treatment was replaced by twice daily oral gavage with Ruxolitinib-phosphate (11

mice) or vehicle control (13 mice) on day 8 until day 21. Presence of B220+mIgM- cells in the PB presented as mean percentage $\pm$ SD measured in all mice per respective treatment group. b) Survival of the mice described in a) as Kaplan-Meier plot analyzed with Log-rank test. In the Ruxolitinib group, 4 mice were excluded and censored due to intervention related adverse reactions or due to their use for to the analysis described in e-g). c-d) Scores, determined as described in Methods. c) Mean $\pm$ SD of the scores per indicated treatment group. ** : $p < 0.01$; *** : $p < 0.001$; **** : $p < 0.0001$ by two-way ANOVA, Sidak corrected d) Percentage of all mice per group showing any score > 0 a-d) Two replicate experiments with similar outcome. e-g) Ultrasound scan of the paravertebral lumbar area of tumour-affected mice. The region of the psoas muscle is highlighted as a circle, the area of which is measured. f,g) Bars show the mean $\pm$ SD size (f) of the defined area or the grey values (g) for three mice with score 0 and 3. V: vertebra; ** : $p < 0.01$ by unpaired t test. h) Exemplary histopathology (HE or CAE) of healthy or leukemia bearing mice (score 3, vehicle-treated or score 0, Ruxolitinib-treated). One exemplary mouse per condition. Bar size in the bottom right corners. Top panels show the lumbar vertebral area. Middle panels show enlargements from the top panels depicting leptomeningeal space (left) and spinal nerve roots (right). Asterisk: bone, X: subarachnoid space, arrowhead: nerve root area. Lower panels: CAE staining of BM including inserts at higher magnification. At least three mice per condition were analyzed. CAE: chloroacetate esterase i) Quantification of perimyeloid invasion of tumour cells (X in G)) according to the depicted formula (At: area of tumour infiltration, Asca: area of total spinal canal, Asp: area of the spinal cord) Bars depict Mean $\pm$ SD of three (score 0) or four (score 3) mice. *** : $p < 0.001$ by unpaired t test.

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