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1 **Interleukin 7 receptor is associated with central nervous system infiltration**

2 **and relapse in pediatric B-cell precursor acute lymphoblastic leukemia**

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**Letter to Blood**

Central nervous system (CNS) involvement in pediatric B-cell precursor acute lymphoblastic leukemia (BCP-ALL) is rarely detected at initial presentation[1]. Nevertheless, CNS-relapse most frequently occurs in children who were initially diagnosed as CNS-negative (CNS⁻) and did not have any high-risk characteristics[2]. Therefore all patients receive intensive CNS-directed chemotherapy[3], an approach associated with short and long-term neurological toxicities[4,5]. The CNS microenvironment may contribute to chemoresistance and survival of leukemic cells[6]. Interleukin 15 (IL15) was shown to promote ALL survival in the hostile microenvironments of the CNS[7,8]. IL7 can be detected in the cerebrospinal fluid (CSF) and high levels have been associated with inflammatory CNS disease[9], which supports that IL7 may be produced by stromal cells in that niche upon different stimuli[10]. Also, elevated IL7 plasma levels were detected in BCP-ALL patients[11]. Here we show that IL7R is highly expressed in pediatric BCP-ALL patients that were CNS⁺ at initial diagnosis, and that an upregulation of IL7R may predict CNS-relapse. Using a xenograft model in immunodeficient mice, we show that IL7R is required for leukemic engraftment *in vivo*, and that targeting IL7R with monoclonal antibody reduces CNS leukemic infiltration.

The t(1;19) chromosomal translocation leading to the E2A-PBX1 fusion has been shown to increase IL7R expression[12], and E2A-PBX1 rearranged BCP-ALL cells have a particular propensity to infiltrate the CNS[13,14]. Thus, we first analyzed IL7R expression in a cohort of 61 E2A-PBX1⁺ patients[13] and correlated the data with clinical characteristics. IL7R expression was significantly higher in patients with an elevated white blood cell (WBC) count (Figure 1A), which is also a classical risk factor for CNS disease. Importantly, IL7R expression was also significantly higher in CNS⁺ as compared to CNS⁻ patients (Figure 1B). In contrast, there were no correlations between IL7R expression and sex, age, prednisone response or minimal residual disease (MRD) risk group (Supplementary Figure 1). We next determined IL7R expression in a further cohort of 98 BCP-ALL patients of mixed molecular backgrounds. The cohort contained 26 patients that were initially CNS⁺ and 72 CNS⁻ patients. There were no statistical differences in sex, age, prednisone response, MRD-risk groups and cytogenetics between both groups (Supplementary Table 1).

Importantly, IL7R expression was found to be significantly elevated in CNS$^+$ compared to CNS$^-$ patients (Figure 1C). Multivariate analysis controlling for age and WBC count showed that IL7R expression in the third and fourth quartiles lead to odds ratios (OR) of 5.4 (95% CI 0.997-29.117) and 5.6 (95% CI 1.023-30.842) for CNS positivity, respectively (Supplementary Table 2). These data suggest that increased IL7R expression levels in BM leukemic cells are associated with and may predict CNS disease at initial diagnosis. IL7R expression also significantly correlated with ZAP70, which is another marker for CNS infiltration[15], and combining both markers did not yield superior correlations, which may however be hampered by a low sample size (Supplementary Figure 2A-B). The association of IL7R expression with CNS relapse was then explored using two publicly available datasets[16-18]. ALL cells retrieved from the CSF of children with isolated CNS-relapse showed a significantly higher IL7R expression compared to ALL cells from BM at diagnosis and BM at BM-relapse without CNS involvement (Figure 1D). Most importantly, a high IL7R expression in BCP-ALL cells from BM/peripheral blood at diagnosis was associated with reduced long-term CNS-relapse-free probability rates in the TARGET phase 1 dataset (Figure 1E). It seems that as IL7R expression increases, reflected by increasing z-score, the rate of CNS-relapse also increases (Supplementary Figure 3A-B, Supplementary Table 3). Among different risk factors for CNS-relapse, an upregulation of IL7R was a statistically significant predictor of isolated CNS-relapse in a Cox-proportional hazards model (Supplementary Table 4). Nevertheless, increased IL7R expression was not associated with an increased risk for BM-relapse or relapses with BM involvement (Supplementary Figure 3C-D). Interestingly, there was a significant association between IL7R upregulation and E2A-PBX1 (30% of IL7R overexpressors had this translocation, Supplementary Table 5).

These findings indicate that IL7R may be used as a diagnostic and prognostic marker without accessing the CNS compartment for diagnosis of CNS leukemia.


We next injected 13 patient samples into NSG mice in duplicates, and mean fluorescence intensity (MFI) of IL7R was determined. Xenografts were sub-grouped into IL7R$^{Hi}$/IL7R$^{Lo}$ relative to median MFI (Supplementary Table 6). CNS infiltration for 11 xenografts was analyzed[13]. 8/12 (67%) mice injected with IL7R$^{Hi}$-cells were

CNS-positive, whereas only 2/10 (20%) mice bearing IL7R^Lo-cells were CNS-positive (Supplementary Figure 4A). Selected IL7R^Hi blasts in this experiment showed a tendency to have higher basal levels of ERK, p-ERK and p-AKT compared to IL7R^Lo (Supplementary Figure 4B). To test whether blocking IL7R *in vivo* can prevent ALL engraftment and homing to the CNS, we down-regulated IL7R expression by RNA-interference using an IL7Rα–specific shRNA in the human cell line 697, which expresses high levels of IL7R. Down-modulation of IL7R led to a marked decrease of blast percentages in the spleen, BM and CNS as compared to mice injected with the respective control (Figure 2A-B). To investigate whether inhibition of IL7R signaling using ruxolitinib can interfere with the engraftment of leukemic cells with a high expression of IL7R *in vivo*, we injected E2A-PBX1^+ BCP-ALL cells from one pediatric patient into NSG mice and monitored the survival of recipient mice under ruxolitinib treatment with and without concomitant chemotherapy. We found that mice treated with ruxolitinib showed only a minor prolongation in survival in comparison to untreated control and that ruxolitinib was markedly less efficient than standard chemotherapy. In addition, ruxolitinib treatment did not decrease leukemic infiltration in the CNS (data not shown). The combination of ruxolitinib and chemotherapy did not result in additional benefits (Figure 2C). Opposite to previously published data[19], our results indicate that ruxolitinib is not efficient for preventing the engraftment of human ALL cells *in vivo*. This might be caused by a poor bioavailability of ruxolitinib in mice and/or an insufficient inhibition of IL7R signaling, as ruxolitinib inhibits mainly JAK1/2 and not JAK3 that can be activated by IL7R signaling. Furthermore, the amount of IL7 available *in vivo* may have overridden the downstream inhibition by ruxolitinib. We therefore next tested whether inhibiting the IL7R with a blocking antibody would substantiate our previous findings in a further experiment with an E2A-PBX1^+ patient sample *in vivo*. Antibody treatment significantly prolonged the survival of xenograft mice as compared to treatment with an isotype control antibody (Figure 2D). In addition, IL7R antibody treatment strongly reduced spleen size and leukemic infiltration in spleen, BM and, most importantly, in the CNS (Figure 2E-G). Ruxolitinib as a single agent or as addition to the antibody treatment had no beneficial effects (Figure 2E-G). It is worth noting that *in vitro* antibody treatment downmodulated IL7R signaling through AKT and induced apoptosis as indicated by an upregulation of cleaved caspase-8 (Supplementary Figure 5A-B).

These findings support the view that targeting IL7/IL7R signaling may be an effective approach in BCP-ALL therapy[20]. So far, anti-IL7R antibodies have been investigated in preclinical mouse models of multiple sclerosis to target T cells that require IL7R expression for their homeostasis[21], indicating a toxic effect of the antibody in T cells. Our study points to IL7R as a main target for BCP-ALL treatment and that further investigation of the anti-IL7R antibodies for immunotherapy of BCP-ALL may lead to improved therapeutic approaches especially for patients with CNS involvement.

## Author Contributions

A. A. designed experiments, analyzed data and wrote the manuscript. L. L., A. V., A. K., F. V., and C. V. performed experiments and analyzed data. G. C. and M. S. provided ALL materials. F. S. K-M. D., and L-H. M. provided materials. A. C. and C. H. provided dataset analyses. D. M. S. and E. H. designed experiments and discussed the research direction. D. M. S. wrote the manuscript. H. J. initiated, designed, discussed the research direction and wrote the manuscript. All authors discussed the manuscript.

## Disclosure of Conflict of Interests

The authors have no conflict of interest to declare.

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**Figure Legends**

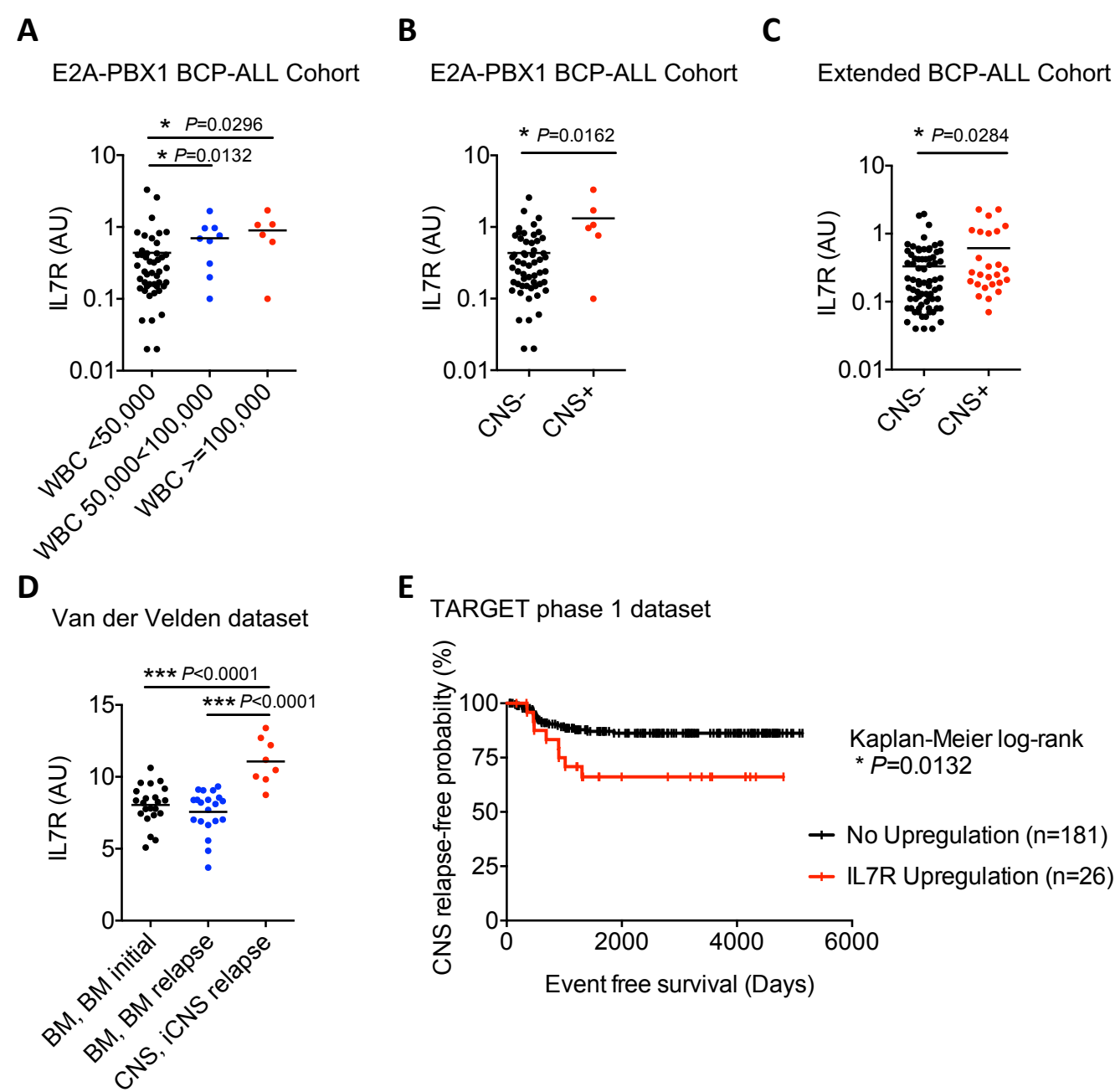**Figure 1: IL7R expression is associated with CNS disease and CNS-relapse in pediatric BCP-ALL patients.**

Correlation analysis of IL7R expression in 61 E2A-PBX1 positive pediatric patients with white blood cell (WBC) count (A) and CNS status (B). Unpaired t-test, two-sided *P*-value. (C) Correlation analysis of IL7R expression in 98 pediatric BCP-ALL patients of mixed cytogenetics and CNS status. Further definitions are provided in Supplementary Table 1. Unpaired t-test, two-sided *P*-value. (D) IL7R expression in ALL cells retrieved from the CSF of 8 children with CNS-relapse of BCP-ALL as well as from the BM of 22 patients at diagnosis, and cells from the BM of 20 patients at the time of isolated BM-relapse. Dataset van der Velden et al 2016[16]. Unpaired t-test, two-sided *P*-value. (E) Kaplan-Meier survival curve showing reduced isolated CNS (iCNS) relapse-free probability in children with upregulated IL7R gene expression in diagnostic BM (n=131) or peripheral blood (n=76) samples of children with high risk ALL. IL7R Upregulation was defined as a z-score for gene expression ≥ 1.2; TARGET phase 1 dataset.

**Figure 2: Inhibition of IL7R delays leukemogenesis in xenograft mice**

(A) NSG mice were xenografted with 697 cells bearing an shRNA against the IL7Rα (shIL7Rα) or a control shRNA (shGFP). Animals were sacrificed at day 26 upon detection of >75% leukemic blasts in the peripheral blood or clinical leukemia (loss of weight or activity, organomegaly, hind-limb paralysis) in first control mice. Spleen (Sp) and bone marrow (BM) infiltration by human leukemic blasts in control and treated animals. (B) CNS infiltration as determined by histology. The arrows indicate human leukemic blasts in an example for the semiquantitative scoring employed[13]. (C) 1 x 10^6 E2A-PBX1 positive patient cells were xenografted into NSG mice. Xenografted mice were treated with vehicle only, ruxolitinib only, chemotherapy only (dexamethasone, vincristine and PEG-asparaginase) or a combination of ruxolitinib and chemotherapy (n=7 per group). Mice were sacrificed upon appearance of leukemic symptoms. Statistics for survival were performed according to the Mantel-Cox log-rank method. *P1*: control vs. Ruxo, *P2*: control vs. chemo, *P3*: control vs. Ruxo/Chemo. (D) 1 x 10^6 E2A-PBX1 positive patient cells were xenografted into

8

256  NSG mice. Xenografted mice were treated with an anti-IL7R antibody or an isotype

257  antibody (n=7 and n=6 per group, as indicated). The experiment was ended on day

258  135. Statistics for survival were performed according to the Mantel-Cox log-rank

259  method. (E-G) 1 x $10^6$ E2A-PBX1 positive patient cells were xenografted into NSG

260  mice. Xenografted mice were treated with control antibody, ruxolitinib, with an anti-

261  IL7R antibody or with both ruxolitinib and the antibody (n=7 per group). One mouse

262  of ruxo/anti-IL7R group died during the experiment and accordingly was excluded.

263  The experiment was ended on day 65 and spleen sizes (E), the percentages of Sp

264  and BM blasts (F), and CNS infiltration (G) were assessed (Fisher´s exact test, two-

265  sided). Treatment protocol: 60 mg/kg of ruxolitinib (LC Laboratories) was

266  administered Monday through Friday by oral gavage. Chemotherapy was

267  administered as previously published[15,22]. 1 mg/kg of anti-IL7R antibody (monoclonal

268  mouse IgG1, clone 40131, R&D Systems) or isotype control antibody were

269  administered on day 0, +3, +7, +21, +35, +48 and +56 post-injection.

**A**

E2A-PBX1 BCP-ALL Cohort



**B**

E2A-PBX1 BCP-ALL Cohort



**C**

Extended BCP-ALL Cohort



**D**

Van der Velden dataset



**E**

TARGET phase 1 dataset



Kaplan-Meier log-rank
* *P*=0.0132

No Upregulation (n=181)
IL7R Upregulation (n=26)

**Figure 1**

**A**

Sp    BM

*    *

Blasts (%)

shGFP  shIL7Rα  shGFP  shIL7Rα

**B**

CNS-    CNS+    CNS++

|         | shGFP    | shIL7α    |
|---------|----------|-----------|
| **CNS+/++** | 6 (100%) | 1 (17%)   |
| **CNS-**    | 0 (0%)   | 5 (83%)   |

**$P$=0. 0.0034

**C**

Kaplan-Meier log-rank
***$P_1$=0.0002
***$P_2$=0.0009
***$P_3$=0.0004

Survival (%)

Event free survival (Days)

— Control (n=7)
— Ruxo (n=7)
— Control /Chemo (n=7)
— Ruxo/Chemo (n=7)

**D**

Survival (%)

Kaplan-Meier log-rank
*** $P$=0.0005

— Isotype Ab (n=6)
— anti-IL7R Ab (n=7)

Event free survival (Days)

**E**

Control

anti-IL7R Ab

Ruxo

Ruxo/anti-IL7R Ab

**F**

Sp    BM

% of Blasts

** **
*** ***
*** ***
** **

Control  Ruxo  anti-IL7R Ab  Ruxo/anti-IL7R Ab  Control  Ruxo  anti-IL7R Ab  Ruxo/anti-IL7R Ab

**G**

|         | Control Ab | anti-IL7R Ab |
|---------|------------|--------------|
| **CNS+/++** | 7 (100%)   | 1 (14%)      |
| **CNS-**    | 0 (0%)     | 6 (86%)      |

**$P$=0. 0.0012

**Figure 2**