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IL1RAP is expressed in several subtypes of pediatric acute lymphoblastic leukemia and can be used as a target to eliminate ETV6::RUNX1-positive leukemia cells in preclinical models

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Author contributions
HÅ and TF designed the study. HÅ, MR, and CS performed the experiments. HÅ, HL, CS, and TF analyzed the data. HÅ wrote the manuscript. HL, MR, CS, and TF critically commented on the manuscript.

Conflicts of interest
TF is a cofounder and board member of Cantargia AB (Medicon Village, Lund), developing therapeutic IL1RAP antibodies. Cantargia AB is the owner of the intellectual property rights for agents targeting IL1RAP for use in the treatment and diagnosis of neoplastic hematologic disorders. The other authors report no potential conflicts of interest to declare.

Data sharing statement
Original data can be made available upon a written request to the corresponding authors.
Acute lymphoblastic leukemia (ALL) is the most common childhood cancer type and in most cases the leukemic cells display a B-cell precursor (BCP) immunophenotype. Although effective, the cytotoxic agents that constitute the backbone in therapy are associated with short and long-term side effects negatively influencing the health and well-being of the growing child. Current treatment protocols extend up to 2.5 years, which may lead to adherence problems that significantly increases the risk of relapse that, in similar to primary disease in infants and adults, has a less favorable prognosis. Newer treatment strategies include the CD22 antibody-drug conjugate inotuzumab ozogamicin, CD19/CD3-bispecific antibodies, and chimeric antigen receptor (CAR) -T cells against CD19 that have shown impressive initial response rates in relapsed/refractory BCP-ALL but a lower long-term remission rate partly attributed to loss of surface marker expression on the leukemic cells and insufficient T cell persistence. Hence, new therapeutic options are needed, and preferably ones that specifically target the leukemic cells while sparing healthy bone marrow (BM) cells. We have previously shown that the Interleukin 1 receptor accessory protein (IL1RAP) is upregulated on the cell surface of chronic myeloid leukemia (CML) and acute myeloid leukemia (AML) cells and that it can serve as a target for therapeutic antibodies in preclinical models. The findings in our studies on myeloid malignancies, as well as the initial results from IL1RAP antibodies in clinical trials for solid tumors demonstrating that IL1RAP antibodies can be safely administered, prompted us to study the potential of IL1RAP as a target for therapy also in BCP-ALL.

To investigate the expression of IL1RAP across different genetic subtypes in BCP-ALL, we used our previously generated RNA-sequencing dataset of 195 pediatric BCP-ALL cases and a set of normal B-cell precursors (Figure 1A). In BCP-ALL
samples that harbored \textit{TCF}::\textit{PBX1} or \textit{DUX4}-rearrangements the \textit{IL1RAP} expression was similar to or lower than normal B-cell progenitors. The majority of hyperdiploid cases also showed low \textit{IL1RAP} expression whereas those with rearrangements of \textit{KMT2A} displayed higher but variable levels. A significantly higher \textit{IL1RAP} expression was found in \textit{BCR}::\textit{ABL1}-positive cases and Philadelphia chromosome-like BCP-ALL (3.8-fold and 3.1-fold compared to normal B-cell progenitors). \textit{ETV6}::\textit{RUNX1}-positive BCP-ALL displayed the highest gene expression of \textit{IL1RAP} with the mean level being 4.3 times higher than in normal B-cell progenitors and also in the closely related group of \textit{ETV6}::\textit{RUNX1}-like BCP-ALL the mean level was relatively high (3.6-fold; Figure 1A).

To validate that the gene expression of \textit{IL1RAP} corresponded to a similar cell surface IL1RAP protein expression, we performed flow cytometric analysis of 22 primary BCP-ALL BM samples and five normal BM samples. Consistent with the gene expression data, all 13 samples with \textit{ETV6}::\textit{RUNX1} and one of the \textit{BCR}::\textit{ABL1}-positive samples showed high IL1RAP cell surface expression, whereas the expression was low or absent in six out of seven samples with \textit{IGH}::\textit{DUX4} or \textit{TCF3}::\textit{PBX1} (Figure 1B-C and Online Supplementary Figure S1A). The level of IL1RAP expression was significantly higher in the CD19+ and in the more immature CD19+CD34+ leukemic cell populations of the \textit{ETV6}::\textit{RUNX1}-positive cells, than in corresponding normal BM cells (Figure 1C). From a targeting perspective, the higher IL1RAP expression on CD19+CD34+CD38− cells in the \textit{ETV6}::\textit{RUNX1}-positive cases compared to corresponding cells harboring \textit{IGH}::\textit{DUX4} or \textit{TCF3}::\textit{PBX1} is interesting to note, as this population has been reported to be enriched for leukemia-initiating cells in \textit{ETV6}::\textit{RUNX1}-positive BCP-ALL (Figure 1C).\textsuperscript{12,13} Two \textit{ETV6}::\textit{RUNX1}-positive cell lines, REH and AT, and the P190 \textit{BCR}::\textit{ABL1}-positive cell line SUP-B15
also showed high cell surface expression of IL1RAP (Figure 1D and Online Supplementary Figure S1B-D).

To test the therapeutic potential of IL1RAP as a target on ETV6::RUNX1-expressing BCP-ALL cells, we first performed antibody-dependent cellular cytotoxicity (ADCC) experiments using IL1RAP antibodies. ADCC is an important mode-of-action of therapeutic antibodies, in which the antibodies specifically bind to their target on the cell surface and through Fc-mediated binding to immune effector cells direct them to killing of the target-expressing cells.\textsuperscript{14} Notably, two monoclonal IL1RAP antibodies, mAb81.2 and mAb3F8, efficiently induced ADCC in a dose-dependent manner in three ETV6::RUNX1-positive primary samples and in REH and AT1 cells whereas the effect was very weak in the ETV6::RUNX1-negative primary sample (Figure 2A-B). As we have previously shown that AML and CML cells upon IL1 stimulation respond with cellular expansion and increased NFKB activation,\textsuperscript{6,7} we investigated if ETV6::RUNX1-positive BCP-ALL cells would react similarly. To address this, we expanded ETV6::RUNX1-positive BCP-ALL samples by serial transplantation in immunodeficient NSG mice. Four out of seven BCP-ALL samples showed engraftment and subsequent analysis verified the retained IL1RAP surface expression and sensitivity to IL1RAP antibodies in ADCC assays, and RNA-sequencing confirmed a conserved global gene expression profile, indicating that the patient-derived xenograft (PDX) -samples ALL2x, ALL3x, ALL4x, and ALL7x maintain relevant properties and thus constitute pertinent BCP-ALL models (Online Supplementary Figure S2). In short-term cultures of the PDX-samples, the addition of IL1 or IL33 did not significantly affect the total number of viable cells (Figure 2C). The PDX-cells, like REH and AT1, did not show cell surface expression of IL1R1 and IL1RL1 (Online Supplementary Figure S3A-B), but as the expression levels of these
receptors may be below the detection limit for flow cytometry, but sufficient to convey signals upon IL1 or IL33 stimulation, we performed phospho-flow cytometric analysis with NFKB phosphorylation as a marker for IL1RAP-mediated signaling. Upon stimulation with IL1, a partial response was noted only in ALL7x, whereas IL33 did not affect the NFKB phosphorylation in any of the samples (Figure 2D). Pre-incubation of ALL7x cells with mAb3F8 or the IL1 receptor antagonist (IL1RA), both known to block IL1 signaling, reduced the response to IL1 thereby confirming that NFKB phosphorylation was a specific effect of IL1 stimulation (Figure 2E and Online Supplementary Figure S3C-D). Results from primary ALL4 and ALL7 were similar to their respective PDX-sample (Figure 2F and Online Supplementary Figure S3E). Thus, although IL1RAP-mediated signaling cannot be excluded as biologically important for ETV6::RUNX1-expressing BCP-ALL cells, the potency of a novel IL1RAP-targeting therapy likely primarily relies on the high IL1RAP surface expression demonstrated here to attract agents with cytotoxic potential.

To study if IL1RAP could serve as a therapeutic target on ETV6::RUNX1-expressing cells in vivo, we transplanted PDX-cells or REH cells into unconditioned NOD/SCID mice as these, in contrast to the irradiated NSG mice used for the expansion of primary BCP-ALL cells, retain some functional immune cells that can act as effector cells upon treatment with therapeutic antibodies. Whereas the PDX-samples failed to engraft sufficiently in the unirradiated NOD/SCID strain, transplantation with REH cells led to a reproducible disease with the mice displaying a mean of 75% BM engraftment at day 42-44 after transplantation (Figure 3A). In vivo treatment studies were performed as schematically outlined in Figure 3B. First, a dose titration experiment with three doses of the IL1RAP antibody mAb81.2 ranging from 1 to 100 µg/dose was performed to determine the dose needed to obtain therapeutic effects.
At the end of experiment day 34 after transplantation, mice given the isotype control antibody displayed a mean level of 69% leukemic cells in BM (Figure 3C). In contrast, a dose of 10 µg IL1RAP antibody clearly reduced the frequency of leukemic cells (mean 28%) and for mice treated with 100 µg IL1RAP antibody, no leukemic cells could be detected (Figure 3C). Based on these results, 50 µg antibody per dose was selected for the next in vivo experiments in which six mice received IL1RAP antibody and seven the isotype control antibody. When euthanized 35 days after transplantation, mice treated with IL1RAP antibodies displayed very few leukemic cells in their BM compared to mice given isotype control antibodies (mean 0.1% vs 35%; Figure 3D). To investigate if targeting the BCP-ALL cells with IL1RAP antibodies also translates into increased survival, in the next experiment the mice were euthanized first upon signs of disease. Notably, mice treated with IL1RAP antibodies displayed a significantly increased survival compared to control mice (median 57 vs 38 days; Figure 3E). Despite the longer disease latency, the IL1RAP antibody treated mice had a lower leukemic cell burden in BM compared to control mice (mean 43% vs 81%; Figure 3F). To investigate if the leukemic cells had lost their expression of IL1RAP during treatment, a flow cytometric analysis was performed on BM cells. Leukemic cells from mice treated with mAb81.2 displayed retained albeit a slightly lower expression of IL1RAP than control mice, indicating a preferential targeting and killing of IL1RAP high-expressing cells. However, the harvested leukemic BM cells were equally sensitive to ADCC-killing mediated by IL1RAP antibodies (Figure 3G-H and Online Supplementary Figure S3F). We conclude that treatment with IL1RAP antibodies significantly reduces leukemia burden and prolongs survival in mice engrafted with human *ETV6::RUNX1*-expressing BCP-ALL cells.
In summary, we here show that IL1RAP constitutes a target for antibodies that can induce killing of *ETV6::RUNX1*-positive BCP-ALL cells by ADCC and that treatment with IL1RAP antibodies in mice engrafted with human *ETV6::RUNX1*-positive BCP-ALL cells reduces leukemia burden. These results suggest that IL1RAP provides a novel therapeutic target in pediatric *ETV6::RUNX1*-positive BCP-ALL with a possible extension to other genetic subtypes, together representing about one third of all BCP-ALL cases.
References


Figure legends

Figure 1. *ETV6::RUNX1*-positive BCP-ALL cells express IL1RAP

A. The two genetic BCP-ALL subtypes defined by expression of *ETV6::RUNX1* or *BCR::ABL1*-rearrangements, as well as the transcriptionally closely related *ETV6::RUNX1*-like and Philadelphia-like (*BCR::ABL*-like) subtypes, display a significantly higher gene expression of *IL1RAP* compared to normal B cell precursors in the dataset of 195 pediatric BCP-ALL cases. B-other refers to the BCP-ALL cases not defined by any of the aforementioned aberrations or hyperdiploidy (HEH), *TCF3::PBX1*, or rearrangements of *KMT2A* (*MLL*) or *DUX4*. The Wilcoxon signed rank test was used to determine statistically significant differences between groups (** p<0.01; *** p<0.001; **** p<0.0001). B. Flow cytometric analysis of IL1RAP expression on primary BM cells from a representative patient with *ETV6::RUNX1*-positive BCP-ALL (ALL4). Isotype antibody-stained cells were used as control. C. Flow cytometric analysis of IL1RAP expression on BM cells from 13 patients with *ETV6::RUNX1*-positive BCP-ALL (*ETV6::RUNX1*+), seven patients with *IGH::DUX4*-positive or *TCF3::PBX1*-positive BCP-ALL (*ETV6::RUNX1*), and five healthy donors (NBM). The graph shows the geometric mean fluorescence intensity (MFI) of IL1RAP in each group. The dotted line represents the mean of all isotype control stained mononuclear cells (MNCs). The Mann-Whitney test was used to determine statistically significant differences between groups (* p<0.05; **** p<0.0001). D. Flow cytometric analysis of IL1RAP expression in leukemic cells and T cells from three patients with *ETV6::RUNX1*-positive BCP-ALL (ALL5, ALL8, and ALL12) and one patient with *ETV6::RUNX1*-negative BCP-ALL (ALL18). As a comparison, the two *ETV6::RUNX1*-positive cell lines AT1 and REH were included. The data is presented
as geometric MFI for IL1RAP-antibody-stained cells divided by geometric MFI for isotype control antibody-stained cells.

**Figure 2. IL1RAP antibodies direct NK cells to killing of ETV6::RUNX1-positive BCP-ALL cells that lack a general response to IL1 or IL33 stimulation.**

**A.** Primary BCP-ALL cells were incubated overnight in presence of increasing concentrations of the IL1RAP antibodies mAb81.2 or mAb3F8 or a corresponding hlgG1 isotype control antibody, and primary human NK cells at a 10:1 effector to target cell ratio. The results are presented as the number of viable target cells in wells with antibody divided by the number of viable target cells in wells without antibody. ALL5, ALL8, and ALL12 are positive for ETV6::RUNX1 whereas ALL18 represents a negative sample. The ADCC-assay was performed with NK cells from two different donors, and the data is presented as mean with error bars representing range. **B.** The ADCC-assay was performed as described above, with REH and AT1 as target cells. The samples were set in duplicates and repeated three times with NK-cells from three different donors. The data is presented as mean with the error bars representing SEM. Students’ t-test was used was used to determine statistically significant differences between each IL1RAP antibody concentration and the corresponding isotype antibody control (* p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001). **C.** ETV6::RUNX1-positive PDX BCP-ALL cells (second passage) from four patients were cultured in serum-free media in presence or absence of IL1 or IL33. The analysis was performed in two biological and two technical replicates. The absolute number of viable cells following 72 hours of culturing is presented. The dotted line represents the number of seeded cells. Error bars represents SEM. **D.** NFkB phosphorylation in the same four PDX BCP-ALL samples following 15 min
stimulation with 10 ng/mL IL1 or IL33 was analyzed by phospho-flow. In the histograms, “no ck” refers to sample without cytokine. Isotype antibody-stained cells were used as control. An AML sample (AMLx) was included as positive control for NFKB phosphorylation in response to IL1 stimulation. **E.** Phospho-flow analysis of ALL7x cells that were stimulated with IL1 in the absence or presence of 10 μg/mL of the IL1 signaling blocking antibody mAb3F8. The level of pNFKB from three paired replicates is presented, with the individual replicates marked as blue, red, or yellow. **F.** NFKB phosphorylation in primary ALL4 and ALL7 cells following stimulation with IL1 or IL33 analyzed by phospho-flow. “No ck” refers to sample without cytokine. Isotype antibody-stained cells were used as control.

**Figure 3.** IL1RAP antibodies induce killing of \textit{ETV6::RUNX1}-expressing BCP-ALL cells in vivo

**A.** Unconditioned NOD/SCID mice were engrafted with REH cells by tail vein injections. At day 42-44 after transplantation the mice (n=5) were euthanized due to disease and displayed a mean of 75% REH cells in BM as determined by flow cytometry. The spleens contained <1% REH cells. **B.** Schematic overview of the antibody treatment model that was used in all in vivo treatment experiments. The antibodies, mAb81.2 or a corresponding mIgG2a isotype control, were distributed biweekly by i.p. injections starting day 3 after transplantation. The first antibody dose was given as a bolus of double amount antibody. **C.** REH-engrafted NOD/SCID mice were treated with 1, 10 or 100 µg/dose mAb81.2 or with 100 µg/dose of the isotype control antibody (n=2 per group). The graph shows the mean frequency of REH cells in the BM at the end of experiment day 34 after transplantation. Error bars represents range. **D.** REH-engrafted NOD/SCID mice were treated with 50 µg mAb81.2 (n=6) or
an isotype control antibody (n=7) per dose, corresponding to approximately 2 mg/kg bodyweight. At day 35 after transplantation the mice were euthanized. The graph shows the frequency of REH cells in BM. The Mann-Whitney test was used for statistical analysis. E. For studies of survival, REH-engrafted NOD/SCID mice were treated with 50 µg/dose IL1RAP antibody mAb81.2 (n=7) or isotype control antibody (n=6) and euthanized upon signs of disease. Mice given control antibody had a median survival of 38 days (range 37-58 days) and mice treated with mAb81.2 survived a median of 57 days (range 56-78 days). The Log-rank (Mantel-Cox) test was used to determine statistically significant difference between groups. F. The frequency of REH cells in BM. G. The expression of IL1RAP on leukemic BM cells. H. BM cells from two mice treated with the IL1RAP antibody and two mice treated with control antibody were incubated overnight in presence of 10 µg/mL of the IL1RAP antibodies mAb81.2 or mAb3F8 or an isotype control antibody, and primary human NK cells. The result is presented as the number of viable target cells in wells with antibody divided by the number of viable target cells in wells without antibody. The ADCC-assay was performed in duplicates with NK cells from two different donors. REH cells from the cell culture was included for comparison. Unless otherwise stated, the data in Figure 3 is presented as mean with error bars representing SEM, and students’ t-test was used to determine statistical significance following the Shapiro-Wilk test to ascertain Gaussian distribution. * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001.
IL1RAP is expressed in several subtypes of pediatric acute lymphoblastic leukemia and can be used as a target to eliminate *ETV6::RUNX1*-positive leukemia cells in preclinical models

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Supplementary material
Supplementary Figure S1. Cell surface expression of IL1RAP on primary BM samples of different BCP-ALL genetic subtypes.

A. Flow cytometric analysis of primary leukemic BM cells from 13 patients with *ETV6::RUNX1*-positive (E::R; ALL1-ALL13), two patients with *BCR::ABL1*-positive (B::A; ALL14-ALL15), five patients with *IGH::DUX4*-positive (I::D; ALL16-ALL20) and two patients with *TCF3::PBX1*-positive (T::P; ALL21-ALL22) BCP-ALL. B. Flow cytometric analysis of IL1RAP expression on *ETV6::RUNX1*-positive REH and AT1 cells. C. Using the BD Quantibrite assay, an estimate of the total number of IL1RAP molecules on the cell surface of AT1, REH, and the four primary samples ALL5, ALL8, ALL12 and ALL18 was made based on the assumption of a 1:1 fluorochrome to antibody ratio. D. Flow cytometric analysis of IL1RAP expression on the two BCR::ABL1-expressing cell lines TOM-1 and SUP-B15. In all cases, isotype antibody-stained cells were used as control.
A. One to two million MNCs isolated from BM samples of ETV6::RUNX1-positive BCP-ALL patients (ALL2, ALL3, ALL4 and ALL7) were transplanted by tail vein injections into sublethally irradiated NSG mice (n=7). Around seven months after transplantation the mice were sacrificed due to disease and displayed a mean of 85% and 82% BCP-ALL cells in BM and spleens, respectively. Error bars represents SEM.

B. The xenografted BCP-ALL cells display cell surface expression of IL1RAP as shown by flow cytometry. Isotype antibody-stained cells were used as control.

C. Two of the xenografted samples (ALL2x and ALL3x) were incubated overnight with the IL1RAP antibodies mAb81.2 or mAb3F8 or a corresponding hIgG1 isotype control antibody and human NK cells. The result is presented as the number of viable target cells in wells with antibody divided by the number of viable target cells in wells without antibody. The ADCC-assay was performed with NK cells from two different donors, and the data is presented as mean with error bars representing range.

D. RNA-sequencing of BCP-ALL cells confirmed a retained global gene expression profile in the xenografted BCP-ALL cells ALL2x, ALL3x, ALL4x and ALL7x. The PDX-samples cluster with the primary ETV6::RUNX1-positive samples in the dataset of 195 pediatric BCP-ALL cases by Lilliebjörn et al. Dim-blue and green boxes represents the ETV6::RUNX1-positive samples in the BCP-ALL subtype row. ALL2 is represented by orange boxes, ALL3 by purple boxes, ALL4 by green boxes and ALL7 by blue boxes in the Xenografted samples row. The xenografted samples are marked by black boxes in the Xenograft row.
Supplementary Figure S3. Response to stimulation with IL1 or IL33

A. REH and AT1 cells does not express IL1R1 or IL1RL1 as shown by flow cytometry. KU812 cells were included as positive control for IL1R1 and IL1RL1 antibody staining. Isotype antibody-stained cells were used as control. B. Flow cytometric analysis shows lack of expression of IL1R1 and IL1RL1 on ALL2x, ALL3x, ALL4x and ALL7x. Isotype antibody-stained cells were used as control. C. Phospho-flow analysis of NFKB phosphorylation in ALL7x cells following stimulation with IL1 in the absence or presence of 10 ng/mL IL1RA. Two paired samples are shown, presented as blue or red. D. Extended flow cytometric analysis showing that the pNFKB-positive ALL7x-cells display essentially the same immunophenotype as the non-responsive cells when stimulated with IL1. E. Flow cytometric analysis of IL1R1 and IL1RL1 expression on the two primary ETV6::RUNX1-positive BCP-ALL samples ALL4 and ALL7. Isotype antibody-stained cells were used as control. F. Flow cytometric analysis of the REH cells in BM from two mice treated with IL1RAP antibody and two mice treated with control antibody as well as REH from the cell culture. Isotype antibody-stained REH cells from the cell culture were used as control (light blue). The geometric mean fluorescence intensity for each sample is presented along with the histograms.