LETTER TO THE EDITOR

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

Acute lymphoblastic leukemia (ALL) is the most common type of childhood cancer and in most cases the leukemic cells display a B-cell precursor (BCP) immunophenotype. Although effective, the cytotoxic agents that constitute the backbone of therapy are associated with short- and long-term side effects that negatively influence the health and well-being of the growing child.[1-3] Current treatment protocols extend up to 2.5 years, which may lead to problems with adherence to treatment significantly increasing the risk of relapse that, similarly to primary disease in infants and adults, has a less favorable prognosis.[4] Newer treatment strategies include the CD22 antibody-drug conjugate inotuzumab ozogamicin, CD19/CD3-bispecific antibodies, and chimeric antigen receptor (CAR) T cells against CD19, which have led to 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.[5] Hence, new therapeutic options are needed, and preferably ones that specifically target the leukemic cells while sparing healthy bone marrow cells. We have previously shown that interleukin 1 receptor accessory protein (IL1RAP) is upregulated on the surface of chronic myeloid leukemia and acute myeloid leukemia cells and that it can serve as a target for therapeutic antibodies in preclinical models.[6-9] Our studies on myeloid malignancies, as well as the initial results from clinical trials of IL1RAP antibodies for the treatment of solid tumors demonstrating that IL1RAP antibodies can be safely administered,[10] 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).[11] In BCP-ALL samples that harbored *TCF::PBX1* or *DUX4* rearrangements, *IL1RAP* expression was similar to or lower than that in normal B-cell progenitors. The majority of hyperdiploid cases also showed low *IL1RAP* expression whereas those with rearrangements of *KMT2A* displayed higher but variable levels. A significantly higher *IL1RAP* expression was found in *BCR::ABL1*-positive cases and Philadelphia chromosome-like BCP-ALL (3.8-fold and 3.1-fold, respectively, compared to normal B-cell progenitors). The highest expression of *IL1RAP* was found in *ETV6::RUNX1*-positive BCP-ALL, with the mean level being 4.3 times higher than in normal B-cell progenitors. The mean level was also relatively high (3.6-fold) in the closely related group of *ETV6::RUNX1*-like BCP-ALL (Figure 1A).

To validate that the gene expression of *IL1RAP* corresponded to a similar cell surface expression of IL1RAP protein, we performed flow cytometric analysis of 22 primary BCP-ALL bone marrow samples and five normal bone marrow samples. Consistent with the gene expression data, all 13 samples with *ETV6::RUNX1* and one of the *BCR::ABL1*-positive samples showed high IL1RAP cell surface expression, whereas the expression was low or absent in six out of seven samples with *IGH::DUX4* or *TCF3::PBX1* (Figure 1B, C; *Online Supplementary Figure S1A*). The level of IL1RAP expression was significantly higher in the $CD19^+$ and more immature $CD19^+CD34^+$ leukemic cell populations of the *ETV6::RUNX1*-positive cells than in corresponding normal bone marrow cells (Figure 1C). From a targeting perspective, the higher IL1RAP expression on $CD19^+CD34^+CD38^-$ cells in the *ETV6::RUNX1*-positive cases, compared to corresponding cells harboring *IGH::DUX4* or *TCF3::PBX1*, is interesting to note because this population has been reported to be enriched for leukemia-initiating cells in *ETV6::RUNX1*-positive BCP-ALL (Figure 1C).[12,13] Two *ETV6::RUNX1*-positive cell lines, REH and AT, and the P190 *BCR::ABL1*-positive cell line SUP-B15 also showed high cell surface expression of IL1RAP (Figure 1D; *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 bind specifically 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.[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 they had only a very weak effect in the *ETV6::RUNX1*-negative primary sample (Figure 2A, B). As we have previously shown that the response of acute myeloid leukemia and

chronic myeloid leukemia cells to interleukin (IL)-1 stimulation is cellular expansion and increased NFκB activation,[6,7] we investigated whether *ETV6::RUNX1*-positive BCP-ALL cells would react similarly. To do 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. Furthermore, 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 Supple-*

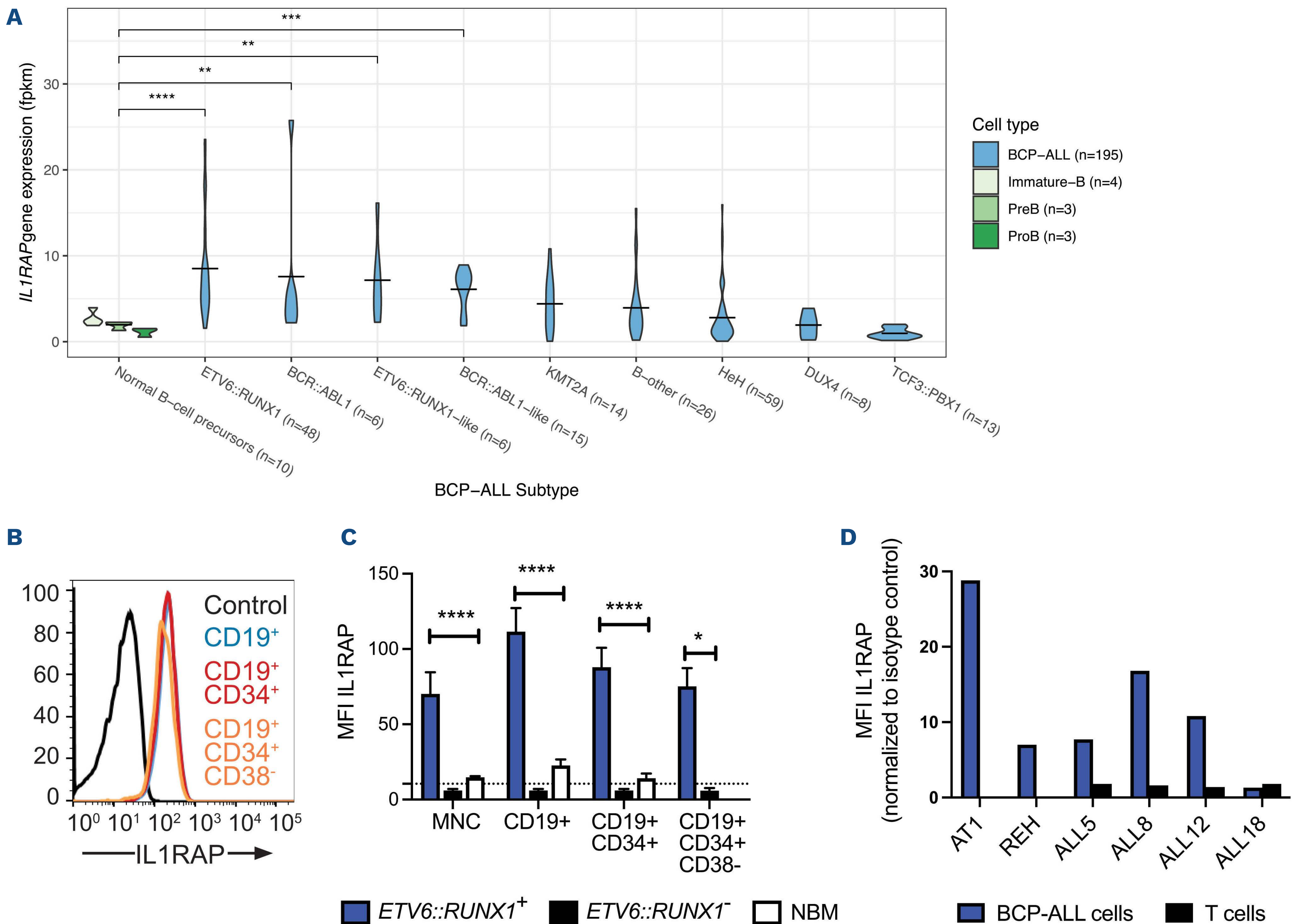

Figure 1. *ETV6::RUNX1*-positive B-cell precursor acute lymphoblastic leukemia cells express IL1RAP. (A) The two genetic subtypes of B-cell precursor acute lymphoblastic leukemia (BCP-ALL) defined by expression of *ETV6::RUNX1* or *BCR::ABL1* rearrangements, as well as the transcriptionally, closely related *ETV6::RUNX1*-like and Philadelphia-like (*BCR::ABL*-like) subtypes, displayed a significantly higher gene expression of *IL1RAP* compared to normal B-cell precursors in the dataset of 195 cases of pediatric BCP-ALL. B-other refers to 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 bone marrow cells from a representative patient with *ETV6::RUNX1*-positive BCP-ALL (ALL4). Isotype antibody-stained cells were used as a control. (C) Flow cytometric analysis of IL1RAP expression on bone marrow 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. 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 are presented as the geometric MFI for IL1RAP-antibody-stained cells divided by the geometric MFI for cells stained with the isotype control antibody. Fpkm: fragments per kilobase of exon per million mapped reads; MNC: mononuclear cells; NBM: normal bone marrow.

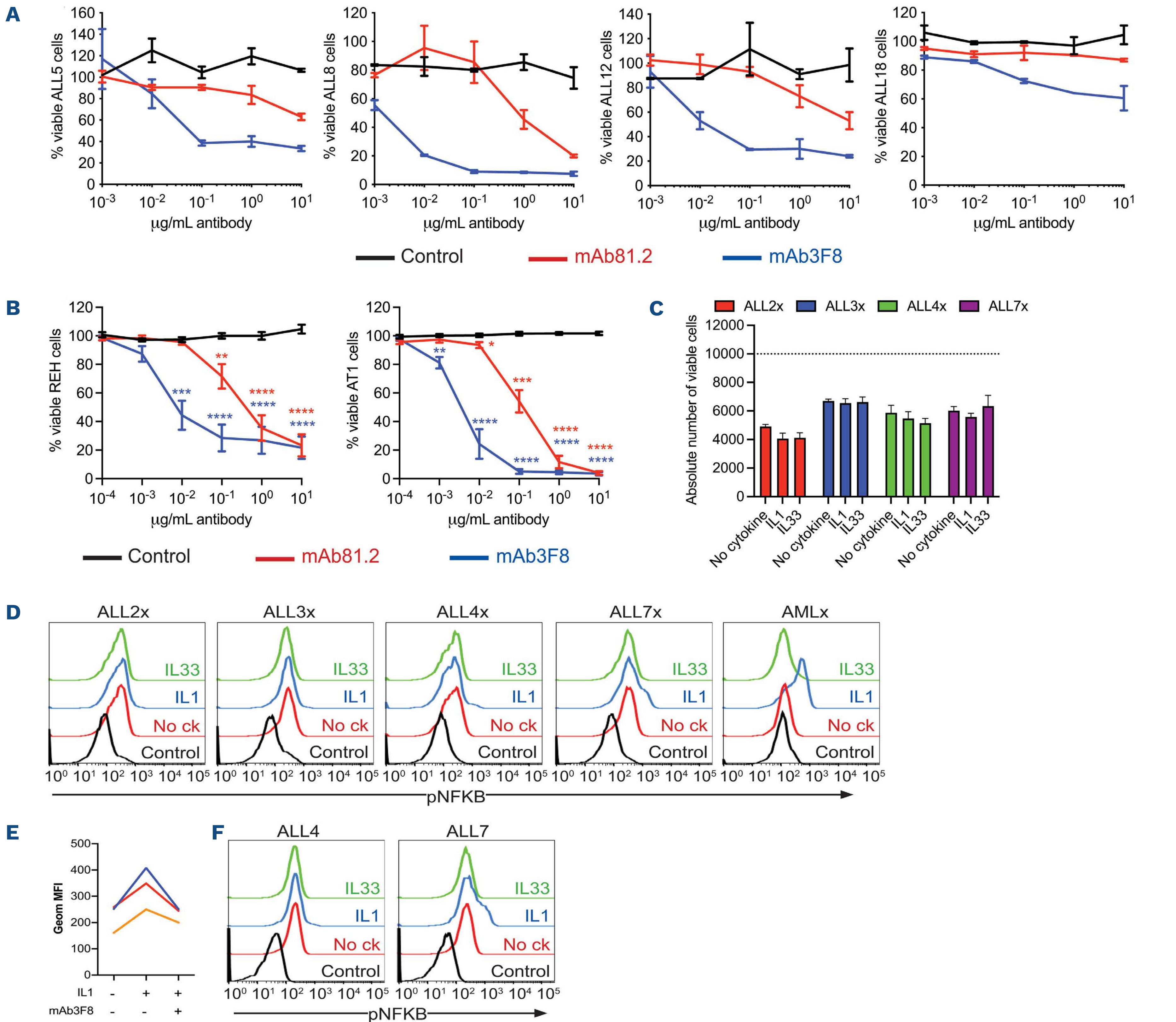

Figure 2. IL1RAP antibodies direct NK cells to killing of *ETV6::RUNX1*-positive B-cell precursor acute lymphoblastic leukemia cells that lack a general response to IL1 or IL33 stimulation. (A) Primary B-cell precursor acute lymphoblastic leukemia (BCP-ALL) cells were incubated overnight in the presence of increasing concentrations of the IL1RAP antibodies mAb81.2 or mAb3F8 or a corresponding hIgG1 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 antibody-dependent cellular cytotoxicity (ADCC) assay was performed with NK cells from two different donors, and the data are presented as the mean with error bars representing the range. (B) The ADCC assay was performed as described above, with REH and AT1 as target cells. The samples were set in duplicate and repeated three times with NK cells from three different donors. The data are presented as the mean with error bars representing the standard error of mean (SEM). A Student *t* test was used to determine statistically significant differences between the effect of 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 patient-derived xenograft (PDX) BCP-ALL cells (second passage) from four patients were cultured in serum-free media in the 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 culture is presented. The dotted line represents the number of seeded cells. Error bars represent the SEM. (D) NFκB phosphorylation in the same four PDX BCP-ALL samples following stimulation for 15 min with 10 ng/mL IL1 or IL33 was analyzed by phospho-flow. In the histograms, "no ck" refers to samples not stimulated with a cytokine. Isotype antibody-stained cells were used as the control. An acute myeloid leukemia sample (AMLx) was included as a positive control for NFκB 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 pNFκB from three paired replicates is presented, with the individual replicates marked as blue, red, or yellow. (F) NFκB phosphorylation in primary ALL4 and ALL7 cells following stimulation with IL1 or IL33 analyzed by phospho-flow. "No ck" refers to samples not treated with a cytokine. Isotype antibody-stained cells were used as the control.

mentary 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 the REH and AT1 cells lines, did not show cell surface expression of IL1R1 (*Online Supplementary Figure S3A, B*), but as the level of expression 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 NFκB phosphorylation as a marker for IL1RAP-mediated signaling. Upon stimulation with IL1, a partial response was noted only in ALL7x, whereas IL33 did

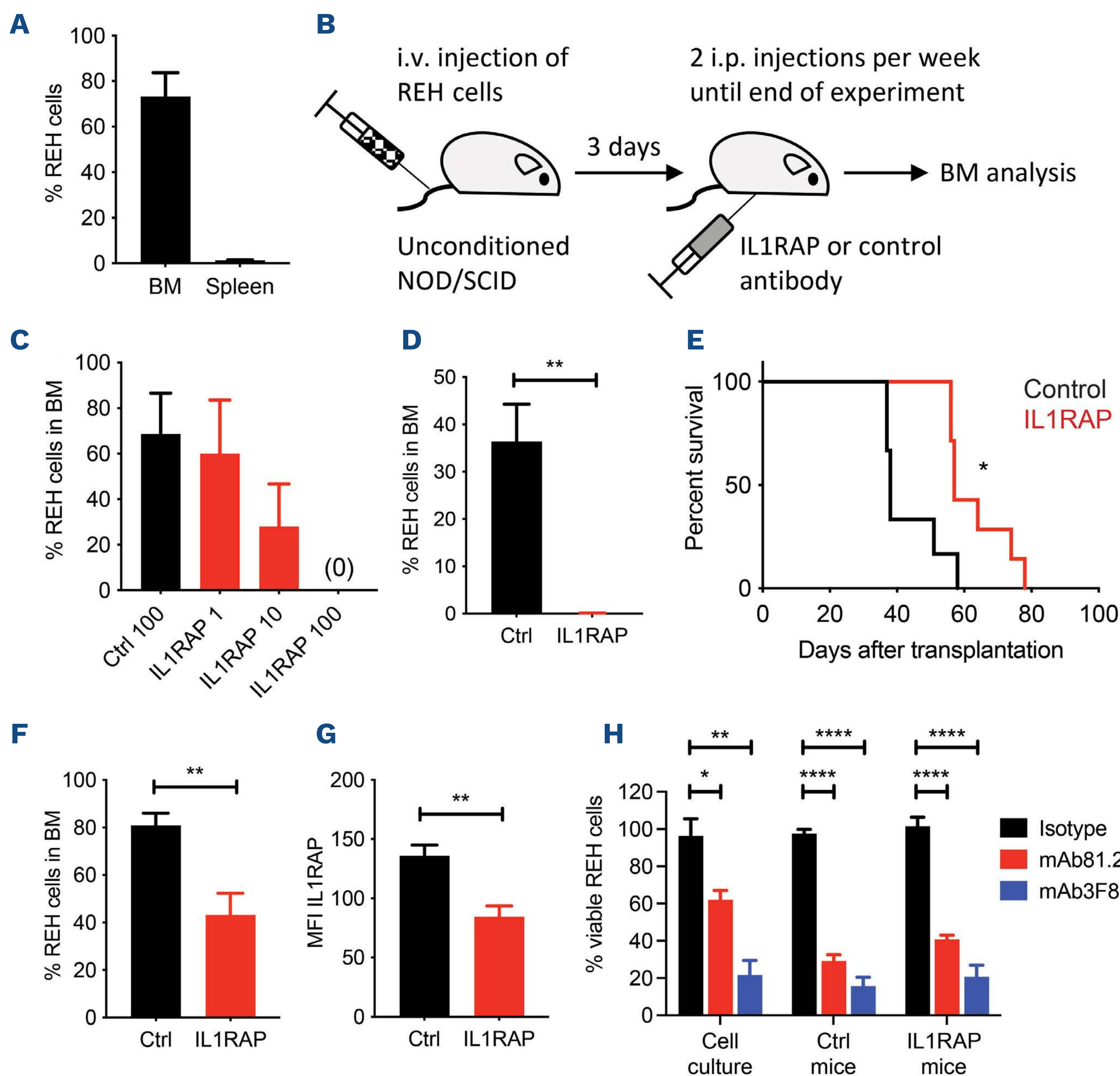

Figure 3. IL1RAP antibodies induce killing of *ETV6::RUNX1*-expressing B-cell precursor acute lymphoblastic leukemia cells *in vivo*. (A) Unconditioned NOD/SCID mice were engrafted with REH cells by tail vein injections. At day 42-44 after transplantation the mice (n=5) were euthanized due to disease and displayed a mean of 75% REH cells in bone marrow (BM) as determined by flow cytometry. The spleens contained <1% REH cells. (B) A 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 intraperitoneal injections starting on day 3 after transplantation. The first antibody dose was given as a bolus of a double amount of 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 the experiment on day 34 after transplantation. Error bars represent the 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. On day 35 after transplantation the mice were euthanized. The graph shows the frequency of REH cells in the 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 the 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 differences 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 IL1RAP antibody and two mice treated with control antibody were incubated overnight in the presence of 10 μg/mL of the IL1RAP antibodies mAb81.2 or mAb3F8 or an isotype control antibody, and primary human NK cells. 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. An antibody-dependent cellular cytotoxicity assay was performed in duplicate with NK cells from two different donors. REH cells from the cell culture were included for comparison. Unless otherwise stated, the data in Figure 3 are presented as the mean with error bars representing the standard error of mean. A Student *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. i.v.: intravenous; i.p.: intraperitoneal; Ctrl: control.

not affect the NFκB 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,[7] reduced the response to IL1 thereby confirming that NFκB phosphorylation was a specific effect of IL1 stimulation (Figure 2E; *Online Supplementary Figure S3C, D*). Results from primary ALL4 and ALL7 samples were similar to those of their respective PDX samples (Figure 2F, *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 whether 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.[15] 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% bone marrow engraftment at day 42-44 after transplantation (Figure 3A). *In vivo* treatment studies were performed as outlined schematically 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 the experiment on day 34 after transplantation, mice given the isotype control antibody displayed a mean level of 69% leukemic cells in bone marrow (Figure 3C). In contrast, a dose of 10 μg IL1RAP antibody clearly reduced the frequency of leukemic cells (mean 28%) and no leukemic cells could be detected in mice treated with 100 μg IL1RAP antibody (Figure 3C).

Based on these results, a dose of 50 μg antibody 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 had very few leukemic cells in their bone marrow compared to the number in mice given isotype control antibodies (mean: 0.1% *vs*. 35%) (Figure 3D). To investigate whether targeting BCP-ALL cells with IL1RAP antibodies also translates into increased survival, in the next experiment the mice were euthanized upon the first signs of disease. Notably, mice treated with IL1RAP antibodies had a significantly increased survival compared to control mice (median: 57 *vs*. 38 days) (Figure 3E). Despite the longer disease latency, the mice treated with IL1RAP antibodies had a lower leukemic cell burden in bone marrow compared to control mice (mean: 43% vs. 81%) (Figure 3F). To determine whether the leukemic cells had lost their expression of IL1RAP during treatment, a flow cytometric analysis was performed on bone marrow cells. Leukemic cells from mice treated with mAb81.2 retained expression of IL1RAP, albeit at a slightly lower level in control mice, indicating a preferential targeting and killing of IL1RAP high-expressing cells. However, the harvested leukemic bone marrow cells were equally sensitive to ADCC mediated by IL1RAP antibodies (Figure 3G, H; *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 show here 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, which together account for about one-third of all BCP-ALL cases.

Authors

Helena Ågerstam,[1,2] Henrik Lilljebjörn,[1] Marianne Rissler,[1] Carl Sandén[1] and Thoas Fioretos[1,2]

[1]Division of Clinical Genetics, Department of Laboratory Medicine, Lund University, Lund and [2]Department of Clinical Genetics and Pathology, Office for Medical Services, Region Skåne, Lund, Sweden

Correspondence:
H. ÅGERSTAM - helena.agerstam@med.lu.se
T. FIORETOS - thoas.fioretos@med.lu.se

https://doi.org/10.3324/haematol.2022.281059

Received: March 16, 2022.
Accepted: September 27, 2022.
Prepublished: October 13, 2022.

©2023 Ferrata Storti Foundation
Published under a CC BY-NC license

Disclosures

TF is a cofounder and board member of Cantargia AB (Medicon Village, Lund), which develops 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 do not have any potential conflicts of interest to declare.

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.

Acknowledgments

We thank Cantargia AB for making the IL1RAP antibodies available.

Funding

This work was supported by the Swedish Children's Cancer Foundation, the Swedish Cancer Society, the Swedish Research Council, the Knut and Alice Wallenberg Foundation, the Magnus Bergvall Foundation, the Royal Physiographic Society, the Medical Faculty of Lund University, and Region Skåne (ALF-medel).

Data-sharing statement

Original data can be made available upon a written request to the corresponding authors.

References

1. Kunstreich M, Kummer S, Laws HJ, Borkhardt A, Kuhlen M. Osteonecrosis in children with acute lymphoblastic leukemia. Haematologica. 2016;101(11):1295-1305.
2. Nielsen SN, Eriksson F, Rosthoej S, et al. Children with low-risk acute lymphoblastic leukemia are at highest risk of second cancers. Pediatr Blood Cancer. 2017;64(10).
3. Tuckuviene R, Ranta S, Albertsen BK, et al. Prospective study of thromboembolism in 1038 children with acute lymphoblastic leukemia: a Nordic Society of Pediatric Hematology and Oncology (NOPHO) study. J Thromb Haemost. 2016;14(3):485-494.
4. Malard F, Mohty M. Acute lymphoblastic leukaemia. Lancet. 2020;395(10230):1146-1162.
5. Si Lim SJ, Grupp SA, DiNofia AM. Tisagenlecleucel for treatment of children and young adults with relapsed/refractory B-cell acute lymphoblastic leukemia. Pediatr Blood Cancer. 2021;68(9):e29123.
6. Ågerstam H, Hansen N, von Palffy S, et al. IL1RAP antibodies block IL-1-induced expansion of candidate CML stem cells and mediate cell killing in xenograft models. Blood. 2016;128(23):2683-2693.
7. Ågerstam H, Karlsson C, Hansen N, et al. Antibodies targeting human IL1RAP (IL1R3) show therapeutic effects in xenograft models of acute myeloid leukemia. Proc Natl Acad Sci U S A. 2015;112(34):10786-10791.
8. Askmyr M, Ågerstam H, Hansen N, et al. Selective killing of candidate AML stem cells by antibody targeting of IL1RAP. Blood. 2013;121(18):3709-3713.
9. Järås M, Johnels P, Hansen N, et al. Isolation and killing of candidate chronic myeloid leukemia stem cells by antibody targeting of IL-1 receptor accessory protein. Proc Natl Acad Sci U S A. 2010;107(37):16280-16285.
10. Robbrecht D, Jungels C, Sorensen MM, et al. First-in-human phase 1 dose-escalation study of CAN04, a first-in-class interleukin-1 receptor accessory protein (IL1RAP) antibody in patients with solid tumours. Br J Cancer. 2022;126(7):1010-1017.
11. Lilljebjörn H, Henningsson R, Hyrenius-Wittsten A, et al. Identification of ETV6-RUNX1-like and DUX4-rearranged subtypes in paediatric B-cell precursor acute lymphoblastic leukaemia. Nat Commun. 2016;7:11790.
12. Castor A, Nilsson L, Åstrand-Grundström I, et al. Distinct patterns of hematopoietic stem cell involvement in acute lymphoblastic leukemia. Nat Med. 2005;11(6):630-637.
13. Hong D, Gupta R, Ancliff P, et al. Initiating and cancer-propagating cells in TEL-AML1-associated childhood leukemia. Science. 2008;319(5861):336-339.
14. Salles G, Barrett M, Foa R, et al. Rituximab in B-cell hematologic malignancies: a review of 20 years of clinical experience. Adv Ther. 2017;34(10):2232-2273.
15. Shultz LD, Schweitzer PA, Christianson SW, et al. Multiple defects in innate and adaptive immunologic function in NOD/LtSz-scid mice. J Immunol. 1995;154(1):180-191.